Characterisation of Transcripitional Mediator Subunit, MED17 and its Regulation by Cyclins

Shamindra Gerald Direkze

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University of London

The Wolfson Institute for Biomedical Research
Cancer Research UK Viral Oncology Laboratory
Cruciform Building
Gower Street
London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Mediator is a transcription co-factor complex that co-operates with transcriptonal activators to enhance gene specifc transcription and is conserved between yeast, *Drosophila* and man. The MED17 subunit of Mediator (formerly known as TRAP80/CRSP6/DRIP80) has been characterised as a transcriptional activator interacting with a number of transcription factors, such as heat shock factor and p53. Expression of MED17 in yeast and *Drosophila* is essential to cell viability possibly due to its function as a global transcriptional regulator.

In a yeast-2-hybrid screen with a viral cyclin as bait, MED17 was identified as an interacting clone. Due to the oncogenic potential of viral cyclins, effects of human MED17 on p53 regulated transcription were investigated. Functional characterisation of MED17 effects on p53 showed that it repressed p53 mediated transcription in luciferase reporter assays. Further, a MED17 constitutively expressing line generated in non-transformed mouse cells inhibited apoptosis and demonstrated other features of p53 functional loss. Human MED17 still activates heat shock regulated transcription, as previously described for the *Drosophila* homologue. Analysis of other transcription factors regulated by MED17 was investigated by gene expression microarray analysis of the MED17 cell line, revealing a putative co-activator function in ß-catenin regulated transcription. Also studied was the interaction of MED17 with cellular homologues of viral cyclin. Cyclin/cdks phosphorylate MED17, with cyclin A/cdk2 specifically phosphorylating MED17 to enhance its expression.

This investigation reveals a novel repressor function for MED17 on p53 mediated transcription and links cell cycle regulators to the transcriptional activities of MED17/Mediator and p53.

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Abbreviations

APS	Ammonium persulphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CAK	cdk activating kinase
Cdk	cyclin dependent kinase
Cdki	cyclin dependent kinase inhibitor
CO-IP	co-immunoprecipitation
CTD	C-terminal domain
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis ß aminoethylether N-N-N-N tetraacetic acid
FCS	foetal calf serum
G418	neomycin sulphate
GTF	general transcription factors
GST	glutathione-S-transferase
HAT	histone acetyl transferase
HDAC	histone deacetylase
Hepes	N'-(2-hydroxyethyl) piperazine N'-(2-ethanesulphonic acid)
HHV8	human herpesvirus 8
HVS	*Herpesvirus saimiri*
IP	immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
KSHV	*Kaposi's sarcoma-associated herpesvirus* (HHV8)
KS	Kaposi's sarcoma
ß-ME	ß-mercaptoethanol
MCD	Multicentric Castleman's disease
mRNA	messenger ribonucleic acid

ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PI	propidium iodide
pol	polymerase
PMSF	phenyl-methyl-sulphonyl fluoride
PCR	polymerase chain reaction
PEL	Primary effusion lymphoma
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
STAT	signal transducer and activator of transcription
TAF	TATA binding protein associated factor
TBP	TATA binding protein
TR	thyroid hormone receptor
TRE	thyroid hormone receptor response element
Tween-20	polyoxyethylene-sorbitan monolaurate
UAS	upstream activator sequence
vcyclin	viral cyclin of KSHV
V-cyclin	viral cyclin of HVS
Y-2-H	yeast-2-hybrid screen

Scientific units

bp	base pairs
°C	degrees centigrade
g	gram
Kb	Kilobases
Kda	Kilodalton
M	Moles
m	milli
µ	micro
n	nano
rpm	revolutions per minute
v/v	volume for volume
w/v	weight for volume

Single amino acid code

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Chapter 1: Introduction

1.0 Transcription by RNA polymerase II

Transcription is the process of synthesising RNA for which the activity of the enzyme RNA polymerase (RNA pol) is central. Three types of RNA pol, I, II and III, exist and differ in their functions. RNA pol I and III are responsible for the majority of cellular transcriptional activity, synthesising ribosomal (rRNA) and transfer RNA (tRNA) respectively. RNA pol II, however, is involved in the transcription of genes and the synthesis of pre-messenger RNA (pre-mRNA). RNA pol II is a multi-subunit complex containing, Rpb1, a C-terminal domain motif (CTD) protein that is phosphorylated by regulatory kinases (Young, 1991). The CTD consists of a heptapeptide repeat (YSPTSPS) and is critical to the function of RNA pol II. Phosphorylation of specific serine residues in the CTD occurs during transcription and is associated with the regulation of RNA pol II activity. Specifically, serine 2 and serine 5 of the heptapeptide repeat are phosphorylated during transcription initiation and elongation. In the formation of the pre-initiation complex, which consists of RNA pol II and the general transcription factors, bound to DNA upstream of a transcription initiation site, serine 5 is phosphorylated. The subsequent phosphorylation of serine 2 is associated with the transition of RNA pol II to the elongation phase of transcription (Kobor and Greenblatt, 2002;Palancade and Bensaude, 2003).

Concurrent with the process of transcription are the capping, splicing and poly-adenylation modifications to the sequence of the transcribed mRNA

(Proudfoot et al., 2002;Zorio and Bentley, 2004). The process of capping involves the addition of a methylated guansine cap to the 5' end of the mRNA when it is between 22-40 base pairs long. The cap stabilizes the mRNA, protecting it from exonuclease activity, as well as facilitating the transport of the mRNA to the ribosomes for translation. In humans three enzymes are responsible for this reaction, human mRNA capping enzyme 1 (HCE1), HCE1A and HCE1B, whose guanyltransferase and triphosphatase catalytic activity is aided by interaction with phosphorylated serine 5 of the CTD.

mRNA splicing also occurs during transcription. Mammalian genes typically contain on average nine introns which are spliced from the mRNA by the spliceosome complex to generate a continuous open reading frame by removing exons and joining introns. Splice sites are initially recognised by the U1snRNP subunit of the spliceosome during transcription which recruits other members of the complex. The elongation rate of the transcript may in fact regulate splicing by transcribing multiple splice sites to which the spliceosome can be used to generate alternative transcripts. The coupling of elongation with splicing may be mediated by an interaction between spliceosome proteins and the CTD of RNA pol II.

The poly-adenylation at the 3' end of the mRNA is required for termination of transcription and the release of RNA pol II from the mRNA on completion of transcription. This "poly-A tail", similar to the 5' cap also protects the transcript from exonuclease activity. The addition of the poly-A tail is initiated by cleavage of the 3' end at a conserved mRNA sequence coupled with the recruitment of

poly-A polymerase. Studies in yeast indicate the CTD of RNA pol II seems to be required for both the cleavage and the poly-A extension by phosphorylation of serine 2 of the heptapaptide repeat in the CTD which is important for binding of accessory factors (Ahn et al., 2004).

The general transcription factors (GTF), TFIIA, B, D, E, F and H are a distinct set of factors required for transcriptional activity of RNA pol II whereas RNA pol I and RNA pol II are recruited to pre-initiation complexes by their own distinct GTFs. GTFs have been identified through biochemical purification and study of their transcription activating function *in vitro*. Together with RNA pol II they are collectively referred to as the basal transcription machinery, which is conserved among eukaryotes. The basal transcription machinery mediates transcription initiation and elongation required for basal transcription from a core gene promoter region. Gene specific transcription is activated by the basal transcription machinery on addition of sequence specific activator proteins binding their cognate DNA enhancer elements which are upstream of the core promoter. Similarly transcriptional repression can also be mediated by repressor proteins binding to their specific elements and inhibiting the activity of the basal transcription machinery. The addition of these DNA regulatory sequences introduces a high degree of complexity in the regulation of eukaryotic transcription.

1.1 DNA regulatory sequences involved in transcription

Levine and Tjian noted that diversity in the types of DNA regulatory sequences present in the promoter regions of genes positively correlated with the mechanistic complexity of transcription in higher order organisms (Levine and Tjian, 2003). A typical simple eukaryote promoter, as would be found in yeast, consists of a core promoter, upstream activator sequence (UAS) and a silencer region all located in the 5' region upstream of the transcription initiation site. The core promoter typically consists of a TATA box which mediates binding to the TATA binding protein (TBP), a subunit of the TFIID complex. Located distal to this is the UAS, binding site for usually two or three different sequence specific transcription factors which regulate the activity of TBP.

Compared with a eukaryote promoter a metazoan promoter demonstrates a higher degree of complexity. A typical metazoan promoter consists of these basic regulatory sequences as found in eukaryotes in addition to regulatory elements located both upstream and downstream of the transcription initiation site (Fig 1.1). The metazoan basic core promoter contains initiator sequences as well as a promoter element which is downstream of the transcription initiation site. This promoter element is thought to be particularly important in transcriptional regulation of genes which do not contain a TATA box. The proximal promoter elements, also known as response elements, functions like the UAS in promoter regions of lower order organisms but additionally contains binding sites for a transcriptional repressor as well as two activators, typically. Multiple enhancer sites which direct tissue specific gene expression patterns are

specific to metazoan transcription. Enhancer sequences can be located many kilobases upstream or downstream from the transcription initiation site and contain binding sites for multiple transcription factors. Due to their distant location, transcription factors from adjacent genes have the potential to inappropriately activate transcription. Insulator sequences block the interaction of distal enhancers with the proximal promoter to prevent this. These additional DNA sequences in the typical metazoan gene promoter provide sites for the interaction of transcription regulatory proteins.

Broadly, two components contribute to transcriptional complexity in Metazoans. Firstly, DNA regulatory sequences in the promoter regions of genes enhance the complexity of the metazoan transcription by permitting the binding of sequence specific DNA binding proteins which have the capacity to positively or negatively regulate transcription. In addition, an increased number of transcription factors encoded in the genomes of higher organisms which enhances complexity still further. The human genome is thought to encode over 3000 transcription factors in contrast to the genomes of *C.elegans* and *Drososphila* which are thought to encode about 1000 transcripton factors each (Lander et al., 2001). Transcription factors and the co-activator proteins recruited by them introduce another level of regulation in transcription by integrating multiple signalling pathways that govern transcriptional programmes. Such signals may be derived from the environment, the cell cycle and/or tissue specific pathways which in turn activate many transcription factors whose combined effect influences the transcriptional status of particular gene loci.

In summary, biological complexity is associated with the enhanced complexity of transcriptional mechanisms. Contributing to the complexity of these mechanisms are increased numbers of DNA regulatory sequences with the concomitant increase in DNA binding transcription factors as well as co-factors encoded by the expanding genomes of higher order organisms. Elucidating these transcription mechanisms is required to understand how the cellular environment manipulates biological processes, via the transcription of specific genes.

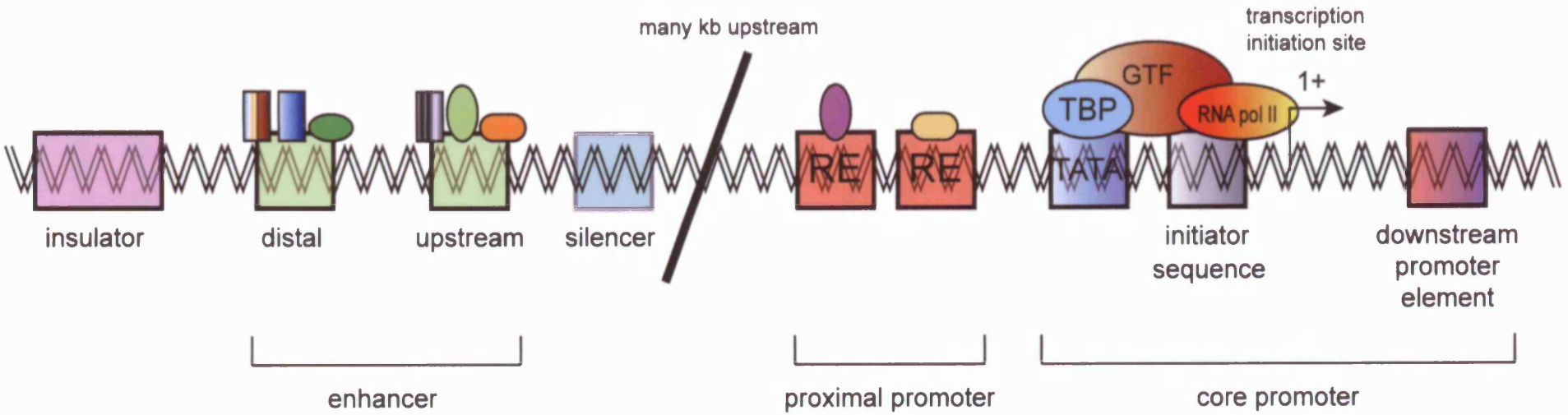
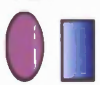


Fig 1.1 DNA regulatory sequences involved in Metazoan transcription

Diverstity of DNA regulatory sequences increases with high order organisms. Comparing between a simple eukaryote, such as yeast, and a metazoan, only the silencer region and TATA box are common between the two organisms.

RE - response element
GTF - general transcription factors
TBP - TATA binding protein

 - transcription factors

1.2 Transcription factors and co-factor complexes

The activation of gene specific transcription, as opposed to basal transcription, requires the binding of sequence specific transcription factors and the recruitment of protein complexes which aid transcription. These complexes have been termed co-activators or co-repressors according to their transcriptional activity. Transcription factors tether co-factor complexes to DNA, which regulate transcription by various mechanisms. Transcription co-factor complexes are broadly classed according to their mechanism of transcriptional regulation. These involve remodelling/modifying chromatin structure or the modulation of basal transcription machinery activity.

1.3 Chromatin modification in transcription regulation

The interaction of DNA with transcription factors and co-factors is affected by its higher order structure within the cell. DNA is packaged within the cell as chromatin. The core units of chromatin are nucleosomes, each one consisting of 146 base pairs of DNA wrapped around a protein octamer scaffold, containing two molecules each of histone H2A, H2B, H3 and H4 (Ehrenhofer-Murray, 2004). The stacking of these nucleosomes allows the formation of chromatin. Nucleosomes themselves are progressively packaged to form higher order structures known as euchromatin and heterochromatin, which reflect DNA in either a transcriptional permissive or inhibited state, respectively. Both chromatin modification and remodelling contribute to the regulation of transcription by altering chromatin structure.

Chromatin modification refers to the post-translation modification of histones which undergo acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation. This extensive repertoire of modifications may in fact directly affect transcriptional activity through the recruitment of specific activators to modified histones, in what is known as the histone code. These modifications may also be implicated in other processes of DNA metabolism such as its replication and repair. Of the modifications, acetylation is the most studied and is generally associated with transcriptional activation. These reversible reactions are catalysed by histone acetyl transferases (HATs). Gcn5 was the first HAT to be identified in yeast, that was associated with transcriptional activation (Brownell et al., 1996). The identification of HATs in mammals, such as CREB-binding protein (CREB) and p300, was through their association with transcriptional activators. Other mammalian HATs have been isolated in complexes with homology to the yeast HAT complex Gnc5p. Two mammalian HAT complexes, containing P/CAF and hGCN5, have been identified and contain subunits homologous to yeast Gnc5p in addition to other novel proteins suggesting that histone acetylation is a well conserved mechanism of transcription regulation from yeast to humans.

Mechanistically, acetylation of lysine residues on the histones reduces the positive charge of the amino group of lysine and alters its interaction with the negatively charged DNA, preventing the formation of heterochromatin. HATs can exhibit specificity to particular histones and even particular lysine residues within their N-termini. For example, yeast Gcn5p preferentially acetylates H4, whereas

human counterparts P/CAF and hGNC5 acetylate H3 (Kuo et al., 1996). Specificity of lysine residue acetylation can also be altered by HAT associated proteins, as demonstrated by yeast Gcn5 when associated with either the Spt-Ada-Gcn5 acetyltransferases (SAGA) or adaptor (ADA) co-activator complexes, both of which can extend the set of lysine residues acetylated on histone H3 by Gcn5 (Grant et al., 1999). HAT activity may also be directed by the recruitment of transcription factors to particular acetylated histones, as proposed by the histone code. Acetylated histones may serve to recruit bromodomain containing proteins such as P/CAF whose bromodomain interacts specifically with an acetylated lysine in a histone H4 peptide (Dhalluin et al., 1999;Kanno et al., 2004). Similarly the two tandem bromodomains of TAF(II)250, a subunit of the TFIID complex, bind with high affinity to two acetylated lysines of histone H4 (Jacobson et al., 2000). In a model of acetylated histone directed transcription, activators may recruit HATs to chromatin to acetylate histone H4, thus facilitating the binding of TAF(II)250. This interaction may serve to recruit other members of the basal transcription machinery to the core promoter. In such a model, co-operation between HATs and transcription factors serves to enhance the relaxing of chromatin and hence facilitate the activation of transcription.

A novel mechanism of transcription regulation has recently been described involving the acetylation of transcription factors by HATs. P300 can acetylate p53 at C-terminus lysine residues to enhance its sequence specific binding to DNA (Luo et al., 2004). The acetylation of nuclear receptors such as androgen receptor, oestrogen receptor α and the peroxisome proliferation-

activated gamma receptor (PPARγ) to alter their sensitivity to ligands and their promoter specificity has also been reported (Fu et al., 2004b). These effects of acetylation on transcription factors implicate HATs activity as having a direct effect on sequence specific transcription factors. These additional effects of acetylation suggest that HATs are generally associated with the activation of transcription which targets transcription at both the level of the chromatin as well as transcription factors, where its activity may more subtly regulate transcription.

Counteracting the activity of the HATs are two classes of histone deacetylases (HDAC) which catalyse the deacetylation of histones to repress transcription. Classification of the HDACs is also based on their similarity to the yeast histone deacetylases with class I HDACs resembling yeast Rpd3 whereas class II HDACs are similar to yeast HAD1. Mammalian class I HDACs consist of HDAC1,2 ,3 and 8, while class II include HDAC 4,5,6,7,9 and 10 (de Ruijter et al., 2003). HDACs require other co-factors to function. These HDAC/co-factor complexes usually consist of proteins that modulate the activity of the HDAC as well as DNA binding proteins which target the HDACs to specific gene promoters. Examples of such complexes are the Sin3, SMRT (silencing Mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor). The Sin3 complex contains mSin3A, HDAC1 and 2. SMRT and N-CoR are distinct complexes but are highly related, both utilising HDAC3 to repress transcription mediated by nuclear receptors (Guenther et al., 2001).

In addition to the classical family, the second family of histone deacetylases include the Sirtuins, which are homologous to the yeast Sir2 protein

(Silent Information Regulator 2). Sir2 catalytic activity requires NAD+ as a co-factor, which links its activity to metabolic pathways (Blander and Guarente, 2004). The Sirtuins include 7 family members and are associated with transcriptional repression of p53 as well as the forkhead transcription factors (Vaziri et al., 2001;Brunet et al., 2004).

The recruitment of HDACs to sites of transcription repression is mediated either by DNA bound transcription factors or methylated DNA. In the later mechanism, methylation of DNA at cytosine residues located 5' to guanosines, termed CpG islands facilitates recruitment of HDAC complexes via methylated-CpG binding proteins to mediate long term transcriptional repression, known as epigenetic silencing. Once recruited, HDAC enzymes repress transcription by removing acetyl groups from lysine residues of histones, enhancing its interaction with DNA thus occluding transcription factor binding. This negative regulatory activity of HDACs complements the transcriptional activator properties of HATs to ensure gene transcription is appropriately activated.

1.4 Chromatin remodelling in the regulation of transcription

Though presented as distinct form of transcriptional regulation, chromatin modification functions alongside chromatin remodelling. Together, these processes alter chromatin architecture and to regulate transcription. Chromatin remodelling differs from its modification as there is no addition of biochemical groups to the histone proteins. Instead nucleosome positions may be altered at a specific gene promoter (known as "sliding") or remodelling of the nucleosome

may occur through altering of DNA-histone interactions (Becker and Horz, 2002). Chromatin remodelling also includes the process of histone eviction, the removal histones, and histone exhange (Boeger et al., 2003;Mizuguchi et al., 2004).

The processes of chromatin remodelling are catalysed by complexes containing an ATPase subunit required for ATP hydroysis, which is required to loosen DNA-histone interactions. The initial discovery in yeast of ATP-dependent chromatin remodelling complexes was that of the switching and sucrose non-fermenting (Swi/Snf) complex containing the Swi2/Snf2 ATPase (Cairns et al., 1994;Cote et al., 1994). Related complexes were also discovered in humans. hBRM/hSnf2a and BRG1/hSnf2b are homologs of Brahma (Brm) a *Drosophila* protein with homology to Swi2/Snf2. hBRM and BRG1 complexes contain proteins homologous to Swi/Snf but also contain novel proteins unique to these complexes (Wang et al., 1996a;Wang et al., 1996b;Papoulas et al., 1998). In addition to Swi/Snf related complexes, there is a second family of ATP-dependent nucleosome remodelling complexes, homologous to the imitation switch (ISWI) ATPase of *Drosophila*. One of the members of this family is the nucleosome remodelling factor (NURF) which was found to enhance GAGA transcription factor binding to DNA to activate transcription (Tsukiyama and Wu, 1995;Tsukiyama et al., 1995).

Chromatin remodelling complexes act as indirect activators by facilitating the interaction of transcription factors with DNA. Their activity is specific to particular genes, for example, the recruitment of Swi/Snf related complexes and activation of transcription by nuclear receptors occurs upon ligand binding to the

receptors (Yoshinaga et al., 1992;Muchardt and Yaniv, 1993;Ichinose et al., 1997;Wallberg et al., 2000). Similarly, the NURF complex is associated with transcriptional activation by the GAGA and Gal4 transcription factors, though no interaction between these transcription factors and NURF has been reported (Kang et al., 2002a).

The activity of the chromatin remodelling complexes is also coupled with chromatin modification. The Swi/Snf complex is recruited to specific acetylated histone lysine residues in a manner dependent on the bromodomains of Swi2/Snf2 and Gcn5 (Syntichaki et al., 2000;Hassan et al., 2002;Agalioti et al., 2002). This implies a synergy between histone acetylation and chromatin remodelling in transcription activation, as seen with both Swi/Snf and ISWI complexes, whose activities require prior histone acetylation (Agalioti et al., 2000;Hassan et al., 2001;Mizuguchi et al., 2001;Reinke et al., 2001).

Conversely, transcriptional repression can also be mediated by the chromatin remodelling complexes. BRG-1 is essential in transcriptional repression mediated by the retinoblastoma protein and its expression is absent in some tumour cell lines, suggesting a tumour suppressor role for this protein (Strobeck et al., 2000). Mechanisms for BRG-1 and BRM mediated repression have not yet been elucidated.

The modification and remodelling of chromatin represents an important initial phase in the regulation of transcription. Chromatin that is permissive to transcription is subject to other forms of transcriptional regulation by the

transcription co-factors, which will be discussed in the next section of this introduction.

1.5 Transcription co-factors associated with the modulation of the basal transcription machinery

Co-factors associated with the basal transcription machinery facilitate transcription by promoting assembly of the GTFs with RNA pol as well as regulating the transcriptional activity of the basal machinery. Such co-factors include TFIID, USA and Mediator, which have all been identified as macromolecular complexes and will be discussed further.

The TFIID co-activator complex

One of the first co-activator complexes to be identified with the basal transcription machinery was TFIID. TFIID is itself a member of the basal transcription machinery and is absolutely required for specific gene transcription by RNA pol II, along with TFIIB and TFIIE, in an *in vitro* system (Sawadogo and Roeder, 1985). These results suggested that transcription activators may function to recruit TFIID to the core promoter where it binds the TATA box to further recruit the GTFs to the transcription pre-initiation complex (Horikoshi et al., 1988;Hai et al., 1988). The interaction between TFIID and the TATA box was found to be dependent on another protein, TATA-binding protein (TBP) which was able to induce basal transcription from TATA containing promoters (Horikoshi et al., 1989).

Further, purification of TFIID by way of TBP revealed other associated proteins known as TBP-associated factors (TAF) (Dynlacht et al., 1991). TAFs may have a role directing transcription from specific promoters as indicated by the interactions of several TAFs with the transcription factors p53 and VP-16 (Goodrich et al., 1993;Lu and Levine, 1995;Thut et al., 1995). Mutational studies of TAF homologs in yeast and *Drosophila* further suggest their role in gene specific transcription due to defects in transcription of certain subsets of genes (Green, 2000;Chen and Hampsey, 2002). TBP can activate transcription from RNA polymerases, though its specificity for activating RNA pol II may be dictated by different complexes of TAFs (Cormack and Struhl, 1992;Chiang et al., 1993). These activities of the TFIID complex suggest that the GTFs may have an intrinsic gene specific transcription activator property without the recruitment of additional co-activator proteins.

Upstream Stimulatory Activity

Upstream stimulatory activity (USA) was isolated from fractions of mammalian nuclear extracts and comprised of proteins that could enhance transcription by activators as well as induce repression (Meisterernst et al., 1991). Independent proteins from the USA fraction were identified. Among them was positive co-factor 4 (PC4), a general co-activator of transcription, which was shown to enhance transcription by interacting with the activator domain of VP-16 and TFIIA to promote recruitment and stable binding of TFIID to the TATA box (Ge and Roeder, 1994;Kretzschmar et al., 1994;Kaiser et al., 1995). PC4

transcriptional activation can be inhibited by the prior binding of TFIID suggesting that PC4 functions in the early stages of transcription initiation. Further study revealed a repressor function for PC4 in a minimal pre-initiation complex, however upon recruitment of TFIIH and TAF(II)250 to the complex, PC4 was phosphorylated by these kinases and released to permit transcription (Malik et al., 1998).

Negative co-factor (NC), also identified in USA fractions, demonstrated effects on the GTFs to negatively regulate basal transcription. Both NC1 and NC2 compete with TFIIA and TFIIB respectively for binding to TBP to prevent initiation of transcription (Meisterernst and Roeder, 1991;Goppelt et al., 1996;Kamada et al., 2001). Overall the USA fraction contains both positive and negative co-factor elements whose recruitment to and activity on the basal transcription machinery may be guided by specific GTF and TAF proteins.

Mediator complex

The presence of Mediator complexes was suggested by studies of the phenomenon of activator interference, the ability of one activator to inhibit transcriptional activation by another, *in vivo*. Initially, activator interference was attributed to the sequestration factors such as GTFs and RNA pol II by the competing activator. However interference persisted even when these factors were provided in excess but were relieved when a crude yeast fraction was added (Kelleher, III et al., 1990;Flanagan et al., 1991). This fraction was termed

Mediator and was hypothesised to contain factors that bridged activator proteins with the basal transcription machinery.

The Mediator complex was eventually isolated in complex with the RNA pol II holoenzyme and was originally thought to activate transcription through TFIIH mediated phosphorylation of RNA pol II CTD (Thompson et al., 1993;Kim et al., 1994). Subunits of the complex: suppressor of RNA pol B (SRB) 2, SRB4. SRB5 and SRB6 had been previously identified in genetic screens for extragenic suppressors of RNA pol II CTD truncations. Cells lacking these SRBs have phenotypes similar to that of cells with a deletion of a large portion of the CTD (Koleske et al., 1992). The discovery of these subunits in a complex with RNA pol II further linked Mediator to the regulation of its CTD.

The mechanism of Mediator activation of RNA pol II dependent transcription is still largely unknown. Although Mediator has been shown to interact with RNA pol II, recruitment of RNA pol II to the transcription machinery may be just one mechanism employed. Mediator associates with only 2% of the RNA pol II in yeast suggesting that it functions at a minority of RNA pol II regulated genes or perhaps that its interaction is transitory. A "Mediator cycle" has been hypothesised whereby Mediator recruits RNA pol II to the transcription initiation site and once phosphorylated by TFIIH, Mediator is released to initiate another round of transcription (Thompson *et al.*, 1993).

The importance of Mediator in transcription is illustrated by mutational studies in yeast of the genes encoding proteins identified in the complex. Many of these genes are essential for viability, an example being *SRB4*, whose

expression is essential for global RNA pol II activity. Studies in a SRB4 negative background however revealed that transcription could be restored with the expression of other Mediator subunits fused to a DNA binding domain (DBD). Transcription could not be restored by similar DBD fusions to SRB5 and SRB6, which were shown to interact with SRB4 (Lee et al., 1999). These studies support a model of recruitment or tethering of the Mediator complex to DNA thus allowing the complex to promote transcription.

Mediator subunits are also associated with negative regulation of transcription. Identified as recessive suppressors of CTD truncation phenotypes, negative regulators SRB8, SRB9, SRB10 and SRB11 have also been isolated as subunits of Mediator (Song et al., 1996;Carlson, 1997). SRB10/cdk8 and SRB11/cyclin C form an active kinase able to phosphorylate the CTD of RNA pol II prior to formation of the transcription initiation complex which inhibits its transcriptional activity (Hengartner et al., 1998). Additionally, Mediator/cyclin C/cdk8 may function as a repressor by phosphorylating the cyclin H component of cyclin H/cdk7, the kinase present in TFIIH, to inhibit transcription activation (Akoulitchev et al., 2000).

The similarity of phenotypes obtained in genetic screens between Mediator subunits has led to the hypothesis that Mediator is composed of subcomplexes which are further divided in to modules and submodules. By analysing the interactions of the Mediator proteins and basal transcription factors, yeast Mediator has been divided into 2 sub-complexes of Rgr1 and SRB4. The Rgr1 subcomplex is further divided into Gal11 and Med9/10 modules as well as

SRB10/11 submodule (Kang et al., 2001). This organisation of Mediator may regulate its transcriptional specificity and activity, through sub-complexes/modules acting individually or in co-operation to regulate RNA pol II activity.

The mammalian Mediator complexes have been identified by various methods which include, co-purification with yeast Mediator homologues and ligand bound nuclear receptors as well as direct purification of the complexes from fractions of nuclear extracts. These Mediator complexes include the Srb/Med co-activator complex (SMCC), thyroid receptor associated protein (TRAP) complex, PC2, vitamin D receptor interacting protein (DRIP) and co-activator required for Sp-1 (CRSP) and have been shown activate transcription *in vitro* in systems consisting of sequence specific activators, USA and crude nuclear fractions containing the GTFs and RNA pol II. Transcriptional repression properties have also been described for the Mediator complexes. Repressing such complexes include negative regulator of activated transcription (NAT) and SMCC (Sun et al., 1998). Though identified independently of one another, all these complexes contain protein subunits conserved between the yeast and mammalian Mediator complexes, though some complexes appear to have subunits specific to that complex.

Despite the addition of other subunits in the mammalian Mediator complexes, the overall structural conformation of the complex remains conserved between yeast and mammalian cells as seen by analysis of yeast, murine and the human CRSP and TRAP complexes by electron microscopy and 3

dimensional reconstruction (Asturias et al., 1999;Dotson et al., 2000;Taatjes et al., 2002). This basic structure consists of three modules, broadly described as a head, middle and tail, which form a pocket with which RNA pol II interacts. Further studies have revealed that Mediator conformation may change dependent on specific interacting transcriptional activators, as seen for CRSP (Taatjes et al., 2004). A conformational change may relate to its interaction with RNA pol II in the transcriptional pre-initiation complex and more subtly regulate transcriptional activation.

1.7 Transcriptional regulation by nuclear receptor

Mediator activates transcription in a multi-step process involving multiple transcriptional co-factors. Studies of the mammalian Mediator complex, TRAP have established a model for transcriptional activation by the thyroid hormone receptors (TR), a nuclear receptor transcription factor interacting with the TRAP complex (Ito and Roeder, 2001). In the absence of the stimulating ligand, the thyroid hormone receptor α exists in a heterodimer with the retinoic acid X receptor (RXR). This heterodimer interacts with the thyroid hormone response element and actively represses transcription by recruiting the co-repressor complex, SMRT. SMRT mediates repression through the recruitment of HDACs (Chen et al., 1996). Upon ligand binding, repression is relieved by the rapid recruitment of p160/SRC (steroid receptor co-activator) proteins, such as SRC-1, TIF2 and RAC3. The intrinsic HAT activity of some of the p160/SRC proteins, as well as their recruitment of HATs, CBP and pCAF, facilitate histone acetylation

and the relaxing of chromatin structure to allowing binding of TRAP to the liganded receptor, and ultimately transcription activation (Sharma and Fondell, 2000;Sharma and Fondell, 2002) (Fig 1.2).

The recruitment of both p160/SRC member proteins and TRAP to the liganded receptor is aided by interactions between specific protein motifs present on these proteins as well as the nuclear receptors. On the nuclear receptors, ligand dependent activation domains (AF-2) of the receptors mediate interaction with LXXLL motifs present on the p160/SRC member proteins. Recruitment of the TRAP complex is also facilitated by a two LXXLL motifs present on subunit TRAP220. In the absence of TRAP220, the TRAP complex can not activate transcription by the liganded receptor (Ito et al., 2000). TRAP220 also interacts with other nuclear receptors in a ligand dependent manner, such as the androgen, oestrogen and peroxisome proliferation-activated receptors, to activate transcription (Kodera et al., 2000;Wang et al., 2002;Kang et al., 2002b). These findings suggest TRAP220 is required for transcription activation by a number of nuclear receptor transcription activators.

This model of nuclear receptor activated transcription includes a chromatin remodelling phase during activation, as has been shown for TRβ by chromatin assembly and transcription assays *in vitro* (Lee et al., 2003). Further, oestrogen and androgen receptors also interact with chromatin remodelling complexes, via the BRG-1 protein of the Swi/Snf complex, to enhance transcription by these steroid hormone receptors (DiRenzo et al., 2000;Marshall et al., 2003). It is therefore suggested that Mediator functions in a multistep process to activate

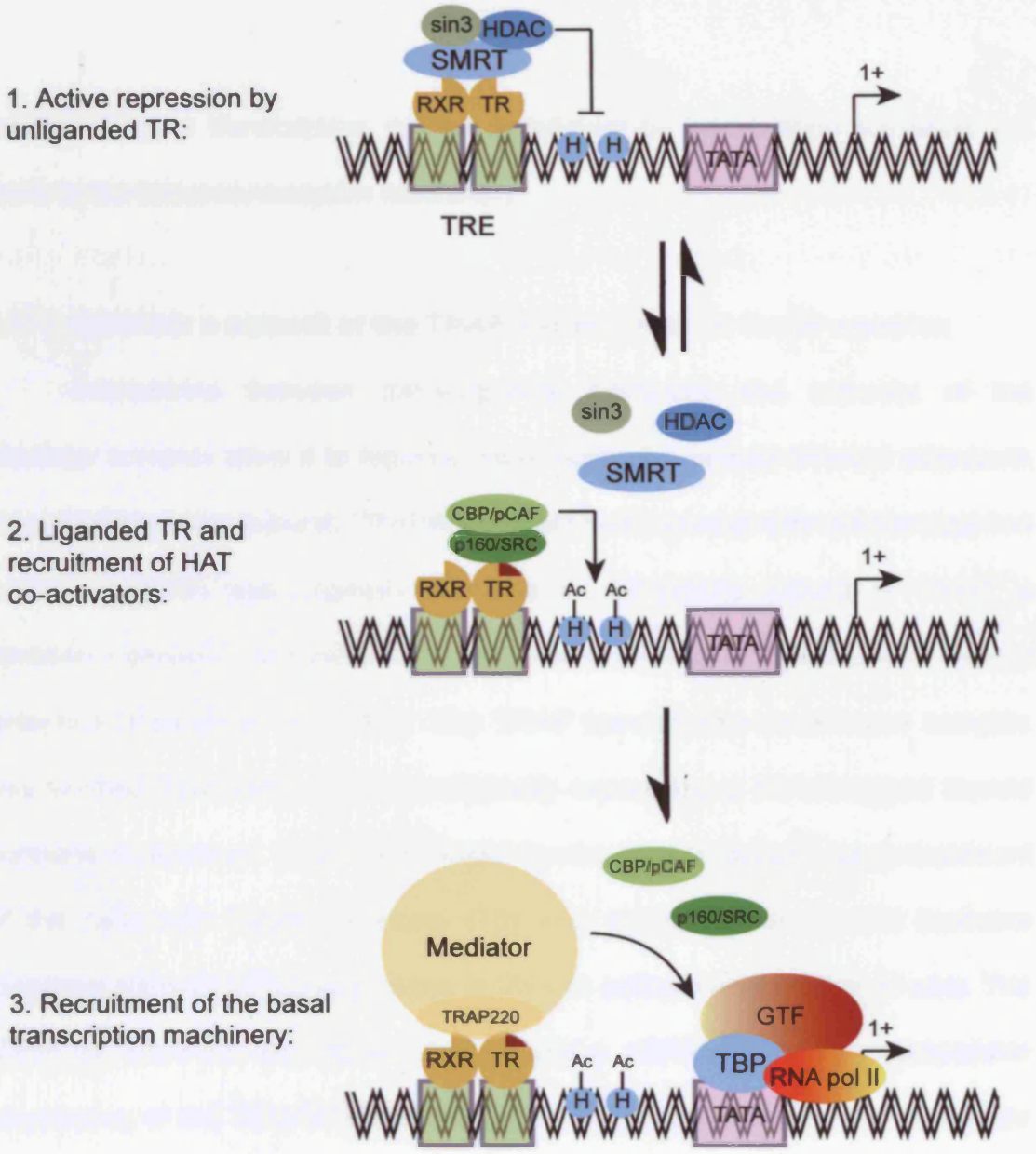


Fig 1.2 Multistep model for the activation of transcription by the thyroid hormone receptor

TR - thyroid hormone receptor
RXR - retinoic X receptor
TRE - thyroid hormone response element
SRC - steroid receptor co-activator
SMRT - silencing mediator for retinoic and thyroid hormone receptors
GTF - general transcription factors
Ac - acetylation
H - histone
1+ - transcription intiation site

nuclear receptor transcription, whose recruitment by transcription activators link them to the basal transcription machinery.

1.7 TRAP80: a subunit of the TRAP transcription co-factor complex

Interactions between transcriptional activators and subunits of the Mediator complex allow it to regulate transcription by several different activators. One such Mediator subunit, TRAP80, interacts with several different transcription factors. TRAP80 was originally identified as an 80Kda subunit of TRAP, a Mediator complex involved in transcriptional activation of ligand bound thyroid receptors (Fondell et al., 1996). The TRAP transcription co-activator complex was purified from HeLa cells constitutively expressing a FLAG-tagged thyroid hormone receptor α1. TRAP is recruited by the thyroid receptor upon treatment of the cells with thyroid hormone (T3) and assembled on thyroid hormone response element (TRE) sequences in DNA to activate transcription *in vitro*. The cDNA of TRAP80 was cloned from a HeLa cDNA library by micropeptide sequencing of the TRAP80 protein using PCR primers based on the nucleotide sequence of these peptides (Ito et al., 1999). PCR products were then sequenced and BLAST searches used to obtain matching human cDNA clones. TRAP80 cDNA was initially predicted to encode a protein of 717 amino acids which contained leucine zipper motif, a region which can mediate protein-DNA and/or protein-protein interactions. The original sequence for TRAP80 was later replaced by a newer sequence that encoded a protein of 651 amino acids but contained all the protein motifs as previously described. This study also revealed

the presence of the TRAP80 protein in another transcription co-factor module, SMCC. SMCC was identified by similar methods to the TRAP complex. Tagged yeast Mediator protein FLAG-srb10, a human homologue of cdk8 (cyclin dependent kinase 8) was used to immunopurify SMCC which contained TRAP subunits TRAP220, TRAP170 and TRAP100 but not TRAP80 (Gu et al., 1999). TRAP80 polypeptides and other TRAP subunits were however identified by mass spectrometry of SMCC and TRAP, which demonstrated the shared subunits of the two complexes (Ito *et al.*, 1999).

1.8 TRAP80 and associated human Mediator complexes

Proteins of similar size to TRAP80 have also been identified as a component of related mammalian transcriptional co-activator complexes, DRIP (vitamin D receptor interacting protein) and CRSP (co-factor required for SP-1 activation) (Rachez et al., 1998;Ryu et al., 1999). The CRSP co-activator complex is a co-factor involved in Specificity protein-1 (Sp-1) transcriptional activation from which CRSP6 (also known as CRSP77) was independently cloned. These transcription co-factor complexes and others often share common subunits though nomenclature for these subunits differs between the complexes. Recently, a unified nomenclature for the subunits of the Mediator, the transcriptional co-activator complex has been adopted, in which TRAP80/CRSP6/DRIP80 has been named MED17 and will be adopted hereafter (Bourbon et al., 2004).

1.8 Conservation of the MED17 gene between species

Homologues of human and yeast Mediator subunits have been identified in *Drosophila* by genomic searches for conserved genes. This has also been aided by the identification of *Drosophila* Mediator complex by affinity purification via dMED6, one of the Mediator subunits conserved between yeast and human (Park et al., 2001a). dMED17 was subsequently identified as a homologue of MED17 through searches of the *Drosophila* expressed sequence tag and genomic databases and was confirmed as a subunit of the *Drosophila* Mediator complex by Western blot and mass spectrometry (Park et al., 2001a). Evolutionarily closer to human Mediator, the mouse Mediator complex also contains a subunit, p78, that is equivalent to MED17 (Jiang et al., 1998;Ito *et al.*, 1999). As shown in Table 1.1, MED17 homologs from other species, especially mammalian, show a high degree of conservation of amino acid sequence between species (see Appendix Fig 7.1 for amino acid alignments). This high degree of homology, is also seen with other Mediator subunits, and suggests that the transcriptional functions of Mediator are conserved among species.

Species	Name	Genbank Accession number	Identity %	Similarity %
Homo sapien	TRAP80	AF117657	-	-
Homo sapien	Crsp6	NM_004268	100	100
Homo sapien	DRIP80	AF105421	98	99
Mus musculus	Crsp6	NM_144933	96	98
Rattus norvegicus	Similar to crsp6	XM_217086	96	98
Gallus gallus	Crsp6	NM_00106280	90	94
Drosophila melanogaster	TRAP80	AF289995	41	62
Apis mellifera	Similar to ENGSANGP 00000021505	XM_394516	41	60
Anopheles gambiae	ENSANGP 000000021505	XM_319901	39	59
Schizosacchromyces pombe	SpSrb4	CAB10081	9	25
Sacchromyces cerevisiae	Srb4	L12026	10	26
Caenorhabditis elegan	Y113G7B.18	CAB76740	8	23

Table 1.1 Species homologues of human MED17

Analysis was done of MED17 homologues from various species for similarity and identity to the human TRAP80 protein using the Clustral W programme. Included in this analysis are homologs from other human Mediator complexes in addition to yeast and worm homologues identified by Boube *et al.*, 2000.

1.9 MED17 interactions and Mediator function in mammalian systems

The study of Mediator function in transcription has predominately been investigated through the use of *in vitro* reconstituted transcription systems supplemented with purified co-factors. Initial characterisation of the transcriptional activating properties of Mediator on Gal4-p53 were done using *in vitro* transcription assays with purified GTFs and PC4 co-activator, but limiting TFIIH (Gu *et al.*, 1999). Importantly, in similar transcription assays, transcriptional repression properties were also seen with Gal4 activated transcription in conditions where TFIIH was not limited. Repression was specific to PC4 activated transcription but was not dependent on the presence of the pol II CTD. A potential mechanism for repression of PC4 activated transcription by SMCC was suggested by kinase assays which showed SMCC was able to phosphorylate PC4, which was thought to inactivate its transcriptional activity. The interaction of SMCC with wild type p53 and not a transactivation mutant was also shown, implying that transcription activation of p53 by SMCC was dependent on this interaction with p53 transactivation domain. Overall, the study showed that the mechanisms of transcriptional activation and repression by SMCC were independent of RNA pol II CTD and its phosphorylation by TFIIH, indicating alternative mechanisms of SMCC regulated transcription.

Many Mediator subunits are conserved between the various Mediator complexes. Investigation of the TRAP and SMCC complexes revealed a high degree of similarity in their subunit composition as well as their interactions with DNA bound transcription factors. These conserved subunits include MED17.

Both TRAP and SMCC activated transcription mediated by the thyroid hormone receptor from TRE and by Gal4 fusions to p53, VP-16 and AH (Gal4 activator) *in vitro* from a GAL promoter element (Ito *et al.*, 1999). Furthermore, TRAP80 and TRAP220 were shown to interact with p53 *in vitro*. A wild type p53 fusion to GST was able to pull down MED17, however MED17 failed to interact with a GST-p53 point mutant at residues 22 and 23, suggesting the N-terminus of p53 mediates this interaction. This region of p53 contains its transactivation domain. The same mutation in p53 also eliminated its interaction with SMCC. Pull down assays also showed a direct interaction between MED17 and wild type VP-16, a herpesvirus transcription factor. This interaction was lost when the activation domain of VP-16 was deleted. These results suggest that the transcriptional activities of TRAP and SMCC on Gal4-p53 and Gal4-VP-16 are mediated, in part, by the direct interaction of the transactivation domains of these DNA bound transcription factors with the MED17 subunit. This interaction with the transactivation domain of transcription factors is also a feature of *Drosophila* Mediator/MED17 regulation of HSF transcription, as is described in the next section.

1.11 Functional characterisation of *Drosophila* MED17 (dMED17) and its role in heat shock factor regulated transcription

dMED17 was identified in both searches of *Drosophila* genome and by mass spectrometry of dMED6 co-purifying proteins. *Drosophila* Mediator is functionally equivalent to human Mediator complexes in both size and transcriptional activation with *Drosophila* Mediator also activating VP-16

transcription *in vitro*. In addition, specific interaction between dMED17 and VP-16 was shown in GST-pull down assays.

Further elucidation of the transcriptional targets of *Drosophila* Mediator revealed additional transcription factors that interact with the Mediator complex, including heat shock factor (HSF), dorsal, bicoid, Krüppel and Fushi-tarazu. Transcriptional activation by Dorsal and heat shock factor in soluble nuclear fractions was inhibited by depletion of Mediator with anti-dSOH1 (dMED31) antibody, showing that Mediator is required for transactivation via these transcription factors. A more direct role for dMED17 in the activation of heat shock transcription was suggested by *in vitro* binding assays with HSF. GST-HSF was able to pull down dMED17, dMED31 and CDK8 from a soluble nuclear extract derived from *Drosophila* embryos (Park *et al.*, 2001a). A direct interaction between *in vitro* translated dMED17 and GST-HSF was shown, with dMED17 specifically interacting with the C-terminus of HSF, containing its transactivation domain. This interaction did not occur with any other Mediator subunit tested, including dTRAP220 (dMED1) and dTRAP100 (dMED24). Furthermore, dMED17 co-localised with HSF at heat response elements upon stimulation with heat shock. This step was distinct from the recruitment of the RNA pol II holoenzyme to the HSP70 promoter. These interactions and localisation studies support the idea that Mediator, via the dMED17 subunit, is recruited to HSF/HSE sites where it activates transcription by phosphorylation of the CTD of RNA pol II using the cyclin C/cdk8 subunits (Park et al., 2001b). This central role for dMED17 suggested by artificial recruitment assays with a gene reporter containing a

region of the HSP70 promoter that was specifically transactivated by Gal4-MED17 but not by any of the other Gal4-Mediator subunit fusions tested (Park et al., 2003). Chromatin immunoprecipitation (ChIP) also showed enhanced occupancy of the HSP70 promoter by Mediator upon transfection of Gal4-MED17. The current hypothesis is that dMED17 activates heat shock factor regulated transcription through recruitment of the Mediator complex.

1.12 Other putative transcriptional targets of MED17

The potential of Mediator to activate a number of transcription factors has been hinted at by studies in *Drosophila*. Mediator was shown to interact with various transcription factors, whose transactivation domains activated transcription of a reporter gene when fused to Gal4. These transcription factors included VP-16 and HSF, which have previously been shown to specifically interact with the MED17 subunit of Mediator, but also included three others previously unknown Mediator interacting transcription factors from *Drosophila*, Dif, Armadillo and Notch. Of these newly identified transcription factors, dMED17 interacted directly with Dif, a homologue of the p65 subunit of NF-κB, in GST pull down assays whereas GST-Armadillo and Notch interacted with dMED17 in soluble nuclear fractions, but did not interact in the GST-pull down assays suggesting other proteins bridged the interaction with these transcription factors. Characterisation of Dif mediated transcription by MED17 showed that transcription of *drosomycin*, a Dif responsive gene was lost when dMED17

expression was eliminated by RNAi. This study suggests a role for MED17 in the activation of NF-κB transcription in mammalian cells.

The interactions of MED17 with this variety of transcription factors together with direct studies of the Mediator complex have led to the proposal of a MED17 module on the Mediator complex. Fractionation of the *Drosophila* Mediator from SL2 cell nuclear extracts by gel filtration has revealed Mediator exists in three different forms, denoted C1, C2 and C3, all of which contain MED17 (Gu et al., 2002). The largest form, C3 at 2 Mda, activates transcription *in vitro*. C2 is a smaller complex of 1.5 Mda and is thought to be a sub-complex of C3 generated by partial disruption of the complex during purification. The C1 complex at 0.5 Mda is the smallest and is a distinct module of Mediator which is unable to activate transcription even in a system containing highly purified general transcription factors and RNA pol II. Interestingly C3, but not C1, interacts with RNA pol II, possibly explaining the inability of C1 to activate transcription. The C1 and C3 forms do, however, interact with Armadillo, Dif, HSF and Notch. These results imply a co-activator role for C3 module and as yet an undetermined transcriptional function, but possibly a repressor function, for C1.

1.13 A role for MED17 in *Drosophila* embryogenesis

The interaction of dMED17 with such a variety of transcription factors, implicates it in the regulation of diverse transcriptional pathways, hence MED17 expression could be essential to *Drosophila* development. dMED17 as well as dMED13 were implicated in *Drosophila* embryogenesis through P-element

screen for genes affecting the function genes *Scr* and *Pb* homologs of Hox-A5/B5 and Hox-A2/B2 respectively (Boube et al., 2000). *Scr* and *Pb* are regulators of cell identity whose over-expression is associated with developmental defects. Both genes also co-operate in the specification of the adult mouthparts. Initially dMED13 was identified as a regulator of *Pb*. Mutation of the dMED13 locus (also known as *pap*) by p-insertion, phenocopies Pb over-expression suggesting Pap functions as a repressor of Pb expression or function. These studies also included an investigation on the effects of dMED13 on developmental abnormalities induced by *Scr* gain of function alleles, namely sex combs. Here, double deletion of *dMED13* and *dMED17* loci increased distal sex comb formation via enhanced *Scr* activity, thus demonstrating co-operation between the two Mediator proteins in the repression of *Scr* activity. However, these effects were not attributed to increased expression of the Scr protein but rather through a mechanism parallel or downstream of Scr activity (Boube *et al.*, 2000).

This same study also demonstrated the importance of dMED17 expression in *Drosophila* development. P-insertion mutation of *dMED17* locus resulted in larvae dying at the second-instar stage. A requirement of MED17 for cell viability was also demonstrated by clonal analysis whereby mitotic recombination producing *dMED17*$^{-/-}$ cells resulted in the loss of cell viability. These *Drosophila MED17* $^{-/-}$ phenotypes were attributed to global transcriptional failure. Similarly, expression of Srb4, the yeast MED17 homologue is also essential yeast viability due to its role in global transcription (Holstege et al.,

1998). More generally, a role for Mediator in *Drosophila* development has also been shown by analysis of Mediator gene transcripts by Nothern blots, with an increase in Mediator transcripts, including dMED17, seen with development from embryo to larvae to pupae stages (Park *et al.*, 2001a). These studies of MED17 suggest that MED17 has a global transcriptional regulatory function but also demonstrates selectivity in the regulation of specific transcription factors.

1.14 MED17: Summary

TRAP80, now known as MED17, is a subunit of Mediator complexes involved in both transcriptional activation and repression. Homologs of MED17 have been identified in the Mediator complexes of yeast and *Drosophila*. Functional studies of these homologs implicate the MED17 subunit as a global regulator of transcription. dMED17, the best studied of the homologs, appears to also to have a co-activator function specific to HSF transcription factor. Several other transcription factors have been demonstrated to interact with MED17 suggesting MED17 may also have further specific transcription regulatory functions. Notably the p53 tumour suppressor protein has been shown to interact with MED17 implicating it as a regulator of p53 mediated transcription. *In vitro* transcriptional studies of the Mediator complex show that it activates p53 dependent transcription, suggesting a co-activator function for MED17. The processes involved regulating p53 dependent transcription are discussed further in the next section of this introduction.

1.15 The p53 tumour suppressor protein

The transcription of genes that mediate cell cycle arrest and apoptosis is regulated, in part, by the activity of the p53. p53 was first isolated as a 53Kda protein interacting with the large T-antigen of simian virus-40 (SV-40), whose expression was enhanced in SV-40 transformed cells (Linzer and Levine, 1979;Lane and Crawford, 1979). These findings and the observation that p53 could co-operate with the Ras oncogene in the transformation of embryonal cells fitted with a oncogenic function for p53 (Parada et al., 1984;Eliyahu et al., 1984). However, sequence variability between different p53 clones revealed that a point mutation (A135V) allowed p53 to co-operation with Ras in transformation (Hinds et al., 1989). Wild type p53 was, in fact, shown to have a suppressing role in transformation mediated by Ras and E1A (Finlay et al., 1989). It was therefore hypothesised that wild-type p53 was possibly an 'anti-oncogene' but when mutated, contributed to tumourigenesis, as was frequently found in tumour samples (Hollstein et al., 1991;Levine et al., 1991). Early experiments revealed that p53 expression suppressed the growth of tumour cells, however the expression of p53 mutant alleles allowed unrestrained growth (Baker et al., 1990). In similar experiments, induction of apoptosis was observed in a murine myeloid leukaemia cell line upon expression of wild type, but not mutant, p53 (Yonish-Rouach et al., 1991). The discovery of these effects of p53 suggested that it was a regulator of cell cycle arrest and apoptosis.

Further characterisation of the p53 protein revealed transcriptional activation properties which were localised to the N-terminus of the protein (Fields

and Jang, 1990;O'Rourke et al., 1990). Target genes for transactivation by p53 were identified by the presence of sequence specific response elements in the promoter regions of genes (Kern et al., 1991;Zambetti et al., 1992). One of the first p53 regulated genes to be identified was muscle creatine kinase (MCK) whose transcription was activated via p53 response element identified within the gene's enhancer region (Weintraub et al., 1991). Additional p53 transcriptional targets have since been identified via these response elements and include such genes as *p21 Cip* and *Mdm2* (El Deiry et al., 1993;Juven et al., 1993). Moreover, mutatants of p53 similar to those found in tumours were found to inhibit transcriptional activation by the p53 tetramer, acting as dominant negatives to prevent its interaction with the response elements (Raycroft et al., 1990). Similarly, viral oncoprotein large T-antigen of SV-40, inihibited p53 transcriptional tetramerisation *in vitro* (Farmer et al., 1992). These data indicated that the wild type p53 protein was in fact a suppressor of proliferation whose normal transcription function could altered by mutation or by interaction with viral oncoproteins during the process of tumourigenesis.

In addition to the transcriptional activating properties, repression by p53 has also been observed in the absence of p53 response elements. Genes such as *c-fos*, *interleukin-6* and *bcl2*, are mediators of cell growth and cell survival whose transcription is repressed by p53. Both the activator and repressor functions of p53 contribute in the induction of apoptosis by enhancing transcription of pro-apoptotic genes while simultaneously inhibiting transcription of pro-survival genes. The role of p53 in the induction of apoptosis was observed

in experiments with p53 null mice whose thymocytes showed increased resistance to some apoptotic stimuli (Lowe et al., 1993;Clarke et al., 1993). Furthermore, p53 null mice were found to be more susceptible to spontaneous and carcinogen-induced tumours, which is also observed in mice haploinsufficient for wild type p53 allele (Harvey et al., 1993). The importance of p53 in the development of human tumours is demonstrated by the cancer susceptibility syndrome, Li-Fraumeni, where the spontaneous development of a range of tumours has been attributed to inactivating mutations of the p53 locus (Malkin et al., 1990).

Outside Li-Fraumeni syndrome, p53 is also important in the development of sporadic tumours. Over 50% of all tumours have been found to harbour mutations of the p53 locus, making it one of the most frequently mutated genes in cancer and represents a key suppressor of oncogenic transformation in both murine and human cells (Rangarajan et al., 2004).

1.16 The p53 response and its regulation

The p53 gene is conserved in human, mouse, *Drosophila* and *C.elegans* genomes, illustrating its importance as a regulator of cell proliferation (Jin et al., 2000;Schumacher et al., 2001). P53 is responsive to cellular insults such as DNA damage, hypoxia or oncogene expression which can induce p53 and activate its transcriptional properties to trigger cell cycle checkpoints, DNA repair, cellular senescence or apoptosis. The loss of p53 function results in the deregulation of these processes leading to genomic instability and the

inappropriate survival of genetically damaged cells, thus contributing to oncogenesis. The p53 transcription factor receives a multitude of signalling stimuli to co-ordinate a cell response to a variety of stress responses. How p53 activity is regulated by these stimuli is not fully understood, but determinants such as cell type, nature and strength of the p53 activating stimulus and its levels of expression are thought to have an impact on the p53 transcriptional response (Fridman and Lowe, 2003).

Many of the activities of p53 can be allocated to protein domains which span the N-terminus, the central core and the C-terminus of the protein. The N-terminus contains a transcriptional transactivation domain and an SH3 domain, which mediates interactions with various transcriptional regulators, as well as mouse double minute (Mdm2), an E3 ubiquitin ligase which catalyses the addition of ubiquitin. The central core contains the DNA binding domain which permits the sequence specific interaction of p53 with response elements. The C-terminal domain of p53 contains nuclear localisation and nuclear export signals, as well as a tetramerisation domain, essential for the transcriptional activity of p53. This tetramerisation domain can be mutated in Li-Fraumeni's syndrome (Varley, 2003). This differs in sporadic tumours where mutations of the p53 locus most frequently involve the central DNA binding region of the protein, some of the most common being mutations of amino acid residues 175, 248 and 273 (Levine et al., 1991).

Together these domains of p53 regulate the transcriptional activity of the protein by affecting its interaction with transcription co-factors as well as

localisation to target genes. Another form of p53 regulation mediated by these protein domains involves its post-translational modification by a number of different processes which are further discussed in the next section.

1.17 Post-translational modification of p53 and their effects on transcriptional activity

Post-translational modifications of p53 alter its expression and localisation of p53 as well as directly affecting its transcription activity. Of these modifications, acetylation and phosphorylation have been extensively studied and both are associated with stabilisation of p53 expression. Upon cell stress, acetylation of p53 occurs at lysine residues its N and C-terminus and is catalysed by HATs, such as p300, CBP and PCAF as well as more recently described, the Hepatitis C core protein, implicating viral proteins in the acetylation of p53 (Liu et al., 1999;Wang et al., 2003c). In addition to the stabilisation of p53, acetylation is also thought to enhance the sequence specific binding of p53 to its response elements (Luo et al., 2004). Other proposed effects of acetylation involve competitive inhibition of Mdm2 by HATs. Mdm2 catalyses ubiquitylation of p53, targeting it for degradation via the 26S proteasome to prevent its inappropriate activation by maintaining expression of the protein at low levels. Both acetylation and ubiquitylation occur on the same lysine residues in the p53 protein. Acetylation may stabilise p53 expression, preventing Mdm2 mediated ubiquitylation of p53 (Li et al., 2002). Such a mechanism for p53 regulation

suggests how the diverse post-translational modification made to p53 may co-operate in regulating the transcriptional activity of the protein.

As acetylation is a reversible post-translational modification, unsurprisingly, histone deacetyalases have been identified in the deacetylation of the p53 protein as well as being associated with transcriptional repression mediated by the protein. The direct association between HDACs and p53 deacetylation was shown when HDAC-1/2/3 were found to deacetylate p53 to specifically repress p53-dependent activation of a p53 responsive gene reporter (Juan et al., 2000). Transcriptional repression by p53 is associated with a complex of mSin3A, HDAC-1 and p53 which represses transcription of the *MAP4* and *stathmin* genes. This repressor complex formed under hypoxic conditions, suggesting that the complex was specific to certain cell stress conditions (Murphy et al., 1999;Koumenis et al., 2001). HDACs have also been identified with other p53 interacting proteins known to negatively regulate its transcription. Examples include the oncogenic transcription factor, PML-RAR, and Mdm2 (Ito et al., 2002;Insinga et al., 2004). In addition to the "classical" family of HDACs, the Sir2 family of histone deacetylases also repress p53 mediated apoptosis, coupling cell metabolism with apoptosis (Luo et al., 2001).

Like acetylation, phosphorylation is generally associated with the activation of p53. Various kinases involved in the p53 signalling pathway, cell cycle regulation as well as cell signal transduction are able to phosphorylate p53 at particularly serine and threonine residues located in the N and C-terminus of the protein, though some phosphorylated residues have also been identified in its

central core. Many of these phosphoacceptor sites can be phosphorylated by more than one serine/threonine kinase signifying a high degree of redundancy as to the type of kinase which phosphorylates it (Bode and Dong, 2004). Though phosphorylation generally activates p53, mechanisms for how phosphorylation achieves this remain largely unknown. Studies with site mutants have largely not revealed a specific consequence for a particular phosphorylation site, so it may stand that specific combinations or the cumulative effect of phosphorylation events along with other post-translational mechanisms may specifically regulate p53 function. However, phosphorylation of p53 at serine 46 by HIPK2 (**H**omeodomain **I**nteracting **P**rotein **K**inase **2**) strongly correlates with induction of apoptosis via transcription of p53 AIP1 (**p53**-regulated **A**poptosis **I**nducing **P**rotein **1**) (Oda et al., 2000;Hofmann et al., 2002).

In addition to acetylation, phosphorylation and ubiquitylation, p53 undergoes sumoylation, glycosylation, ribosylation and recently discovered, neddylation (Harper, 2004). How each of these modifications regulate p53 is not yet fully understood but does indicate further complexity in the regulation of the p53 transcriptional machinery.

1.18 p53 and associated general transcription factors and co-factors

Like many sequence specific transcription regulators, p53 requires the activity of RNA pol II. Additionally, p53 interacts with a number of general transcription factors in the basal transcription machinery to mediate both transcriptional activation and repression. One of the first general transcription

factors to be identified as interacting with p53 was TBP of the TFIID complex, which induced p53 mediated transcription repression at a minimal promoter containing an initiator sequence and a TATA box but lacking a canonical p53 DNA binding site (Seto et al., 1992). A mechanism for p53/TBP repression may involve disruption of the holoezyme complex or squelching of necessary factors for transcriptional activation, in addition to as yet unidentified factors which specifically target the repressor complex to p53 negatively-regulated genes such as *bcl-2* (Ragimov et al., 1993;Farmer et al., 1996). The interaction between TBP and p53 was mapped to the transactivation domain of p53 and further studies showed that p53 activated transcription, with TBP or TFIID, from promoters containing the p53 response element (Liu et al., 1993;Chen et al., 1993). These data suggest the dual activator and repressor functions of p53, are in part, determined by the presence of the p53 RE in the proximal promoter of p53 regulated genes.

Interactions between p53 and other members of the TFIID complex have also been identified. In *Drosophila*, p53 directly interacts with both TAFII 40 and TAFII 60 to mediate transcriptional activation which is eliminated by point mutations in the p53 transctivation domain (Thut *et al.*, 1995). Similarly, TAFII 31 of the human TFIID complex has been identified as a p53 transactivation domain interacting protein required for p53 transcription (Lu *et al.*, 1995). In addition to interacting with the TAFs, the p53 transactivation domain also interacts with Mdm2, linking transcriptional activity to post-translation modification of p53. TAFII 31 can block the interaction of Mdm2 with p53, preventing its degradation by

ubquitylation (Buschmann et al., 2001). Likewise Mdm2 can mask the transactivation domain preventing TAFII 31 interaction and can also inhibit activation of RNA pol II by an interaction with TBP and TFIIE (Thut et al., 1997). The general transcription factor TFIIH also interacts with p53, though the functional significance of this interaction is not yet known (Xiao et al., 1994). These data indicate that the transcriptional activation by p53 is closely associated with the GTFs of the basal transcription machinery and that disruption of these interactions by post-translational modification of p53 may alter the transcriptional response. Additionally, transcriptional co-factors may also have a role in modifying p53 transcription.

P53 interacts with other co-factors that regulate RNA pol II activity. Among these the chromatin remodelling complex, Swi/Snf, has been found to interact with p53, through interaction with two subunits of the complex, BRG-1 and hSNF5, which co-operate in transcriptional activation (Lee et al., 2002). Other transcription regulatory factors have been identified as positively or negatively regulators of p53 transcription. One such factor is Yin Yang 1 (YY1) which inhibits of p53 acetylation by inhibiting its interaction with p300 (Gronroos et al., 2004). YY1 also promotes p53 degradation by MDM2 (Sui et al., 2004). More recently, a DEAD box RNA helicase protein, p68 has been described as a positive co-factor which is recruited to the *p21* promoter in a p53 dependent manner (Bates et al., 2005).

In sum, p53 interacts with and is regulated by a number of transcriptionally associated proteins. Broadly these proteins can be classed in to components of

the basal transcription machinery as well as transcription co-factors that may be involved in regulating p53 transcription at specific gene targets. Like the many post-translational modifications that occur to the p53 protein, the diversity of interacting transcription co-factors may finely regulate the p53 transcriptional response to diverse cell stress stimuli.

1.19 Summary: p53

Mutation of the p53 tumour suppressor locus occurs in 50% of all tumours, resulting in the inactivation of its transcriptional activity and loss of its anti-tumourigenic activity. The regulation of p53 transcription occurs via a variety of post-translation modifications to the transcription factor as well as interacting co-factors, which modulate p53 activity. Such a complex mechanisms of regulation may be required to integrate the signalling of cell stresses into finely tuning the cell's response to the particular stimulus. This diversity of regulatory mechanisms may also compensate for the inactivation of some p53 response pathways in cancer, which would prevent activation of p53 transcription. Understanding the mechanisms behind the regulation of p53 transcription will lead to a greater understanding of how cell stresses activate p53 and how they may be disrupted in the development of cancer.

1.20 The Cell Cycle

The control of cell proliferation is regulated by the process of the cell cycle. Most adult cells reside in a non-cycling state termed quiescence (G0), however on receiving extracellular signals, such as cell to cell contact and growth factors, cells exit from quiescence and re-enter the cell cycle. The cell must ensure faithful duplication, which occurs through four distinct, ordered cell cycle phases known as G1, S, G2 and phase M.

During normal cell proliferation, cells exit from G0 and enter to G1 (**g**ap**1**) phase where they grow in size and prepare for DNA replication by inducing expression of genes required for DNA synthesis. The process of DNA synthesis occurs in S-phase (**s**ynthesis) when the cellular DNA and organelles are replicated in preparation for cell division. This phase is followed by the G2 phase (**g**ap**2**) where the cell machinery is prepared for the M-phase (**m**itosis) where segregation of replicated DNA and cytokinesis forms the daughter cells. Following this, cells become sensitive to external signals and may enter in to another round of the cell cycle or may enter polyploidy.

A critical point in the "decision" to enter another round of replication is the G1 restriction point, and is therefore one of the most tightly regulated restriction points of the cell cycle. As demonstrated in normal cells in culture, factors such as nutrients and cell density can influence the progression through the G1 phase or in their absence induce cell cycle withdrawal into quiescence (Pardee, 1974). The G1 restriction point in tandem with other cell cycle check points maintains genomic integrity and allows regulated cell proliferation. Cancer cells exhibit a

lack of regulation by the cell cycle check points, permitting uncontrolled proliferation resulting from the activation of oncogenes and/or the loss of tumour suppressors. Understanding the molecular mechanisms occurring at cell cycle check points is fundamental to understanding the deregulation of the cell cycle in cancer.

1.21 Cyclin/cdks and the regulation of cell cycle check points

Study of the cell cycle in yeast has given a great deal of insight in to regulation of the mammalian cell cycle and its check points. One of the initial discoveries of cell cycle regulation in yeast was that of cdc2, a serine/threonine kinase which is responsible for the transition between G1/S and G2/M phases. A human homologue to the yeast cdc2 was discovered through a screen of human cDNA screen for complementation of *cdc2* mutant lethality (Lee and Nurse, 1987). Other Cdc2 homologues were discovered in a number species, representing a common mechanism of cell cycle regulation. Work in *Xenopus* oocytes revealed the presence of cdc2 in a complex known as Maturation Promoting Factor (MPF) and was essential for oocyte meiosis and mitosis. MPF contained a protein that was regularly destroyed with each egg cleavage cycle and was hence termed "cyclin". A cyclin-like protein, cdc13, was found to interact with cdc2 in yeast and though originally thought to be a substrate of cdc2, cdc13 was actually found to regulate the catalytic activity of its binding partner as well as its nuclear localisation (Booher et al., 1989). Other cdc2-like proteins were identified on the basis of conserved motifs, PSTAIRE and the T-loop, within the

protein. With the discovery of these complexes, the nomenclature of the cdc2 family proteins changed to "cyclin dependent kinases" (cdks) based on their interaction with specific cyclin subunits. In yeast, there is only one cdk whereas genomes of higher eukaryotes encode multiple cdks. In human cells, the counterpart of cdc13/cdc2 is now known as cyclin B/cdk1.

Other human cyclins were discovered in complementation studies in yeast as described for cdc2. The D-type family of cyclins (D1/D2/D3), as well as cyclin E, were discovered on their ability to complement *cln* mutants in *S.cerevisiae* (Koff et al., 1991;Lew et al., 1991;Xiong et al., 1991). In contrast, human cyclin A (*CCNA2*) was identified through cloning of a single hepatitis B virus integration site, which was predicted to encode a protein with similar homology to cyclin A of clams and *Drosophila* (Wang et al., 1990). Cyclin A1 was discovered later through its homology with mouse cyclin A1 (Yang et al., 1997). To form a kinase complex, the D-type cyclins predominately bind cdk4 and cdk6, whereas cyclins E and A bind cdk2. The kinase activity of these cyclin/cdk complexes is associated with the particular phases of the cell cycle. The D-type cyclin complexes and cyclin E/cdk2 are recognised as important regulators of the mid G1 restriction point. The activity of cyclin A/cdk2 however is associated with transition through S-phase whereas cyclin B/cdk1 is required for the G2/M phase transition. Regulation of the kinase activity of these cyclin/cdk complexes is essential in maintaing normal cell proliferation.

2.4 Regulation of cdk activity

Cyclins

The catalytic activity of cdks is dependent on its binding to an appropriate cyclin partner to induce conformational changes. The determination of cyclin/cdk structures has given insight as to how these structural changes activate the kinase. Using the example of cyclin A, which binds and activates cdk2, cyclin binding induces structural changes in two regions of the cdk known as the T-loop and PSTAIRE helix. While in an inactive kinase state, the T-loop blocks the substrate binding groove and also orientates away from this groove the PSTAIRE helix, a region of the cdk that binds ATP. Upon interaction with cyclin A1, the T-loop is re-orientated out of the substrate groove and consequently the PSTAIRE helix moves in, to allow coordination of the ATP molecule required for catalysing the kinase reaction (Jeffrey et al., 1995). Cyclin E1 also interacts with cdk2 to activate its kinase activity and is reported to induce similar conformational changes in cdk2 as seen with the interaction with cyclin A1 (Honda et al., 2005). The expression of the cyclin in a cell cycle phase specific manner therefore determines when a cyclin/cdk complex becomes active to phosphorylate its specific substrates.

The specific phosphorylation of substrates during a cell cycle phase may be required for progression through a phase or transition between phases of the cell cycle. How a cyclin/cdk complex targets phosphorylation of specific substrates is thought arise from interactions that occur between the cyclin and the substrate. The presence of the amino acid motif, RXL (also known as the Cy

motif) on a substrate has been associated with phosphorylation by cyclin A/cdk2 (Brown et al., 1999). More recently, using the S-phase promoting cyclin/cdk in yeast, Clb5/cdk1, an interaction between the Cy motif on specific substrates and a hydrophobic patch on the cyclin has been found to be essential to phosphorylation (Loog and Morgan, 2005). Substrate specificity for the D-type cyclins complexes is limited as the retinoblastoma family of proteins were the only identified substrates, up until recently when Smad3 was identified as a substrate of cyclin D/cdk4 complexes (Matsuura et al., 2004). Identification of further substrates of the D-type cyclin complexes may elucidate mechanisms of substrate specificity for these cyclin/cdks.

The activation of the cdk by cyclins is also dependent on the localisation of the cyclin. Many of the cyclin/cdk substrates are nuclear therefore co-localisation ensures that the kinase meets its substrate. D-type cyclins are nuclear proteins which accumulate during the G1 phase of the cell cycle and become cyctoplasmic during interphase, though the mechanisms regulating their localisation are not fully understood (Baldin et al., 1993). Cyclins E and A are also nuclear imported proteins however cyclin B1 and B2 show different patterns of localisation. Cyclin B1 expression is predominately cytoplasmic whereas cyclin B2 is nuclear (Pines and Hunter, 1991;Ohtsubo et al., 1995).

As well as the timely expression of the cyclins during cell cycle phases, equally important is their destruction to inactivate cyclin/cdk activity when passing between cell cycle phases. Degradation of the cyclins is undertaken by the proteasome, after cyclins have been covalently attached to ubiquitin at lysine

residues. Two ubiquitin conjugating enzyme complexes are known to mediate cyclin degredation, Skp/Cullin/F-box (SCF) and the anaphase promoting complex (APC). Cyclin D1 is a substrate of the SCF type complex containing Skp1, Skp2 and Cul1 (Yu et al., 1998). Furthermore, cyclin D1 may be targeted for degradation via phosphorylation of Thr-286 by the glycogen synthetase kinase (GSK)-3ß (Diehl et al., 1997). Cyclins A and B are ubiquitinated by APC in contrast to cyclin E which is ubiquitinated by the SCF complex containing the Fbw7 F-box protein (King et al., 1995;Geley et al., 2001;Koepp et al., 2001). The degradation of the cyclins after completing their function allows co-ordinate transitition between phases of the cell cycle. The requirement for timely degradation of the cyclins is emphasised by the cell cycle abnormailities that occur when their destruction is inhibited. An example is cyclin B1 whose continued expression during M-phase prevents transition into G1 (Wheatley et al., 1997).

Cdk activating kinase (CAK)

Unlike the cyclins, the cdks do not demonstrate such fluctuations in expression during cell cycle phases. However their activity is regulated by other post-translational mechanisms. The phosphorylation of cdks has both positive and negative regulatory influences on cdk activity. CAK mediated phosphorylation of Thr-160, located on the T-loop of cdk2 moves it out of the substrate groove to allow substrate binding. Dephosphorylation occurs by way of the cdk-associated phosphatase, KAP, which inactivates cdk.

CAK itself comprises of cyclin H/cdk7 and is also responsible for the activation cdk4 and cdk1. Apart from its role as a cdk activator, cyclin H/cdk7 has been isolated as component of the TFIIH complex. Though its kinase activity is required for transcription elongation, its ability to phosphorylate the CTD of RNA pol II seems to be dispensable for this function (Akoulitchev et al., 1995;Makela et al., 1995). The presence of this cyclin complex in the transcriptional machinery did however suggest crosstalk between the cell cycle regulators and transcription.

Inhibitory phosphorylation of cdk2 also occurs at Thr-14 and Thr-15 through the actions of the Wee1 and Myt kinases. Phosphorylation at these sites, located in the ATP binding phosphate binding site, is thought to reduce its affinity for ATP required for the kinase reaction. De-phosphorylation of these sites by the cdc25 family of phosphatases (cdc25A/B/C) restores cdk activity.

Cyclin dependent kinase inhibitors (Cdki)

In addition to direct modifications to cyclin/cdk proteins, additional regulatory proteins modulate their activity. The Cdkis represent another well established group of protein involved in cdk regulation. Two families of Cdkis are directly implicated in cell cycle regulation. The inhibitor of kinase 4 (INK4) family of Cdkis consists of p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d). These inhibitors predominately block the activity of cdk4 and cdk6 by preventing their interaction in the activation of D-type cyclins (Ruas and Peters, 1998).

A second family of Cdkis containing p21, p27 and p57 make up the Cip/Kip family which block the activity of cyclin/cdk2 complexes. Structural studies of cyclin A1/cdk2 bound to p27 revealed that p27 inhibited cdk2 activity by occlusion of the ATP binding pocket in cdk2 as well as blocking the substrate binding groove. Contrary to their inhibitory role, a current model for p21 and p27 function has been proposed as D-type cyclin assembly factors that enhance the stability of the cyclin/cdk complex. The kinase inhibitory function of p21 and p27 may predominate at high stoichiometric ratios relative to the cyclin D/cdk complexes. This models offers an explanation for the dual roles of p21 and p27 as both promoters as well as inhibitors of cyclin D/cdk kinase activity.

1.23 Cyclin/cdks and cell cycle checkpoints

Sixteen cyclins and nine cdks have been so far identified in human cells, of which eleven cyclins and six cdks are directly involved in cell cycle regulation. These cyclin complexes are cyclin D1/D2/D3 complexed with cdk4 or cdk6, cyclin A1/A2 complexed cdk2 or cdk1, cyclin E1/E2 with cdk2, cyclin B1/B2 with cdk1 and cyclin H with cdk7. More recently, cyclin C/cdk3 was identified as a cyclin/cdk complex associated with the cell cycle regulation that was able to induce exit from quiescence by phosphorylation Rb (Ren and Rollins, 2004).

Studies of the mammalian cyclin/cdk complexes in cultured cells by over-expression, use of cdk inhibitors and microinjection of antibodies has revealed phases of the cell cycle where the activity of specific cyclin/cdks may be required. The synthesis and assembly of D-type cyclin complexes results from mitogenic

signals and is thought to be necessary in the entry and progression through the G1 phase as observed in cells over-expressing cyclin D1 which show an accelerated G1 (Jiang et al., 1993;Quelle et al., 1993). Inhibition of the D-type cyclin kinase subunit, cdk4, has the converse effect, inducing G1 arrest and exit from cell cycle (Serrano et al., 1993;Tetsu and McCormick, 2003). D-type cyclin/cdk complexes phosphorylate Rb and subsequently release E2F from repression which activates the transcription of cyclins E and A. The D-type cyclins perform a second function during the G1 phase by titrating away Cdkis, p21 and p27, releasing the cyclin E/cdk2 complex from inhibition. Cyclin E/cdk2 functions during the G1/S phase transition to phosphorylate Rb. Inhibition of cyclin E or interacting kinase, cdk2, prevents transition in to S-phase and induces G1 phase arrest (Pagano et al., 1993;Ohtsubo *et al.*, 1995). The cyclin A/cdk2 complex functions in the progression of S-phase where the expression of the cyclin is required for the synthesis of DNA (Girard et al., 1991;Pagano et al., 1992). Substrates of the cyclin A/cdk2 complex include proteins involved in the assembly and function of the cellular machinery required for DNA synthesis. Cyclin B/cdk1 is present in the G2/M-phase transition of the cell cycle and is required for entry into mitosis.

Cell cycle arrest induced by the inhibition of cyclins and their cdks implies that expression and kinase activity of the cyclin/cdk complexes is essential at specific phases in the cell cycle. However, these studies in cultured cells do not reflect observations made in mice nullizygous for specific cyclins and cdks. If cyclin expression was essential to the cell cycle progression, *in vivo*, then it

would be expected that mice null for the cyclins would not be viable. Instead, mice null for cyclin D1 are still viable but are smaller than normal and demonstrate tissue-specific developmental abnormalities (Fantl et al., 1995;Sicinski et al., 1995). Mice nullizygous for cyclin D2 and cyclin D3, respectively, are also viable with limited tissue specific abnormalities indicating that some tissues require expression of specific cyclins for development (Sicinski et al., 1996;Sicinska et al., 2003). Lethality occurs when mice are nullizygous for triple deletions of the cyclin D1, D2 and D3 genes. Deletion of all three cyclins is lethal at embryonal day 16.5 and is attributed to a severe defect in the elaboration of haemopoietic lineages (Kozar et al., 2004). Similarly, mice null for cdk4 and cdk6 show a partial haemopoietic failure resulting in a progressive embryonic lethality which is not seen mice deficient for either cdk4 or cdk6 (Malumbres et al., 2004). However, the phenotype of the $cdk4^{-/-}$ /$cdk6^{-/-}$ mice is similar to that of the D-type cyclin null mice. Lethality is also induced in mice deficient for both cyclin E1 and E2 due abnormal placental development but can be rescued to allow embryos to develop to term, whereas mice singly null mice do not have these abnormalities (Geng et al., 2003;Parisi et al., 2003). $Cdk2^{-/-}$ deficient mice are still viable but have some tissue-specific defects (Ortega et al., 2003). These phenotypes of the cyclin and cdk null mice suggest that there is redundancy in their functions with alternative cyclins and cdks able to compensate for deficiencies. Overall these studies imply that the cyclins are not essential regulators of cell cycle progression but may in fact be rate limiting steps during specific phases of the cycle.

2.5 **Transcriptional regulation at the G1 restriction point**

Regulation of E2F transcriptional activity by pRb at the G1 restriction point is thought to be important in cell growth. Exit from G0/early G1 to late G1 and S-phase requires transcriptional activation of genes regulated by members of the E2F (E2A binding factor) family of transcription factors specific for the next phase of the cell cycle (Johnson et al., 1993;Lukas et al., 1996). E2F proteins in complex with either DP-1 or DP-2 (**d**ifferentiation-regulated transcription factor **p**rotein) regulate the transcription of genes containing a TTTCGCGC concensus sequence within their promoter regions. Such genes include cell cycle regulators cyclin E, cyclin A, cyclin D1, cdc2(cdk1) and cdc25A, enzymes involved in DNA synthesis: dihydrofolate reductase (DHFR), thymidine kinase (TK) and DNA polymerase α; proteins involved in DNA replication: including ORC1 (origin recognition complex), cdc6 and MCM proteins (minichromosome maintenance) (Stevens and La Thangue, 2003). Apoptotic genes also regulated by E2F include APAF-1 (apoptosis protease-activating factor), p14ARF and p73 (Irwin et al., 2000;Ginsberg, 2002). Thus E2F activates pathways involved in promoting growth as well as apoptosis. It is proposed that this concomitant activation of apoptotic genes safe guards against inappropriate high levels of activation of E2F, possibly by oncogenes, and therefore eliminates potential tumour cells.

pRb similarly protects against inappropriate activation of E2F by repressing E2F-regulated transcription thus regulating the passage of cells into late G1. pRb belongs to retinoblastoma family of proteins which also includes the p107/Rb2 and p130/Rb3 proteins. pRb bound to E2F represses its activation by

recruiting chromatin remodelling complexes, including HDACs (histone deacetylase), Swi/Snf complex and histone methyalses. HDAC activity induces remodelling of chromatin structure through the removal of acetylated lysine residues on histones, condensing the structure of transcriptionally active chromatin. pRb also utilises mechanisms of transcriptional repression independent of chromatin remodelling and DNA bound transcription factors. Termed "active repression", Rb can inhibit formation of the pre-initiation complex by E2F, TFIID and TFIIA (Ross et al., 2001).

The phosphorylation status of Rb determines its ability to repress E2F bound to DNA. The hypophosphorylated Rb binds E2F repressing its transcriptional activity, preventing G1progression. This arrest is overcome by the D/cdk complexes as well as cyclin E/cdk complexes which phosphorylate pRb on 14 potential phosphoacceptor sites. The multiple phosphorylation of Rb on serine and threonine residues induces conformational change in Rb which prevents binding to E2F. Phosphorylation by the D-type cyclin kinases initially releases HDAC complexes from binding to Rb and reveals a serine phosphosite phosphorylated by cyclinE/cdk2. The derepression of E2F allows the transcription of cyclin E and via a positive feedback loop cyclin E/cdk further phosphorylates Rb to disrupt its interaction with E2F and thus permit full activation of E2F transcription. With this, the G1 restriction point is by-passed and the cell enters S-phase. Cyclin A has also been implicated as a negative regulator of E2F transcription in S-phase. Cyclin A with cdk2 phosphorylates heterodimeric E2F-1/DP-1 transcription factor to inhibit its DNA binding and

permits entry in to S-phase (Dynlacht et al., 1994;Krek et al., 1995). Such a mechanism limits activation of E2F mediated transcription and the subsequent delay/arrest of cells in S-phase leading the re-initiation of cell cycle and/or apoptosis.

The mechanisms of transcriptional regulation of E2F by the G1/S phase cyclins are dependent on their kinase activity and are directly involved in the control of cell cycle. Other mechanisms of transcriptional regulation of different transcription factors have been demonstrated for the cyclins, as is outlined in the next section.

1.25 Cdk independent transcriptional activities of D-type cyclins

The regulation of E2F transcription by the D-type cyclins, via Rb, is an example of the cell cycle regulatory function of these cyclins which requires the kinase activity imparted by cdk4 and cdk6. However, the D-type cyclins also have other transcriptional regulatory functions that are independent of the cdk activity. Such a role for the cyclins was suggested by findings that cyclin D1 specifically interacted with the oestrogen receptor to activate transcription mediated by this nuclear receptor which was not dependent on cdk activity (Neuman et al., 1997;Zwijsen et al., 1997). Since these findings, other transcription factor and co-factors have been found to interact with the D-type cyclins, in particular cyclin D1 (Coqueret, 2002;Fu et al., 2004a). D-type cyclins have also been found to interact and regulate transcription mediated by the androgen receptor and thyroid receptor as well as the Sp-1 transcription factor, all of which are regulated by the

Mediator complex (Shao and Robbins, 1995;Knudsen et al., 1999;Lin et al., 2002). The interaction of cyclin D1 with these nuclear receptors has both activating and repressing effects on the transcriptional activity of these receptors. The cyclin D1 interaction with the oestrogen receptor stimulates transcriptional activation from oestrogen receptor response elements. This property of the cyclin was dependent on its C-terminal domain, containing a LLXXXL motif, similar to that found on the AF2 domain of the oestrogen receptor. This motif is required for efficient transactivation of the oestrogen receptor by the nuclear co-activator/steroid receptor co-activator (NcoA/SRC-1a) and is utilised by cyclin D1 to interact with SRC-1a to bridge a complex with the oestrogen receptor. Furthermore, this interaction allowed ligand independent □olyploidy□tion of oestrogen receptor-mediated transcription (Zwijsen et al., 1998).

In contrast to its effects on the oestrogen receptor, the cyclin D1 interaction with the androgen and thyroid receptor-ß1 has been demonstrated to induce transcriptional repression. Characterisation of cyclin D1 repression of the androgen receptor showed cyclin D1 competed with p300/cAMP binding protein (P/CAF) for binding to the receptor. P/CAF has previously been shown to interact with cyclin D1 to potentiate oestrogen receptor-mediated transcription (McMahon et al., 1999). Though the C-terminus of cyclin D1 was required for androgen receptor repression, it was not dependent on the LLXXXL motif there. Repression did require the AF-1 domain of the androgen receptor and was partially dependent on the activity of HDACs (Petre et al., 2002). HDAC3 has been reported to interact with cyclin D1 and subsequently this interaction has

been mapped to a central domain on cyclin D1, which when expressed alone is sufficient to repress transcription by both the androgen and thyroid receptor without affecting the oestrogen receptor (Lin *et al.*, 2002;Petre-Draviam et al., 2004).

Another nuclear receptor that is repressed by cyclin D1 is the peroxiosome proliferation activated receptor-γ (PPAR-γ) whose transcriptional activity is linked to adipocyte differentiation. Homozygous deletion of cyclin D1 gene in mouse fibroblasts enhanced PPAR-γ transcriptional responses, which was also demonstrated *in vivo* by a cyclin D1 anti-sense transgenic mouse model (Wang et al., 2003a).

Cyclins E and A have also been implicated in the regulation of transcriptional targets not directly related to their cell cycle control. Cyclin E has been demonstrated to potentiate androgen receptor-mediated transcription on stimulation with di-hydroxy-testosterone, an activity that was independent of its interaction with cdk2 (Yamamoto et al., 2000).

These additional functions of the cyclins suggest that they have more direct role in transcription regulation, specific to nuclear receptor mediated transcription. Furthermore, with the nature of cyclin expression during phases of the cell cycle, the cyclins may co-regulate nuclear receptor mediated transcription in a cell cycle phase dependent manner. These functions of the cyclins along with their more established roles may contribute to the effects of cyclin deregulation as observed in the pathogenesis of cancer.

1.26 Deregulation cyclins and cancer

Many regulatory mechanisms act at the G1 restriction point which is an important barrier to the formation of cancerous cells. It is these mechanisms that are targeted by activating oncoproteins and/or inactivation of tumour suppressors. This can result from the mutation of cellular genes or the expression of viral proteins. The loss of the G1 restriction point predisposes cells to malignant transformation and genomic instability as cell proliferation becomes unchecked and autonomous.

The over-expression of the cyclins is one such mutational event whereby cells acquire autonomy from external signals. Over-expression of cyclin D1 in tumours can result from enhanced mitogenic signalling as well as amplification of the *CCND1* gene locus, encoding the cyclin D1 protein. *CCND1* (also known as PRAD1) was initially identified and observed as a clonally rearranged gene in parathyroid adenomas where its gene locus was juxtaposed to the parathyroid hormone gene promoter to induce cyclin D1 over-expression. This mutation is observed in 20-40% of parathyroid adenomas (Motokura et al., 1991).

Breast tumours also frequently demonstrate over-expression of cyclin D1, by both amplification of the *CCND1* gene and by constitutive over-expression. Mouse models of targeted cyclin D1 over-expression to the mammary gland support the oncogenic potential of cyclin D1 in the formation of breast tumours. Cyclin E is also over-expressed in some breast tumours and is linked with a poorer prognosis (Keyomarsi and Pardee, 1993;Nielsen et al., 1996). A chromosomal rearrangement, t(11;14) (q13;q32) involving the translocation of

CCND1 to the immunoglobin heavy chain promoter has also been observed in lymphomas (Medeiros et al., 1990). Discovered more recently was a nucleotide polymorphism, A870G, of *CCND1* which results in alternately spliced transcript encoding a 55 amino acid C-terminal truncation of cyclin D1. This truncated protein alters the functional properties of the cyclin and is associated with a higher incidence of tumours (Solomon et al., 2003;Wang et al., 2003b).

Other mutations also contribute to the deregulated kinase activity of the D-type cyclins, such as mutation of the p16INK4A and cdk4 loci, which would contribute to the enhanced kinase activity of the D-type cyclins/cdks. The cell cycle regulatory function of cyclin D1 is thought to be responsible for promoting tumourigenesis, though a contributory role by its cdk independent transcription regulatory functions can not be ruled out.

Viruses have also targeted cell cycle regulators by encoding viral proteins able to inactivate or enhance the function of cell cycle regulators for the purposes of the viral life cycle. Some of these viral proteins are homologous to cellular proteins. Members of the oncogenic γ2-herepesvirus family, *Herpesvirus saimiri* (HVS), Kaposi's sarcoma-associated virus (KSHV) and murine herpesvirus-68 (MHV-68) encode functional viral cyclins with homology to cellular cyclins.

1.27 The γ2-herpesviral cyclins

HVS: v-cyclin

The first viral cyclin to be discovered was that of HVS, identified through sequencing of a region of the genome unique to HVS and not present in a related γ1-herpesvirus, EBV (Nicholas et al., 1992). The open reading frame, termed eclf2, encoded a viral cyclin with 24% amino acid identity and 46% similarity to the human cyclin D1 protein (Schulze-Gahmen et al., 1999). Characterisation of v-cyclin revealed it formed a functional kinase when bound to cdk6 via a cyclin box motif present on the cyclin (Jung et al., 1994). Later studies revealed v-cyclin/cdk6 kinase activity to be resistant to cyclin/cdk inhibitors p16INK4A, p21 (Cip) and p27 (Kip), suggesting its kinase activity is unchecked by these proteins in infected cells (Swanton et al., 1997).

KSHV: vcyclin

vcyclin, encoded by ORF72 in the KSHV genome, is a cyclin homologue with 32% amino acid homology to cellular D-type cyclins (Cesarman et al., 1996;Li et al., 1997). Like cellular D-type cyclins, vcyclin binds cdk2 and cdk4 but predominately activates cdk6 and extends its range of substrates to include those of cyclin E/cdk2 and cyclin A/cdk2. Substrates of vcyclin/cdk6 include the D-type cyclin/cdk substrate, Rb (Godden-Kent et al., 1997), cyclinE/cdk2 substrate, p27Cip (Mann et al., 1999;Ellis et al., 1999), cyclinA/cdk2 substrates, origin recognition complex-1 (ORC-1) and Cdc6 (Laman et al., 2001b) as well as cyclin B substrate, histone H1 (Godden-Kent, 1997).

Like HVS cyclin, vcyclin is also resistant to the cdkIs (Swanton *et al.*, 1997). Though required for its full activation, vcyclin/cdk6 can function independently of CAK phosphorylation (Child ES 2001)(Kaldis et al., 2001). These properties of vcyclin/cdk6 suggest that the complex can subvert normal regulatory mechanisms imposed on cellular cyclin/cdks to function as a constitutively active kinase.

The ability of vcyclins to function like the G1/S phase cyclins, with a kinase activity that is unregulated, results in the independence of the cell cycle on growth signalling and the loss of normal cell cycle checkpoints, allowing cells to exit from quiescence and re-enter cell cycle (Swanton *et al.*, 1997;Child and Mann, 2001). The oncogenic potential of vcyclin has been tested in a transgenic mouse model. The vcyclin transgenic mouse developed lymphoma after a long latency, suggesting secondary somatic mutations are important to the tumourigenicity of vcyclin (Verschuren et al., 2004). This is supported by further mouse studies, the progeny of the vcyclin transgenic mice crossed on to a p53 null background developing lymphoma earlier, implying apoptosis as a barrier to oncogenesis when vcyclin is overexpressed (Ojala et al., 1999;Verschuren et al., 2002). In the context of KSHV infection vcyclin is likely to co-operate with the other latently expressed genes of KSHV, like LANA-1 with which it is co-transcribed, that inactivate the p53 pathway, in order to potentiate KSHV induced oncogenesis.

Vcyclin properties also extend to functions previously not ascribed to cyclins. Phosphorylation of cellular bcl-2 is unique to vcyclin/cdk6 and results in

the inactivation of its anti-apoptotic properties (Ojala et al., 2000). Vcyclin may also have functions that are independent of its kinase activity, an example being vcyclin interaction with signal transducer and activator of T-cells 3 (STAT3) to inhibit its DNA binding and transcriptional activation thus inhibiting the growth suppressive effects of oncostatin M signalling, a property similar to cyclin D1 which represses STAT3 in a cdk4-independent manner (Bienvenu et al., 2001;Lundquist et al., 2003). This range of activities demonstrated by vcyclin has been used as a robust model for studying the functions of the G1/S cyclins.

MHV-68: m-cyclin

Sequencing of the MHV-68 genome revealed an ORF similar to that of ORF72 of KSHV encoding vcyclin, demonstrating conservation of the viral cyclin between the γ-2 herpesviruses (Virgin et al., 1997). In contrast to vcyclin, the MHV-68 cyclin, M-cyclin, displays a late lytic gene expression profile and was predicted to encode a smaller protein of about 25Kda. M-cyclin also only binds cdk2 and cdk1 but not cdk4 or cdk6 (Upton et al., 2005). Studies of a transgenic mouse expressing M-cyclin from a *lck*-promoter, targeting protein expression to the T-cell compartment, revealed M-cyclin enhanced cell cycling and proliferation in *ex vivo* cultured thymocytes and in some mice led to the development of lymphoblastic lymphoma (van Dyk et al., 1999). This study suggests functional similarity between M-cyclin and the related herpesvirus cyclins.

1.28 Summary: Cyclins

Cyclins are regulators of cell growth and proliferation that were initially discovered in yeast and *Xenopus* oocytes. Cyclins bound to their cognate kinase subunit, the cdk, form serine/threonine kinases whose activity is regulated by multiple mechanisms. Specific cyclin/cdk complexes phosphorylate proteins that enable and co-ordinate transition and progression through the phases of the cell cycle. Some of these proteins are transcriptional regulators whose function is modulated to alter gene expression and facilitate cell proliferation. Cdk-independent functions for the cyclin D1 in transcription regulation have also been shown in transcription mediated by the thyroid and androgen receptors. Mediator complexes are involved in transcription initiated by both these receptors.

Cancer is often associated with aberrant cyclin function leading to unchecked cell proliferation. Oncogneic herpesviruses, namely HVS, KSHV and MHV-68 of the γ2-herpesviridae, encode functional cyclins that are resistant to cellular regulation and have expanded functions compared to their cellular counterparts. Both cellular and viral cyclins therefore have a role transcriptional regulation that promotes cell proliferation and when abberantly expressed may drive tumourigenesis.

1.29 This Thesis

MED17, a subunit of a Mediator complex regulating transcription by nuclear receptors, interacted with a viral cyclin in a yeast two hybrid screen (Laman, unpublished data). It had been previously shown that MED17 activates p53 mediated transcription *in vitro*. This novel interaction suggested that viral and/or cellular cyclins affect MED17 and thereby regulate the transcriptional activity of p53 tumour suppressor, thus linking cell proliferative signals with transcriptional regulators of cell cycle arrest and apoptosis.

In this thesis, the activity of MED17 on p53 and other potential Mediator regulated transcription factors was explored. In addition, the interaction between cyclins and MED17 was investigated to determine their nature and function. Evidence is provided that MED17 is a repressor of p53-mediated transcription *in vivo* which inhibits apoptosis and induces phenotypes seen in cells with compromised p53 function. Human MED17 activates heat shock factor mediated transcription as has been described for *Drosophila* MED17, demonstrating that this function of MED17 is conserved. Investigating the interaction with the viral cyclin, it was found that MED17 interacted with vcyclin of KSHV as well as cellular cyclins, cyclin D1, cyclin E and cyclin A both *in vitro* and *in vivo*. Furthermore, these interacting cyclins mediate phosphorylation of MED17 and regulates its expression. Overall these data link cyclins to the control of p53 transcriptional activity through an interaction with the MED17 subunit of Mediator, suggesting interplay between cell proliferative signalling and transcriptional regulation of apoptosis.

Chapter 2: Materials and methods

Standard methods

2.1 Cell culture and transfection: Mammalian adherent cell lines

U2OS, MG63 and NIH-3T3 cells were maintained in DMEM supplemented with 10% FCS (Sigma), 5 mg/ml penicillin/streptomycin (Gibco BRL, UK) and cultured at 5% CO_2 in humidified conditions (see Table 2.1). For transfection, cells were seeded at $2x10^5$ in 60mm well dishes using a haemocytometer, 12 hours before applying DNA/Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany), following the manufactures protocol (expression constructs transfected are given in Table 2.2). Fresh media was added after 24 hours and cells were harvested for protein after 48 hours post transfection.

2.2 Cloning of vector and MED17 cell line

NIH-3T3 cells were maintained and transfected in conditions as stated for mammalian adherent cells. Cells were seeded at $1x10^6$ in 10cm Petri dishes before transfection with either 2µg of pOP-RSV-1 (Stratagene) or pOP-RSV-1 MED17 HA, to generate the NIH-3T3 vector and NIH-3T3 MED17 cell lines respectively. 48 hours after transfection cells were trypsinised and re-seeded at $1x10^5$ into multiple 10cm Petri dishes overnight. After 12 hours the cells were washed with PBS and media changed to select for neomycin resistant clones. Selection media consisted of DMEM, 10% FCS and 600µg/ml of G418 (neomycin

sulphate) (Gibco BRL, UK). During selection media was replaced every three days and after 14 days single colonies isolated using cloning rings (Sigma). Colonies were transferred to 24 well plates containing neomycin supplemented media for further selection and expansion. Expanded colonies were screened by Western blotted for expression MED17 HA. For the vector only line, several G418 resistant colonies were pooled. MED17 expressing colonies were further expanded and maintained in 300µg/ml of G418 of supplemented media.

2.3 Yeast culture and transformation

Single colonies were inoculated in to 10ml of YPD medium and cultured for 12 hours at 25°C. Cultures were then diluted to OD_{600nm}=0.3 in 50ml of YPD medium and grown for a further 4 hours. To remove medium, the yeast were then spun and suspended in 25ml of sterile water and spun again and the water aspirated. The yeast pellet was then re-suspended in 2ml of 1x lithium acetate/0.5x TE in preparation for transformation. After incubating at room temperature for 10 minutes, the yeast 100µl of the mixture was combined with 10µl of 10mg/ml sheared salmon sperm DNA as well as 1µg of the expression construct DNA. To this 700µl of 1x Li Ac/ 1x TE/ 40% PEG was added and incubated at 30°C for 30 minutes without shaking. 85µl DMSO was added before heat shocking the transformation mixture at 42°C for 7 minutes. The transformed yeast were washed in a volume of 1ml before re-suspending them in 500µl of 1x TE. Of this volume, 100µl of yeast were plated on to amino acid deficient medium to allow for growth of transformants.

2.4 Preparation of yeast cell lysates

In preparation for lysis and extraction of protein, yeast cultures were grown to OD_{600nm}=0.3. Cultures were then centrifuged at 2500rpm for 5 minutes and the pellet washed with water before centrifuging again. 500µl of ice cold protein sample buffer was added to the pellet in addition to 500µl of glass beads followed by agitation of the sample 4 times for 45 seconds. Lysates were then centrifuged at 13,000rpm for 5 minutes at 4°C to remove debris and the supernatant transferred to a new microfuge tube and boiled with Laemmli protein sample buffer for 5 minutes before loading on SDS-PAGE gel to resolve proteins.

2.7 Cell culture and baculovirus infection: Sf9 cells

Sf9 cells were maintained in culture flasks containing Graces Medium (Gibco BRL, UK) supplemented with 20% FBS (Sigma), L-glutamine and pluronic acid at 26°C in a rotating incubator. Cells were cultured at a density of $5x10^5$/ml. For the production of cyclin/cdk complexes for *in vitro* kinase assays, Sf9 cells were seeded at $1x10^6$/well in 6 well dishes and allowed to adhere for 30 minutes at room temperature after which the media was carefully aspirated and 1ml of baculoviruses encoding the cdk or cyclin were overlayed onto cells, singly or in combination. Infected Sf9 cells were cultured for a further 72 hours before cells were harvested.

2.6 Preparation of Sf9 lysates

Lysates from Sf9 cells expressing cdks with or without cyclins were used for *in vitro* kinase assays. 72 hours after infection, cells were re-suspended by pipetting and centrifuged at 3000 rpm for 5 minutes to pellet cells following which the media was aspirated. 1x Sf9 lysis buffer was added to lyse the cell pellet which was done for 10 minutes on ice. Cell debris was then pelleted by centrifugation. Lysates were either used fresh in kinase assays or alliquoted and snap frozen in a dry ice/ethanol bath and stored at -80°C until required.

2.7 Amplification of baculovirus stocks

For amplification of the baculovirus stocks, Sf9 cells were seeded at $1x10^7$ into 175mm flasks in non-supplemented Graces Medium for 15 minutes before 2ml of baculoviral supernatant was added and incubated for a further hour. After the incubation the viral media was aspirated and fresh 20% FCS supplemented Graces Medium was added and cells were incubated for 72 hours at 26°C. Supernatant containing progeny baculoviruses was extracted and cleared of cell debris by centrifugation before being stored in the dark at 4°C until required.

2.8 Polymerase chain reaction (PCR) and site directed mutagenesis

PCR was used to amplify DNA for cloning of genes and in site directed mutagenesis, to create phosphosite mutants of MED17. PCR was performed using a 96-Plus thermocycler (MWG Biotech, UK). Expand High-Fidelity DNA polymerase (Roche) used was in the reactions. A typical reaction mixture

consisted of: 0.2µM of the sense and anti-sense primer (see Table 2.3), 5µl of 10x polymerase buffer (containing Mg Cl_2), 200µM of dNTP, DNA template in a total volume of 50µl. For the addition of an HA epitope at the C-terminus of the protein, an anti-sense primer was designed to delete the stop codon and place the HA sequence in frame. MED17 HA was PCR amplified from a human foetal cDNA library. A typical amplification protocol would be: 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 4 minutes followed by 1 cycle at 72°C for 10 minutes.

For site directed mutagenesis, Pfu polymerase (Stratagene) was used to amplify whole plasmids using overlapping primers introducing the point mutation. PCR reaction was setup and run according to the manufacturer protocol as set out in the Stratagene Quikchange® Site-Directed Mutagenesis kit.

2.14 Cloning and restriction analysis

Plasmids and DNA fragments were prepared for cloning by digesting with restriction enzymes. Restriction analysis was also used to verify the insertion of the DNA fragment after cloning. A typical 10µl reaction mixture consisted of 200ng of DNA, 1µl of 10x buffer, 2 units of restriction enzyme and in a total volume of 10µl. Reactions were incubated at 37°C for 1 hour except for *Sma I* enzyme which was incubated at 25°C. All restriction enzymes used were obtained from Promega and were used with the supplied buffers.

2.10 Agarose gel electrophoresis

Digested plasmids and DNA fragments were resolved on agarose gels to assess digestion and size of the digested DNA. Typically, 1% gels were made with electrophoresis grade agarose (Sigma) in 0.5x TAE, using the Biorad mingle system. To melted agarose was added ethidium bromide (Sigma) at 0.5µg/ml before casting gels. Set gels were placed in to a mini gel migration trough (BioRad), filled with 0.5xTAE and DNA loaded with a DNA loading buffer. Sizing of bands was done by running a separate well with 1kb ladder (Boehringer-Mannheim). Gels were run at 100v until the loading buffer dye reached the bottom of the gel. Bands were visualised using a UV transilluminator.

2.11 Gel extraction of DNA

DNA fragments of correct size were excised using a clean scalpel. Removal of agrose from DNA fragments was done using the QIAquick gel extraction kit (Qiagen) as per manufacturer's protocol. 30µl of DNA was extracted from the column and was used for DNA ligation or frozen at -20°C.

2.12 DNA ligation

DNA extracted from the agrose was ligated in a total volume of 20µl consisting typically of a ratio of 7:1 of insert to vector 2µl of vector DNA, 14µl of DNA fragment, 2µl of 10x ligase buffer, 1µl of DNA ligase (New England BioLab). Ligation reaction was incubated at room temperature for an hour before transformation into chemically competent bacteria.

2.13 Production of chemically competent FB810 bacterial stocks

20ml of LB broth (Sigma) was inoculated with a single colony of FB810 bacteria and incubated at 37°C for 12 hours. The bacterial culture was then diluted 1:10 and cultured further until the culture had an OD_{600} of 0.5. Cultures were chilled on ice for 15 minutes before being pelleted by centrifugation at 4°C for 20 minutes at 15000 rpm. Bacteria were resuspended in one volume of sterile, chilled. After second centrifugation, the bacterial pellet was resuspended in $1/15^{th}$ of the volume of 50mM $CaCl_2$ in 10% glycerol and incubated for 30 minutes on ice before being aliquoted. The bacteria were then frozen in dry ice/ethanol and stored at -80°C.

2.14 Transformation of bacteria

Chemically competent TOP10 (Invitrogen) *E.coli* bacteria were used to transform DNA ligation reactions following exactly the manufacturer's protocol. For transformation to produce recombinant GST proteins, expression plasmids were transformed in to FB810 chemically competent *E.coli*. 50µl of competent bacteria was alliquoted and incubated on ice to which 20µl of the DNA ligation reaction was added. After 30 minutes incubation on ice the competent bacteria were placed at 42°C for 30 seconds before. Transformed bacteria were then plated on to ampicillin (100µg/ml) supplement agar and allowed to incubate for 12 hours at 37°C. Individual transformants were screened for presence of the clone of interest or used to grow larger cultures for protein purification.

2.15 Plasmid extraction and purification

Small scale extraction and purification of plasmid DNA was done using Qiagen miniprep kits. Single bacterial colonies were inoculated into 5ml of LB broth supplemented with ampicillin at 100µg/ml. Cultures were grown for 12 hours at 37°C in a shaking incubator. Bacteria were then pelleted by centrifugation, resuspended, lysed and neutralised according to the manufacturer's protocol. The supernatant was passed over a affinity column to capture plasmid DNA and after washing the column, DNA was eluted with 50µl of 0.1M Tris pH 0.75. Miniprep DNA was stored at -20°C.

Qiagen Maxi™ kits were used to extract and purify DNA from 500ml bacterial cultures in a similar method to the miniprep kits. DNA was isopropanol precipitated at 4°C for 30 minutes by centrifugation at 30,000 x g. DNA pellets were washed with 70% ethanol and air-dried before being re-dissolved in distilled water. The concentration and purity of DNA was determined by UV spectrophotometry at OD 260nm and OD 280nm.

Protein biochemistry

2.19 Western blotting

Proteins were mixed with protein sample buffer and resolved on SDS-PAGE gels between 6 and 15%, dependent on the molecular weight of the protein. Proteins were then transferred to Hybond PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using semi-dry gel transfer apparatus (BioRad) after which the membrane was incubated in 5% non-fat milk (Marvel) in PBS and 0.05% Tween-20 (Sigma) for 30 minutes at room temperature. Incubation with primary antibodies occurred in 5% non-fat milk in PBS/Tween-20, overnight at 4°C. After the incubation milk was removed from the membrane by washing 3 times for 20 minutes with shaking, at room temperature, using fresh PBS/Tween-20 between washings. A secondary HRP conjugated antibody was then added for an hour at room temperature with shaking. Following this, a further three incubation for 20 minutes with PBS/Tween-20 were done at room temperature to remove excess secondary antibody. Bands were visualised by luminescent detection using ECL+ (Amersham Pharmacia Biotech) and photographic film. A list of primary and secondary antibodies used in this thesis is given in Table 2.4 and Table 2.5.

2.17 Recombinant GST-protein synthesis

FB810 cells transformed with the plasmid of interest were cultured overnight in 20ml of LB broth with ampicillin before being diluted 1:10 in fresh LB and cultured to an OD_{600} of between 0.3 and 0.5. IPTG was added to the culture to a final concentration of 1mM and incubated at 30°C for 2 hours. Cells were pelleted and lysed in Tween lysis buffer with lysozyme (Sigma) at 100µg/ml followed by sonication to extract protein. Lysates were cleared of cell debris by centrifugation at 10,000 rpm for 15 minutes at 4°C before being incubated with 200µl of 50% slurry of GST beads overnight, at 4°C on an orbital rotor. Beads were washed five times in a total of 50 bead volumes with Tween lysis buffer and stored in the buffer at 4°C. Expression and purification of proteins was checked by resolving proteins on a SDS-PAGE gel and visualised with Comassie staining.

2.18 *In vitro* binding assays

GST-proteins bound to beads were washed in a GST binding buffer solution to replace the buffer. Beads were incubated with *in vitro* transcribed/translated protein using the TnT® Quick Coupled System (Promega) according to the manufacturer's protocol. The *in vitro* transcription/translation (IVT) reactions were diluted 5 fold in GST binding buffer to a final volume of 260µl, and centrifuged for 30 minutes at 15,000rpm at 4°C to remove precipitated protein. 50µl of diluted and clarified IVT reactions was added to 25µl of GST affinity beads and incubated for 3 hours at 4°C on an orbital rotor. Beads were pelleted by centrifugation and washed with RIPA buffer 5 times with 40 times the

bead volume. The presence of bound proteins was determined by Western analysis.

2.19 *In vivo* co-immunoprecipitations

DNA plasmids encoding proteins of interest were transfected alone or in combination in U2OS cells seeded at $1x10^6$ cells on a 10cm dish. Cells were harvested 48hrs post-transfection by scraping into 500µl of RIPA lysis buffer with protease cocktail inhibitor (Boehringer Mannheim). Protein concentration was determined by Bradford assay between samples. Prior to immunoprecipitation, lysates were pre-cleared with protein G-beads for 30 minutes at 4°C on an orbital rotor to reduce non-specific binding to the beads. Pre-cleared lysate was incubated with 30µl of protein G-beads and 2µg of specific antibody or isotype control antibody (Upstate Technologies) on an orbital rotor for 90 minutes at 4°C (see Table 2.6 for antibodies). For FLAG tagged protein immunoprecipitations, pre-conjugated FLAG protein on agarose beads (Sigma) were used for immunoprecipitation. After immunoprecipitation, antibody conjugated protein-G beads were washed 3 times with 10 fold bead volumes of the lysis buffer and re-suspended 40µl of Laemmli protein sample buffer. 10µl was resolved on polyacrylamide gel for Western blot analysis.

2.20 *In vitro* kinase assays

Sf9 lysate expressing cdk alone or cyclin/cdk were incubated in kinase assay buffer with GST fusion proteins, 10μCi γ-^{33}P ATP and ATP was added to a final concentration 50μM. Incubations occurred at 30°C for 30 minutes after which GST beads were washed with 500μl of kinase assay buffer, Laemmli protein sample buffer added and proteins resolved on a SDS-PAGE gel. Gels were fixed in 10% acetic acid and 10% methanol for 30 minutes at room temperature before drying to Watman paper (3M). Images were obtained by using a Cyclone™ phosphoimager (Packard).

Gene reporter assays

2.21 Luciferase assays

Luciferase reporters consist of a promoter upstream of a luciferase gene, whose gene product is assayed as a measure of transcriptional activation from that promoter. A list of the promoter constructs with luciferase reporters used in this thesis is given in Table 2.5.

The luciferase enzyme is assayed by inducing a reaction which produces light. Quantification of luminescence is proportional to the amount of the luciferase enzyme and hence a measure of the strength of activation of the reporter. In this reaction, initially the addition of an adenyl group from ATP to the carboxyl group of luciferin ($C_{13}H_{12}N_2S_2O_3$) is catalysed by luciferase to form

adenyl-luciferin and pyrophosphate in a reversible reaction. The emission of light only occurs in a second reaction when adenyl-luciferin reacts with oxygen to produce adenyl-oxyluciferin and light.

Reporter assays were done using a 6 well dish format with samples done in triplicate. Cells seeded at $2x10^5$ were co-transfected with 200ng of reporter luciferase construct as well as 200ng of control plasmid, pEF-LacZ and MED17 and/or p53. Transfected DNA was equalised between samples with pcDNA3 vector only. Cells were harvested for protein 48 hours post transfection into 300µl of 1x reporter lysis buffer (Promega) per well. 20µl of lysate was used for each luciferase assay, to which 100µl of luciferase substrate (Promega) was added. Dispensing of luciferase substrate and luminescence readings were done using Fluroskan Ascent FL (Thermo Electron Corporation) with an integration time of 10 seconds. Results were displayed as a fold change in luminescence relative to the vector only control and normalised to ß-galactosidase values (see section 2.22). Cell lysates were also used for Western blots to check protein expression.

2.22 ß-galactosidase assays

Co-transfection of the pEF-LacZ plasmid allowed normalisation of the luciferase values between samples by assaying for ß-galactosidase activity. A typical reaction consisted of 30µl of reporter cell lysate, 160µl of ONPG (10mM) and 700µl of Z-buffer. The reaction was incubated for 15 minutes at 37°C until yellow and the reaction stopped by the addition of 400µl of Na_2CO_3 (1M). OD_{420} was then measured to obtain ß-gal value. The normalised luciferase value was

determined as a ratio between the raw luciferase value and the ß-galactosidase value.

Cellular staining and analysis

2.23 Propidium iodide (PI) staining

Cells were seeded at $1x10^6$ in 10cm dishes and treatment with nocadozole at 40nM for 48 hours. Cells were then trypsinised, washed in PBS and fixed with ice cold 70% ethanol for a minimum of 2 hours at 4°C. Cells were washed in PBS before adding 50µl of a 100µg/ml stock of Rnase A and 200µl of a 50µg/ml stock of PI and allowed to incubate for 30 minutes at room temperature. The cells were washed again in PBS and filtered before FACS analysis. Using the doublet detection module to gate the single cells from the doublets, polyploidy population of cells were counted as those cells with DNA content greater than 2N.

2.24 Immunofluoresence assay (IFA) and centrosome analysis

$1x10^4$ cells were seeded on to glass cover slips in 60mm dishes. The media was then aspirated and cells washed twice with PBS. Cells were then fixed and permeabilised with ice cold methanol for 10 minutes at -20°C followed by rinsing with cold acetone for 1 minute at room temperature. To prevent non-specific binding of the antibody, permeablised cells were blocked with 1% fraction V foetal BSA (Sigma) in PBS for 30 minutes, changing the blocking

solution twice. Incubation with the γ-tubulin antibody conjugated to CY3 (Sigma) was done in 1% fraction V BSA/PBS for 1 hour at room temperature, using the antibody at a concentration of 1:200. Coverslips were washed a further three times in BSA/PBS over 15 minutes, with Hoescht 33342 dye at a final concentration of 5μM added in the last wash to allow visualisation of nuclei. Coverslips were mounted onto glass slides with anti-fade mounting media (0.1% phenylenediamine in 60% glycerol), allowed to dry and then sealed with nail varnish. Centrosomes and nuclei were visualised using confocal microscopy (Leica system at a magnification of 125x).

2.25 Annexin staining

$1x10^6$ cells lines were seeded on 10cm dishes before treatment with either 1μM staurosporine (Sigma) or 100μM etoposide (Sigma) for 24 hours to induce apoptosis. Cells were harvested by trypsinization and stained with annexin-V conjugated to FITC and PI (Molecular Probes) following manufacturer's protocol. Cells were collected by FACS and gated to separate cells from debris and then sorted according to PI and annexin staining. Cells staining with annexin-V only were quantified to measure induction of apoptosis.

2.26 Matrigel cell invasion assay

Sterile invasion chambers (Chemicon® International) were rehydrated using 300µl of warm serum free DMEM for 1 hour at room temperature. During rehydration of the extracellular matrix layer a suspension of the vector/MED17 cells at $1x10^6$ cells/ml was prepared in serum free medium. Serum free DMEM was aspirated from the upper chamber and replaced with 300µl of the cell suspension. To the lower chamber was added 500µl of supplemented DMEM. The invasion chambers were incubated for 48 hours under standard cell culture conditions after which non-invading cells and the extracellular matrix from the chamber were removed using a cotton-tipped swab. Inserts containing invasive cells visualised by light microscopy after staining for 20 minutes at room temperature and then rinsing in water before drying. Matrigel assays were done in triplicate for both the vector and MED17 cell lines.

Gene Expression Microarray (GEM) and analysis

2.27 RNA extraction

RNA was extracted from cells using an Rneasy kit (Qiagen). $2x10^6$ cells were lysed on 10cm dishes using RLT buffer, supplemented with ß-ME, and harvested by scrapping. Samples were then homogenised by passing crude lysates through a QIAshredder column (Qiagen) by centrifugation and the subsequent clarified lysates were collected, to which 350µl of 70% ethanol was added. This mixture was passed through an Rneasy spin column and washed with buffers RW1 and RPE according to the manufacturer's protocol. RNA was eluted into a clean eppendorf tube with 30µl of Rnase-free water. RNA quality and quantity was analysed using an Agilent Bioanalyser (Agilent Technologies).

2.28 cRNA synthesis, labelling and gene chip hybridisation (see Fig 2.1)

To synthesise labelled cDNA for hybridisation to the gene chip, 10µg of total RNA was used to generate first-strand cDNA using a T7-linked oligo(dT) primer and was followed by cRNA synthesis, using biotinylated UTP and CTP (Enzo Diagnostics), by *in vitro* transcription. The target cRNA synthesised were then processed according to manufacturer's recommendations using an Affymetrix GeneChip© Instrument System. Spike controls were added to 20µg of fragmented cRNA before hybridisation overnight to Affymetrix mouse 430A microarrays (www.affymetrix.com/product/arrays/specific/mouse430.affx). Spike controls are targeted cRNAs derived from different species and are used to

confirm threshold detection of low abundance transcripts which is masked excessive variation in the background intensity, termed noise. After hybridisation, arrays were washed and stained with streptavidin-phycoerythrin before scanning on an Affymetrix GeneChip© scanner. Scanned images were first assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches. 3'/5' ratios for GAPDH were checked to be within acceptable limits and were used to confirm suitable amplification of the RNA as well as checking for sample degradation. Spike controls BioB, BioC, BioD and CreX were all present in increasing intensity as expected confirming threshold detection of low abundance transcripts.

2.29 Microarray data processing (in collaboration with Dr.M.Trotter)

Array probe-set expression values were background corrected, normalised and summarized using default parameters of the RMA model (Irizarry et al., 2003). All array processing was performed using the "*affy*" package of the *Bioconductor* (www.bioconductor.org) suite of software for the *R* statistical programming language (www.r-project.org). The resulting gene expression data set contained processed expression values for 22690 probe-sets (Affymetrix *MOE430A* GeneChip©). The expression set was analysed to assess the significance of differential gene expression between cell lines, which were both done in triplicate arrays.

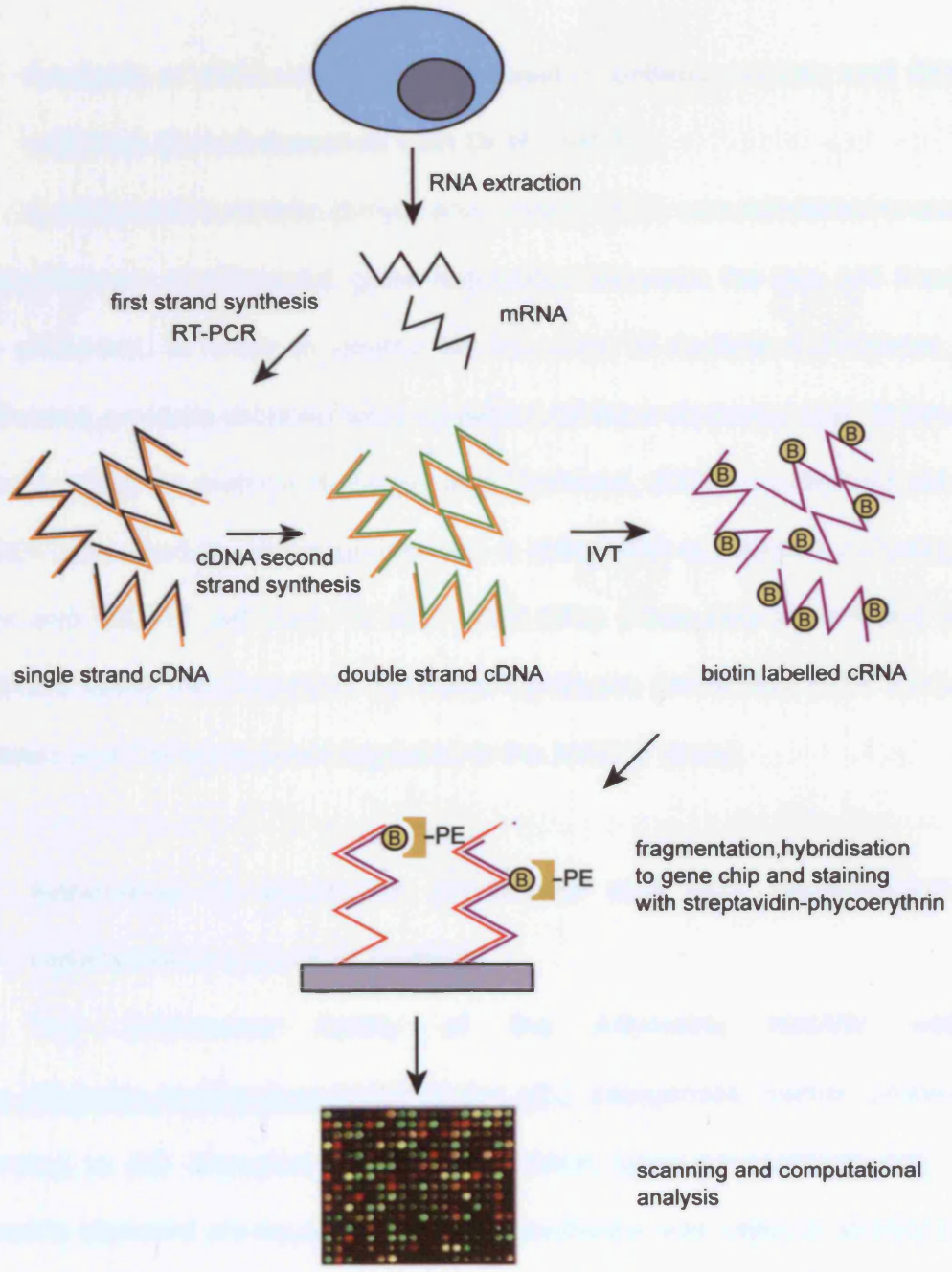
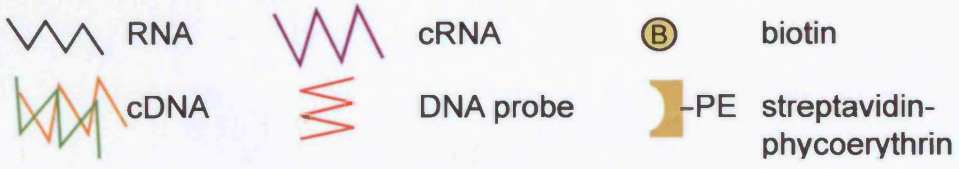


Fig 2.1 Preparation of RNA for oligonucleotide array analysis

RT-PCR - reverse transcriptase polymerase chain reaction
IVT - *in vitro* transcription reaction



2.30 Analysis of differential gene expression between vector and MED17 cell lines (in collaboration with Dr.M.Trotter)

A moderated t-statistic (Smyth and Speed, 2003) was calculated to assess the significance of differential gene expression between the two cell lines for each probe-set. In order to control for the error of multiple hypotheses, the significance *p-values* obtained were corrected for false discovery rate, to become *q*-values, using the method of (Storey and Tibshirani, 2003). A threshold value of $q<0.001$ was used to select genes with a differential expression between the vector and MED17 cell lines. In total, 1858 array probe-sets were found to be significant using this threshold. Of these significant probe-sets 1124 were up-regulated and 734 were down-regulated in the MED17 group.

2.31 Annotation of significant probe-sets and data visualisation (in collaboration with Dr.M.Trotter)

The *GOBrowser* facility of the Affymetrix *NetAffx* website (www.affymetrix.com/analysis/netaffx/index.affx) categorises marker probe-sets according to GO Biological Process annotation (www.geneontology.org). The browser's standard chi-square test of independence was used to identify over-represented GO Biological Process categories among the 1858 significantly changed probe-sets. Heat maps were created for genes present in these biological process categories by exporting RMA gene expression scores into *dChip v1.3* programme (http://biosun1.havard.edu/complab/dchip/).

2.32 Analysis of probe-set data using Ingenuity Pathway analysis

Ingenuity Pathway Analysis is a web-based programme that allows access to a large database consisting of many modelled relationships between proteins and is used to identify pathways involved in biological functions from microarray data. The database is continuously reviewed by literature searches allowing pathways to be updated based on current published data. Ingenuity Pathway Analysis was applied to the MED17 cell line marker probe-sets to identify signal pathways whose regulation may be affected by MED17 over-expression. In addition to clustering of genes by biological function, as done using *GOBrowser*, the Ingenuity Pathway analysis identifies relationships between genes including protein-protein interactions and transcription networks. By using two independent analysis programmes, comparisons can be drawn between the two analyses and with the aim of identifying transcription factors and networks regulated by MED17 identified.

To analyse the marker probe-set data from the microarrays of the MED17 cell line, data was uploaded to the web based application (www.ingenuity.com/product/pathways_analysis.html) in a text document format containing probe set IDs with corresponding *p*-values. Global and network analysis of the data was downloaded and displayed accordingly. Network diagrams displayed in this thesis were generated by the Ingenuity Pathway analysis application.

Real-time Quantitative Polymerase Chain Reaction (Q-PCR)

2.33 Q-PCR general technique

Quantitative RT-PCR was used to determine the relative expression of mRNA from a multiple tissue cDNA panel of human tissues (Clontech). Due to the sensitivity of the technique, extra precaution was taken to prevent contamination of samples. Such measures included, preparation, storage of samples in separate areas as well as autoclaving and filter sterilisation water. Filtered pipette tips and eppendorf tubes were also autoclaved and irradiated at 254nm in a UV cross-linker (Stratagene Stratalinker 2400) prior to use. Designated pipettes were also irradiated prior to use.

Quantification of gene expression was done using the ABI Prism 7700 Sequence Detection System. The Q-PCR technique is based on the principle of detection of the amplified product of the PCR reaction by labelling with a fluorescent dye. The SYBR green I dye achieves this by staining double stranded DNA formed by the amplified sequence. For quantifying the MED17 transcript, the SYBR green I dye was used.

To allow normalisation of the SYBR green signal between wells, an internal reference dye (ROX) is incorporated into the PCR AmpliTaqGold® buffer; its signal is unaffected by the PCR reaction. Normalised reporter signals (R_n) from reactions containing cDNA template (R_n+) and non-template (R_n-) controls are calculated before there is detectable fluorescence from the SYBR green.

The normalised reporter signals from the template and non-template controls are further used to calculate ΔR_n by subtraction of R_n- from R_n+. ΔR_n can be plotted on an amplification plot against cycle number to analyse the amplification of the sequence (Fig 2.2). During early cycles small changes are seen in ΔR_n on the amplification plot, which is known as the baseline. Increases in ΔR_n above this baseline indicate detection of amplified products from the PCR reaction. To determine the threshold cycle (C_T), the cycle at which amplification of the product becomes statistically significant, a threshold is calculated as the average standard deviation of ΔR_n in the early cycles of the reaction, multiplied by an adjustment factor. Ideally on the amplification plot the threshold should lie in the linear range of the plot. From the threshold, C_T is then obtained.

Q-PCR was also done for ubiquitously expressed housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is used to normalise between samples. In a separate reaction, Q-PCR for GAPDH was done and C_T values calculated in the same manner. The difference between C_T values for MED17 and GAPDH was calculated to give ΔC_T. These values were further normalised to one of the samples on the panel of cDNAs by subtracting the ΔC_T of each sample to give $\Delta\Delta C_T$. The final value for the relative expression of MED17 was calculated by expressing the figure as a negative power of 2 ($2^{-\Delta\Delta CT}$).

The hybridisation of primers to one another, to form primer dimers, can affect the fluorescent detection of the primers hybridised to the template cDNA is a false positive result. Formation of primer dimers can be detected using a

dissociation curve programme. The dissociation curve programme initially denatures the cDNA and then heats the reaction from 60°C to 95°C over 20 minutes to induce annealing and hence generates a fluorescent signal when 50% of the primers dissociate. The presence of more than one signal, at different temperatures, during the running of the dissociation curve programme suggests formation of primer dimers, the amplification of more than one product, or the contamination of the template cDNA (Fig 2.3). The dissociation curve programme was run at the end of each reaction.

2.34 Oligonucleotide design for Q-PCR

Primers for Q-PCR (Qiagen-Operon, Cologne, Germany) were designed using the Primer Express® software programme (PE Applied Biosystems, UK) based on default parameters and guidelines. Primers used for Q-PCR are shown in Table 2.3.

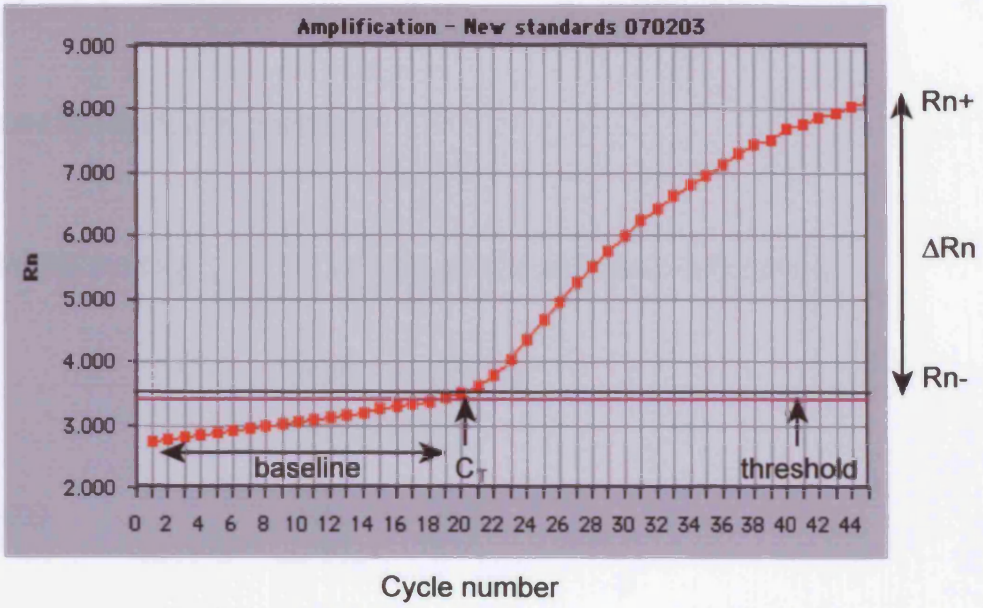


Fig 2.2 Amplification plot illustrating terms used in Q-PCR

C_T - threshold cycle

ΔRn - Change in normailsed reporter signal

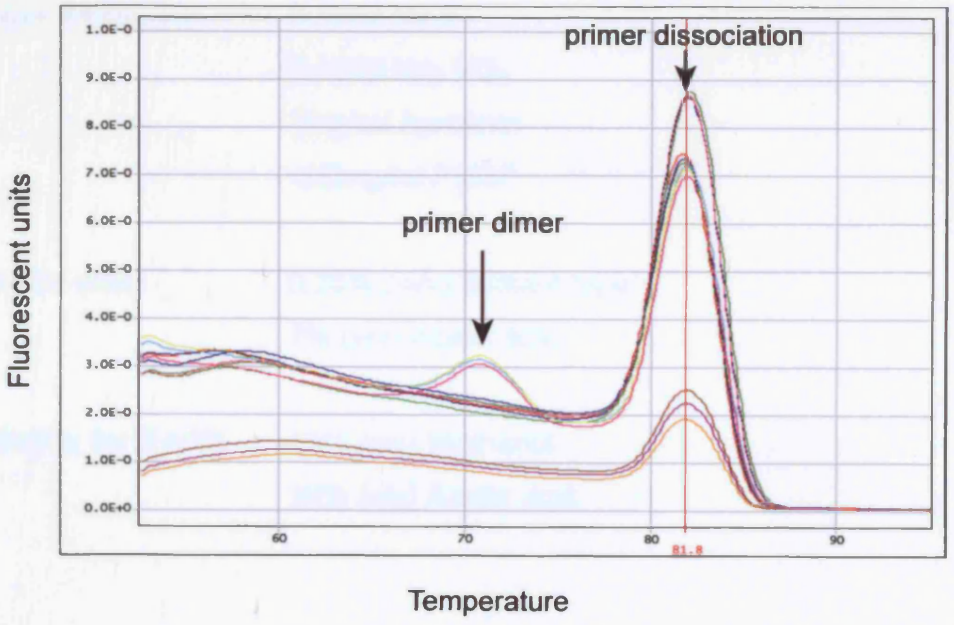


Fig 2.3 Dissociation curve illustrating primer dimer formation

Solutions for standard methods

10x Lithium acetate	1M Li Ac in sterile water and autoclaved.
1x TE	10mM Tris pH 7.5 1mM EDTA
TAE (50x)	242g Tris base 57.1ml Acetic acid 100ml 0.5M EDTA Make to 1L with dH_2O and adjust pH to 8.5
DNA loading buffer	60% (w/v) sucrose solution 0.1% (w/v) bromophenol blue
Protease Inhibitors	0.1mM Na F 0.1mM Na_3 VO_4 2mg/ml Aprotinin 100mg/ml PMSF
Coomasie stain	0.25% (w/v) Brilliant blue 7% (v/v) Acetic acid
Destain/Fix for PAGE	10% (v/v) Methanol 10% (v/v) Acetic acid

Laemmli Sample Buffer (4x)	250 mM Tris pH 6.8 0.02% (w/v) BPB 4% (w/v) 2-Mercaptoethanol 8% (w/v) SDS 40% (v/v) Glycerol

Solutions for Protein Biochemistry

Sf9 lysis buffer	0.1% (w/v) Tween 20 50mM Tris pH 7.5 50mM NaCl 2mM EGTA 1mM EDTA 1mM DTT 50% (v/v) glycerol + cocktail protease inhibitor
Tween lysis buffer	50mM Hepes pH 7.5 150mM Na Cl 2.5mM EGTA 1mM EDTA 1mM DTT 0.1% (w/v)Tween-20 10mM ß-glycerophosphate + cocktail protease inhibitor

RIPA lysis buffer	50mM Tris pH 8.0
	150mM NaCl
	1% (w/v) NP-40
	0.1% (w/v) SDS
	+ cocktail protease inhibitor
GST binding buffer	20mM Hepes pH7.6
	50mM KCl
	2.5mM $MgCl_2$
	0.02% (w/v) NP-40
	1mM dithiothreitol (DTT)
	10% (v/v) glycerol
	1mM PMSF
NETN	20mM Tris pH 8.0
	100mM NaCl
	1mM EDTA
	0.5% (w/v) NP-40
10x Kinase assay buffer	500mM Hepes pH 7.4
	100mM $MgCl_2$
	10mM DTT
100mM ATP stock	60mg ATP
	adjust pH with NaOH to pH 7.0
	Distilled water to 1ml

Solutions for reporter assays

Z- buffer

60mM $Na_2HPO_4.7H_2O$
40mM $Na_2HPO_4.H_2O$
10mM KCl
1mM $MgSO_4$
50mM ß-ME

Add dH_2O to 80% of final volume, adjust pH to 7.0, add remaining volume and store at 4°C.

Table 2.1 Cell lines used in this thesis

Cell line	ATCC number	Morphology	Organism	Growth properties
U2-OS	HTB-96	Osteosarcoma with epithelial morphology, p53 wt	*Homo sapien*	Adherent
MG-63	CRL-1427	Osteosarcoma with fibroblast morphology, p53 mutated	*Homo sapien*	Adherent
NIH-3T3	CRL-1658	Fibroblasts, p16 INK4a and p19 ARF transcriptionally silenced, p53 wt	*Mus musculus*	Adherent
Sf9	CRL-1711	Epithelial	*Spodoptera frugiperda*	Adherent/suspension

Table 2.2 Expression construct used in this thesis

vector	gene	cloning/reference
pCDNA3 (Invitrogen)	MED17 HA (wt)	PCR, *EcoR I/Xho I*
	MED17 HA (288A)	Created by site mutagenesis
	MED17 HA (573A)	
	MED17 HA (647A)	
	MED17 HA (573/647A)	
	MED17 (ΔSP)	
	Cdk4 HA	(van den and Harlow, 1993)
	vcyclin (2xFLAG tagged)	(Swanton *et al.*, 1997)a)
	cyclin D1 (2xFLAG tagged)	
pOP RSV-1 (Stratagene)	MED17 HA (wt)	Sub-cloned from pCDNA3 MED17 HA *Kpn I/Xho I*
pGEX 6P-1 (Amersham Bioscience)	MED17 (wt)	PCR, *EcoR I/Xho I*
	MED17 aa148-651	PCR, *EcoR VI/Xho I*
	MED17 aa148-651 (288A)	Created by site mutagenesis
	MED17 aa148-651 (573A)	
	MED17 aa148-651 (647A)	
	MED17 aa148-651 (573/647A)	
	MED17 aa148-651 (ΔSP)	
pCB6+	p53 (wt)	(Clark et al., 2002)
pEF	LacZ	(Dalton and Treisman, 1992)
pGBD-U1	V-cyclin (wt)	*Bam HI* digest ligated in to *Sal I* digested vector
	V-cyclin aa1-251	*Pst I* restriction from wt
	V-cyclin aa1-168	*Bgl II* restriction from wt
	V-cyclin aa169-254	*Bgl II/Sal I* restriction from wt in to vector *Bam I/Sal I*
	V-cyclin aa216-254	*Pst I* restriction fragment

Table 2.3 Primer sequences used in this thesis

Primer	Use	Sense/anti-sense	Sequence
MED17	MED17 cloning/ sequencing	S	CG**GAATTC**CCACCATGTCCGGGGTGCGCGCAGTGCGG
MED17 HA		A	CGG**CTCGAG**TCAAGCGTAGTCTGGGACGTC GTATGGGTATAGTAGACAAGGGCTAAGTGC
MED17 (S288A)	Site mutagenesis	S	CCAAACCAGGTGCCCCACATTGGCAGACAAAATTAG
MED17 (S288A)		A	GTCTGCCAATGTGGGGCACCTGGTTTGGATTTGG
MED17 (S573A)		S	GCCATCACGGTGGCCGCCCCAAGTGG
MED17 (S573A)		A	CCACTTGGGGCGGCCACCGTGATGGC
MED17 (S647A)		S	GCTTATGTCTGCACTTGGCCCTTGTCTACTA
MED17 (S647A)		A	TAGTAGACAAGGGCCAAGTGCAGACATAAGC
MED17	qRT-PCR	S	CTAAGGAGTAGAGCTGCTGCAACC
MED17		A	ATTTGACCAATGAGCCTGTATCTGA
GAPDH		S	GGAGTCAACGGATTTGGTCGTA
GAPDH		A	ACTCTGGTAAAGTGGATATTGTTGCC
101	MED17 sequencing	A	GCCAGACGCGCCAGATTCTGCGACAT
303		S	CCTGAAGATTACTGTCCTCTTGATGTCC
303		A	GGACATCAAGAGGACAGTAATCTTCAGG
403		S	CCGAGCTTGCAGTTATCTTTGTGC
403		A	GCACAAAGAAATAGATAACTGCAAGCTCGG
501		S	GCAAAGCATATTTTTCTAAGGAGTAGAGC
501		A	GCTCTACTCCTTAGAAAAATATGCTTTGC
601		S	CATGCAGTTCAGCAACTCGCCAAGG
601		A	CCTTGGCGAGTTGCTGAACTGCATG

GAATTC – Eco R1
CTCGAG – Xho I

Table 2.4 Primary Antibodies used for Western blotting

Antibody/catalogue number	Isotype	Company
HA.11	Mouse Ig G	BAbCO, Berkeley, Ca.
HA (12CA5)	Mouse Ig G	Cancer Research UK
p53 (DO-1)	Mouse Ig G	Santa Cruz Biotechnology, Ca.
p53 (PAb421)	Mouse Ig G	Calbiochem
p21 (C-19)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.
Mdm2 (SMP-14)	Mouse Ig G	Santa Cruz Biotechnology, Ca.
Caspase-9 (SA-321)	Rabbit Ig G	BIOMOL
HSP40 (611780)	Mouse Ig G	BD Transduction Laboratories
HSP60 (611562)	Mouse Ig G	BD Transduction Laboratories
ß-actin (Ab-1)	Mouse Ig M	Oncogene
GAL4 (RK5C1)	Mouse Ig G	Santa Cruz Biotechnology, Ca.
Rb (554136)	Mouse Ig G	BD Pharmingen
cdk4 (C-22)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.
cdk6 (C-21)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.
cdk2 (M2)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.
FLAG (M1 monoclonal) (F 3040)	Mouse Ig G	Sigma

Table 2.5 Secondary antibodies used for Western blotting

Antibody/catalogue number	Isotype	Company
Anti-mouse-HRP (sc-2060)	Ig G	Santa Cruz Biotechnology, Ca.
Anti-mouse-HRP (401225)	Ig M	Oncogene
Anti-Rabbit-HRP (323-005-021)	Ig G	Jackson ImmunoResearch Inc.

Table 2.6 Antibodies used for co-immunoprecipitation

Antibody	Isotype	Company
p53 (DO-1)	Mouse Ig G	Santa Cruz Biotechnology, Ca.
FLAG (M1 Agarose) (A4596)	Mouse Ig G	Sigma
cyclin D1 (CC11)	Mouse Ig G	Oncogene
cyclin E (M-20)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.
cyclin A (H-432)	Rabbit Ig G	Santa Cruz Biotechnology, Ca.

Table 2.7 Reporter constructs used in this thesis

vector	reporter promoter	company/reference
pGL3	Bax	(Lomax et al., 1998)
pGL3	Bax (promoter and intron RE)	(Thornborrow et al., 2002))
p21-luc	p21 Cip	(El Deiry *et al.*, 1993)
PG13-luc	p53 RE	(Maestro et al., 1999)
Mdm2-luc	Mdm2	(Ard et al., 2002)
pHSE-Luc	heat shock factor	BD Biosciences
pAP-1-Luc	AP-1	BD Biosciences
TOPFLASH	Lef/Tcf	(Coghlan et al., 2000)

Chapter 3

3.0 Introduction

Mediator is a transcriptional co-factor complex recruited to DNA bound transcription factors, such as the thyroid hormone receptor, to activate or repress gene transcription. It is hypothesised that Mediator complex bridges transcription activators and the basal transcription machinery to activate RNA pol II. Further, Mediator is conserved among many species, suggesting it has an essential role in regulating transcription. In a yeast-2-hybrid screen, MED17, a subunit of the Mediator complex was identified as an interacting protein with a viral cyclin closely related to vcyclin of KSHV.

MED17 is an 80KDa protein which forms one of the core components of the Mediator complex. Studies of human and *Drosophila* MED17 have suggested its interaction with a number of transcription factors and is thought to co-operate in the activation of transcription. Human MED17 has been shown to interact with p53 and VP-16, an interaction that may be required for their activation of transcription by the Mediator complex (Ito *et al.*, 1999). Similarly, the *Drosophila* homologue, dMED17, interacts with the heat shock factor transcription factor and is recruited to sites of active transcription (Park *et al.*, 2001b). These data further suggest a role for MED17 as a transcription co-activator which may function with other Mediator subunits to recruit the basal transcription machinery to specific DNA bound transcription factors. Many of these studies of MED17 and Mediator,

especially the human protein, have been done *in vitro.* No studies have been done specifically investigating MED17 function in mammalian cells.

A yeast-2-hybrid screen using the *Herpesvirus saimiri* cyclin as bait, revealed an interaction with MED17 (Laman et al., 2001a) and suggests possible interactions with cellular D-type cyclins, as well as the viral homolog, vcyclin of KSHV. The known interaction between MED17 and p53 further suggests a relationship between the cell proliferative properties of the cyclins and transcriptional regulation of p53, via MED17. In the absence of any *in vivo* functional data on MED17 in mammalian cells, the aims of this chapter were to investigate the transcriptional properties of MED17 on p53 mediated transcription as well as the consequential effects on cell cycle arrest and apoptosis.

Chapter 3: Results

3.1 Expression of an HA tagged MED17 *in vivo*

An epitope tagged MED17 expression construct was generated to study MED17 function *in vivo*. Expression of an epitope tagged protein allows specific detection of the exogenous protein but not the endogenous protein. Sequences encoding an haemagglutin (HA) epitope were incorporated into oligonucleotide primers which were used to amplify MED17 cDNA from a human foetal cDNA library. These oligonucleotide primers introduced the HA epitope to the C-terminus of the MED17 protein. Initial attempts were made to detect MED17 expression with either a FLAG or HA epitope at the N-terminus, but were unsuccessful. Expression of these proteins was undetectable *in vivo* despite in frame fusions to the epitope. The failure to detect MED17 with an N-terminus epitope may be attributed to this region of MED17 being critical in the post-translational processing of the protein, which could have been affected by the addition of the epitope.

MED17 HA was cloned into a pCDNA3 expression vector and transfected into human osteosarcoma cells, U2OS, to test for expression of the protein. Cell lysates were harvested 48 hours post transfection and analysed by immunoblotting for the presence of the HA epitope. In transcriptional co-factor complexes, TRAP and CRSP, MED17 has been reported to have approximate masses of 80KDa and 77KDa respectively. Immunoblot analysis with HA antibody, 12CA5, of the cell lysates prepared from the transfected U2OS cells

detected a protein of approximately 75KDa, however the protein was also detected in the non-transfected control suggesting this HA antibody was cross reactive with a protein of similar size to MED17 (Fig 3.1A).

Immunoblots were also performed with an alternative HA antibody, HA.11, to avoid cross reactivity with the similarly sized protein, which also detected a protein with a molecular weight in agreement with those previously observed for MED17 in the transcriptional co-factor complexes. Furthermore, no other proteins were detected in the non-transfected and the CDK4 HA transfected positive controls, suggesting specific detection of MED17 HA (Fig 3.1B). Expression of this protein was at a significantly lower level than that for the CDK4 HA transfected positive control which indicated the MED17 protein was not highly over-expressed. Similar levels of expression were seen at 0.5μg to 2μg of transfected DNA. Alternatively, these data could indicate the relatively high turnover of the MED17 protein within the cell. However, these results indicated that MED17 HA was expressed in U2OS cells from a transfected pcDNA expression construct. The HA.11 antibody was therefore used in all subsequent Immunoblots for detection of MED17 HA.

To test expression from the pcDNA MED17 HA, further studies were also conducted in other tumour cell lines, H1299 (p53 null human small cell lung tumour) and MG63 (p53 null human osteosarcoma) (Fig 3.1C and 3.1D). Similar to the results observed in the U2OS cells, a protein in the 75KDa range was specifically detected by Western blotting for HA. These data show that this

MED17 HA expression construct was able to express MED17 in different tumour cell lines and was used for further *in vivo* studies.

3.2 MED17 transactivates a heat shock gene reporter

dMED17 has previously been shown to transactivate heat shock factor (HSF) mediated transcription in reporter assays (Park *et al.*, 2003). To investigate whether MED17 functions as a transactivator of HSF transcription in human cells, reporter assays were performed using a constructs carrying heat shock element (HSE) upstream of the luciferase gene. MED17 was co-transfected with the HSE reporter and a ß-galactosidase expression construct, as a transfection control, into U2OS osteosarcoma cells. Transcriptional activation of the reporter was measured using luciferase assays and normalised to the amount of ß-galactosidase activity. The HSE-luciferase reporter showed a dose dependent activation, to a maximum of 30-fold, in response to transfection of increasing amounts of MED17, compared to the vector alone (Fig. 3.2A).

To confirm the *in vivo* activation of HSE-responsive genes indicated by the reporter assays, cell lysates from the reporter assay were tested for the expression of heat shock proteins. The levels of two differentially regulated heat shock proteins, HSP40 and HSP60 were assayed by Western blotting. HSP40 expression is induced by cellular stress in contrast to HSP60 whose expression is constitutive (Fink, 1999). Both HSP40 and HSP60 expression were moderately enhanced with increasing expression of MED17 (Fig. 3.2B). These data show that MED17, in the absence of cellular stress, enhanced expression of an

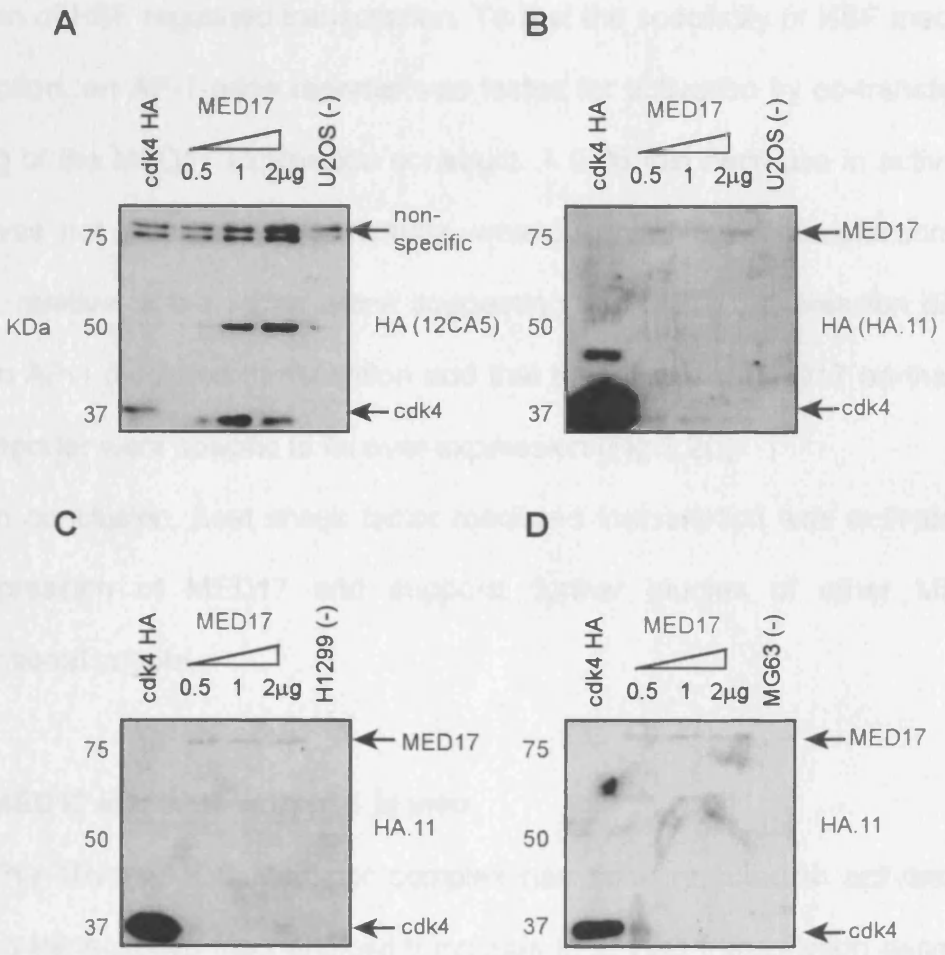


Fig 3.1 *In vivo* expression of HA tagged MED17 in tumour cell lines

Western blots of MED17 HA expressed from a transfected pcDNA3 construct. Increasing amounts of construct were transfected into U2OS, H1299 and MG63 tumour cell lines and protein lysates prepared for Western blot with anti-HA antibody.

(-) untransfected cells, (+) transfected cdk4 HA positive control

A. Detection of MED17 HA with anti-HA antibody 12CA5
B. Detection of MED17 HA with anti-HA antibody HA.11
C and D. MED17 HA expression in tumour cell lines, H1299 and MG63, respectively.

inducible and constitutively expressed HSP, which may be attributed to the activation of HSF regulated transcription. To test the specificity of HSF mediated transcription, an AP-1 gene reporter was tested for activation by co-transfecting with 2μg of the MED17 expression construct. A 0.05 fold decrease in activation, which was not statistically significantly, was observed on co-transfection with MED17, relative to the vector alone suggesting that MED17 expression did not affect on AP-1 mediated transcription and that the effects of MED17 on the heat shock reporter were specific to its over-expression (Fig 3.2C).

In conclusion, heat shock factor mediated transcription was activated by over-expression of MED17 and supports further studies of other MED17 transcriptional targets.

3.3 MED17 interacts with p53 *in vivo*

The TRAP/SMCC Mediator complex has been reported to activate p53 mediated transcription from artificial templates in *in vitro* transcription assays, a function that may require MED17, a Mediator subunit reported to interact with p53 *in vitro* (Ito *et al.*, 1999). However, the *in vivo* function of MED17 in the regulation of p53 transcription has not been studied.

To test whether the interaction between p53 and MED17 occurred in human cells *in vivo*, cell lysates from U2OS cells transfected with MED17 were immunoprecipitated with antibodies to p53, and analysed for the presence of MED17-HA by Western blotting for the HA epitope. MED17 co-immunoprecipitated specifically with p53 from a U2OS cell lysate, but not with an

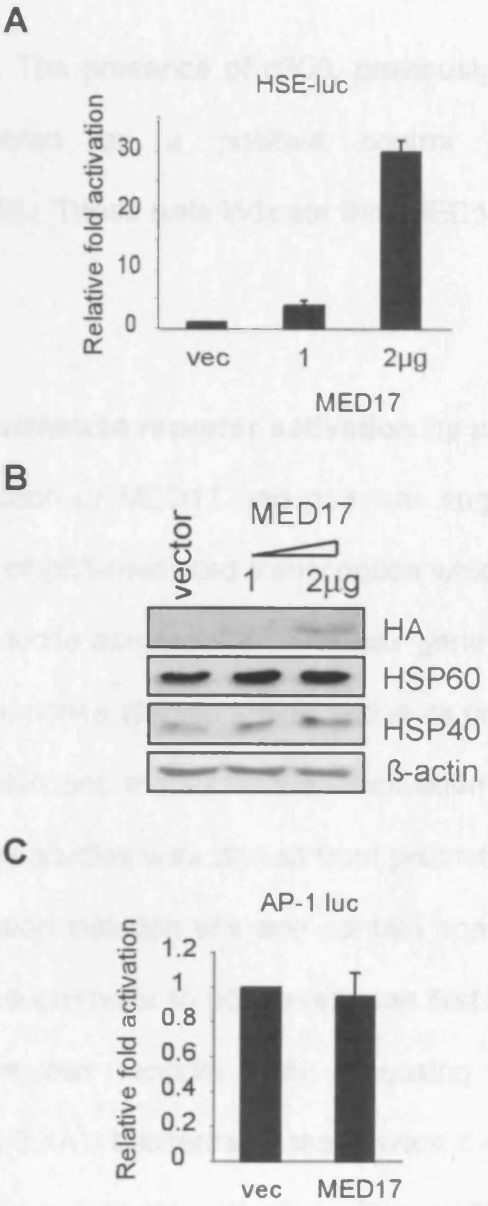


Fig 3.2 MED17 transactivates a heat shock gene reporter

The Drosophila homolog of MED17 has been reported to activate transcription mediated by heat shock factor (HSF). Human MED17 was tested for activation of HSF transcription *in vivo* using a luciferase gene reporter in U2OS cells.

A. Gene reporter assays with a heat shock responsive luciferase reporter, co-transfecting with increasing amounts of MED17.
B. Immunoblots of reporter cell lysates detecting MED17 HA, HSP40 and HSP60 expression.
C. AP-1 (Activator Protein-1) luciferase reporter co-transfected with 2µg of MED17.

isotype control (Fig. 3.3). The presence of p300, previously identified as a p53-interacting protein, served as a positive control for the equivalent immunoprecipitation of p53. These data indicate that MED17 interacted with p53 *in vivo*.

3.4 Analysis of *bax*-luciferase reporter activation by p53 over-expression

The *in vivo* interaction of MED17 and p53 was suggestive of a role for MED17 in the regulation of p53-mediated transcription which was subsequently investigated using a *bax* luciferase reporter. The *bax* gene is a p53 responsive gene and contains p53 response elements (RE) within its promoter region which mediate p53 binding to DNA and the subsequent activation of transcription. The *bax* reporter used in these studies was cloned from promoter sequences 370bp upstream of the transcription initiation site and contain one consensus p53 RE. The responsiveness of this promoter to p53 levels was first tested in U2OS cells by co-transfection of the *bax* reporter with increasing amounts of a p53 expression construct (Fig 3.4A). Luciferase assays were then conducted on the cell lysates to assess transcriptional activation. The addition of 50ng of p53 increased transcriptional activation of the *bax* reporter by 45 fold relative to the vector control. This activation was further increased by 10 fold with transfection of 100ng of p53, increasing reporter activation to 59 fold (+/-5 fold), as compared to the vector control. With transfection of 200ng of p53, reporter activation was decreased, a phenomenon that may be attributed to the squelching of transcription co-activators. Immunoblot analysis of cell lysates used in the

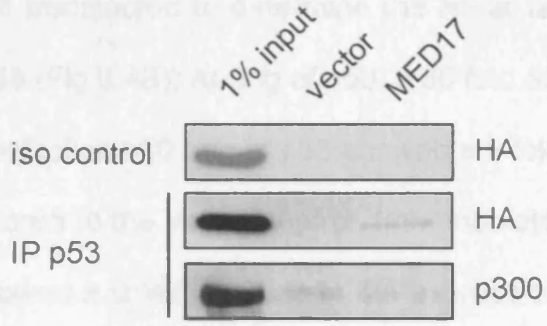


Fig 3.3 MED17 interacts with p53 *in vivo*

Western blot for HA tagged MED17 co-immunoprecipitated with endogenous p53 from U2OS cell lysates. Isotype matched antibody was used as a control in an identical IP reaction. Immunoblotting for p300 was used as a positive control for the immunoprecipitation of p53.

reporter assays show a dose dependent increase in the expression of p53, with increasing amounts of transfected p53 expression construct. These results indicate maximal activation of the bax reporter to approximately 60 fold was achieved when 100ng of p53 was introduced.

This experiment was repeated with a decreasing titration on a log scale in the amount of p53 transfected to determine the linear range of activation of the *bax* reporter on p53 (Fig 3.4B). At 5ng of p53, a 30 fold activation (+/-10 fold) was observed and transfection of 0.5ng of p53 showed a 9 fold activation (+/-1 fold) of the reporter compared to the vector control. Immunoblots of cell lysates from the reporter assay showed a small increase in the expression of p53 between vector only and 0.5ng of p53 compared to other transfected amounts of p53. These data indicate transfection of p53 between 0.5ng and 50ng activated the *bax* reporter within a linear range.

3.5 MED17 represses activation of a *bax*-luciferase reporter

To analyse the effect of MED17 on p53-regulated transcription, the *bax* reporter was co-transfected with a titration of MED17 HA expression construct into U2OS cells. Transfection of 0.25, 0.5 and 1µg of the expression construct resulted in 0.61 (+/- 0.06), 0.65 (+/- 0.08) and 0.56 (+/- 0.04) fold activation of the *bax* reporter, which was equivalent to a 39%, 35% and 44% reductions in reporter activation compared to the vector control (Fig 3.5A). Surprisingly, the reporter did not show a dose dependent decrease in activation with increasing amounts of MED17 HA. Cell lysates prepared for the reporter assays were also

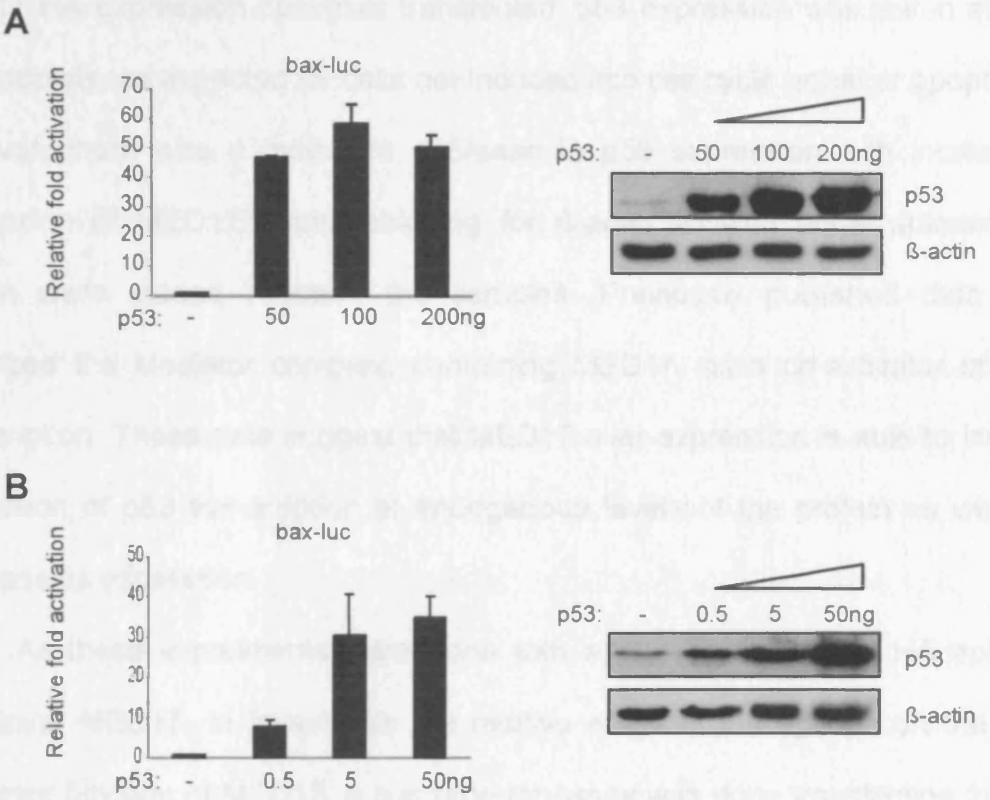


Fig 3.4 Transactivation of a *bax*-luciferase gene reporter on co-transfection with p53

bax gene reporter assays in U2OS cells co-transfecting with a p53 expression construct to determine sensitivity of reporter to enhanced expression of p53. Adjacent to bar charts are corresponding Western blots of cell lysates used in the reporter assays, blotting for p53 and ß-actin.

A. *bax* gene reporter transcactivation on co-transfection with 50, 100 and 200ng of p53
B. *bax* gene reporter transcactivation on co-transfection with 0.5, 5 and 50ng of p53

immunoblotted for expression of MED17 HA, p53 and ß-actin (Fig 3.5B). MED17 HA showed enhanced expression correlating with increasing amounts of the MED17 HA expression construct transfected. p53 expression was low in all the immunoblots, as expected for cells not induced into cell cycle arrest or apoptosis, however, there was a moderate decrease in p53 expression with increasing expression of MED17. Immunoblotting for ß-actin showed equal amounts of protein were loaded between the samples. Previously published data has described the Mediator complex, containing MED17, as a co-activator of p53 transcription. These data suggest that MED17 over-expression is able to induce repression of p53 transcription at endogenous levels of the protein as well as decrease its expression.

As these experiments were done with a with an C-terminal HA epitope containing MED17, to investigate the relative effect of the epitope on the p53 repressor function of MED17, a *bax* reporter assay was done transfecting 1µg of a non-epitope containing MED17 expression construct (Fig 3.5C). Transfection of this construct induced a 0.57 fold activation of the *bax* reporter compared to the vector control, equivalent to a 43% reduction in activation. This repression is similar to that of MED17 HA and suggests that repression of the *bax* reporter was not a consequence of the epitope.

These data suggest that MED17 represses p53 transcription which is contradictory to previous data suggesting an activator function for the protein within the Mediator complex, as tested in an *in vitro* system. Human MED17 has not been previously shown to exhibit transcriptional repression with any of its

interacting transcription factors which may represent more subtle regulation of the transcriptional activities of the Mediator complex by its individual subunits. MED17 was therefore further investigated, using the MED17 HA expression construct, to elucidate its repression effects on p53 transcription.

To investigate the strength of p53 repression by MED17 over-expression on bax-luciferase transactivation, reporter assays were performed in the presence of exogenous p53. A range of 9 to 35 fold of activation of the bax-luciferase reporter was seen with transfection of 0.5ng to 50ng of p53 transfected into cells was previously demonstrated in earlier experiments. Thus 0.5ng of p53 was chosen to sub-optimally activate the bax luciferase reporter and test MED17 repression. U2OS cells were co-transfected with MED17 and p53 expression constructs, and luciferase assays were conducted. In this experiment when 0.5ng of the p53 construct was transfected, the bax-luciferase reporter showed a 0.2 fold increase in activation however on co-transfection of increasing amounts MED17, decreased activation of bax-luciferase reporter in a dose dependent manner was observed. Moreover, reporter activation was decreased to levels below that of the vector only control, decreasing activation 50% relative to the vector only control (Fig 3.5D). These data showed that MED17 decreased activation of the bax reporter in a dose dependent manner when moderately activated by exogenous p53. This dose dependent repression of the *bax* reporter by MED17 may not occur at endogenous levels of p53 due to the relatively low expression of p53 in unstressed cells requiring only moderate levels of MED17 over-expression to repress p53 transcription.

To see if repression of the *bax* reporter correlated with a decrease in the expression of p53 regulated genes, Western blots for p53, p21 and Mdm2 were performed on cell lysates prepared from U2OS cells transfected with the MED17 HA expression construct. Due to the relatively low expression of p53 regulated proteins more cell lysate was prepared, in comparison to that used in the reporter assays. $1x10^6$ cells were transfected with 5µg of MED17, which was proportional to the amounts of MED17 transfected in the reporter assays. Over-expression of MED17 in U2OS cells resulted in the decreased expression of the p53, Mdm2 and p21 proteins relative to ß-actin (Fig 3.5E) and was consistent with decreased transcriptional activity of p53 as observed with the *bax* reporter. Importantly the decreased expression of p53 was not due to enhanced expression of Mdm2, a negative regulator of p53 expression, suggesting a mechanism other than enhanced degradation of p53 was responsible for decreased transactivation.

3.6 p53 expression is necessary for MED17-mediated repression of a *bax*-luciferase reporter

As demonstrated previously, MED17 interacts with p53 and decreases p53 transcriptional activity upon MED17 over-expression *in vivo*. These experiments were conducted in a $p53^{+/+}$ cell line U2OS. To investigate whether the decreases in transcription associated with MED17 over-expression were dependent on p53, the *bax*-luciferase reporter was transfected into MG63 ($p53^{-/-}$) cells. In these cells, over-expression of MED17 HA had no effect on the basal level of activation of the *bax*-luciferase reporter relative to the vector control (Fig

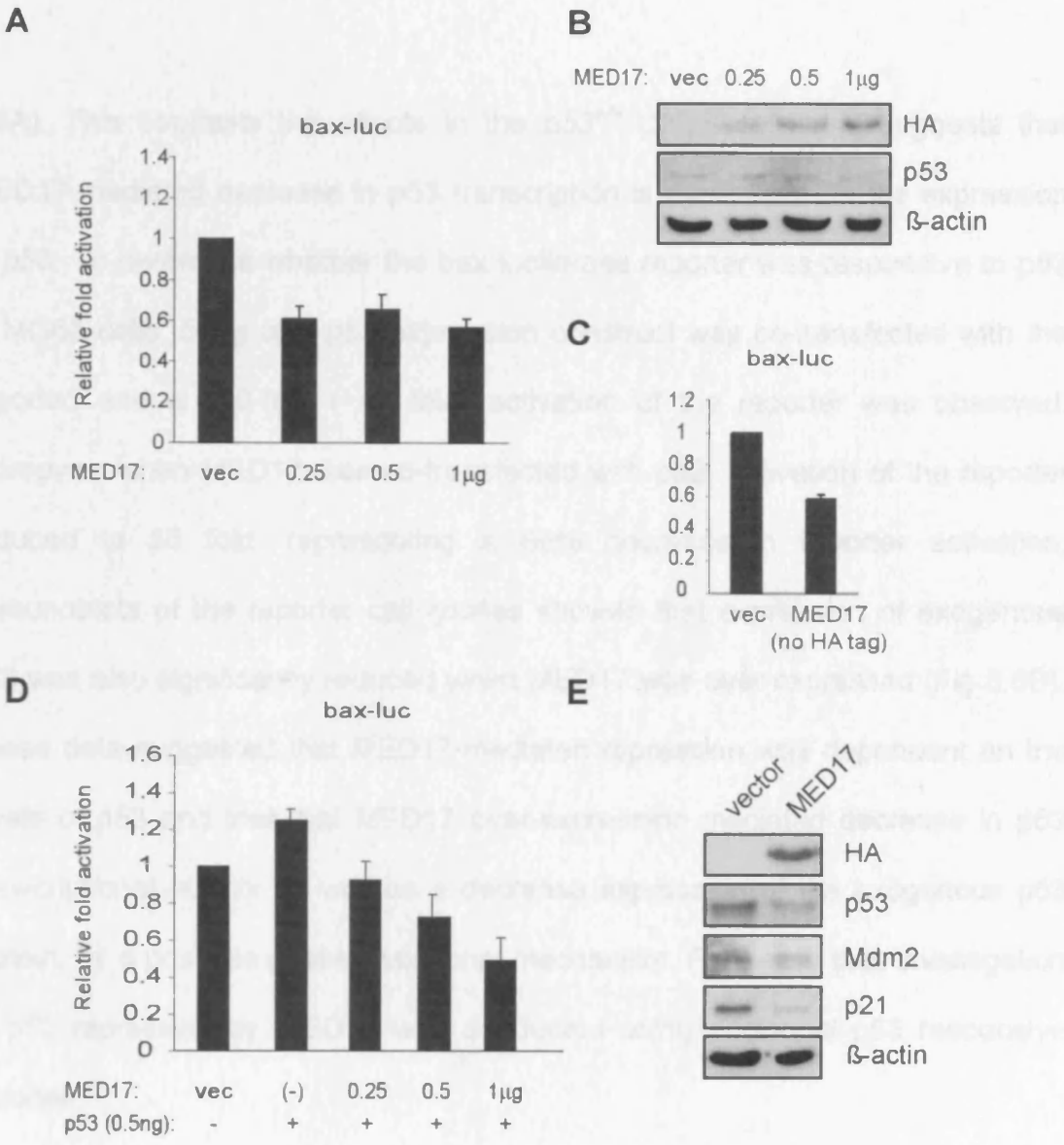


Fig 3.5 MED17 represses a p53 responsive *bax*-luciferase reporter and decreases expression of p53 regulated proteins

Effects of MED17 over-expression on p53 transcription were investiagted in U2OS cells using a *bax*-luciferase reporter. Expression of p53, Mdm2 and p21 were also tested as transcriptional targets of p53.

A. *bax* reporter was co-transfected into U2OS cells with a titration of MED17 HA. Reporter activity is normailsed against a vector only (vec) control.
B. Immunoblots of cell lysates prepared for the *bax* reporter assays with titrations of MED17 HA.
C. *bax* reporter assay in U2OS cells with 1µg of MED17 expression construct containing no HA epitope.
D. *bax* reporter assays with co-transfection of activating amounts of p53 and increasing MED17.
E. Western blot of lysates prepared from U2OS cells transfected with 5µg of MED17 to investigate expression of p53 regulated proteins.

3.6A). This contrasts the effects in the *p53*$^{+/+}$ U2OS cells and suggests that MED17-mediated decrease in p53 transcription is dependent on the expression of p53. To determine whether the bax luciferase reporter was responsive to p53 in MG63 cells, 50ng of a p53 expression construct was co-transfected with the reporter, and a 110-fold (+/-4 fold) activation of the reporter was observed. Moreover, when MED17 was co-transfected with p53, activation of the reporter reduced to 35 fold, representing a 69% decrease in reporter activation. Immunoblots of the reporter cell lysates showed that expression of exogenous p53 was also significantly reduced when MED17 was over-expressed (Fig 3.6B). These data suggested that MED17-mediated repression was dependent on the levels of p53 and that that MED17 over-expression mediated decrease in p53 transcriptional activity as well as a decrease expression of the exogenous p53 protein, by a possible post-translational mechanism. Further to this, investigation of p53 repression by MED17 was conducted using additional p53 responsive reporter.

3.7 MED17 represses transactivation of a p21 and a synthetic p53 responsive gene reporter

p53 responsive genes have been identified through conserved DNA sequences located in their promoter regions, termed p53 response elements, and have been identified in most, but not all p53 regulated genes (El Deiry *et al.*, 1993;Miyashita and Reed, 1995). To investigate MED17 repression of p53, two other p53-responsive reporter constructs were tested; a p21 reporter and an

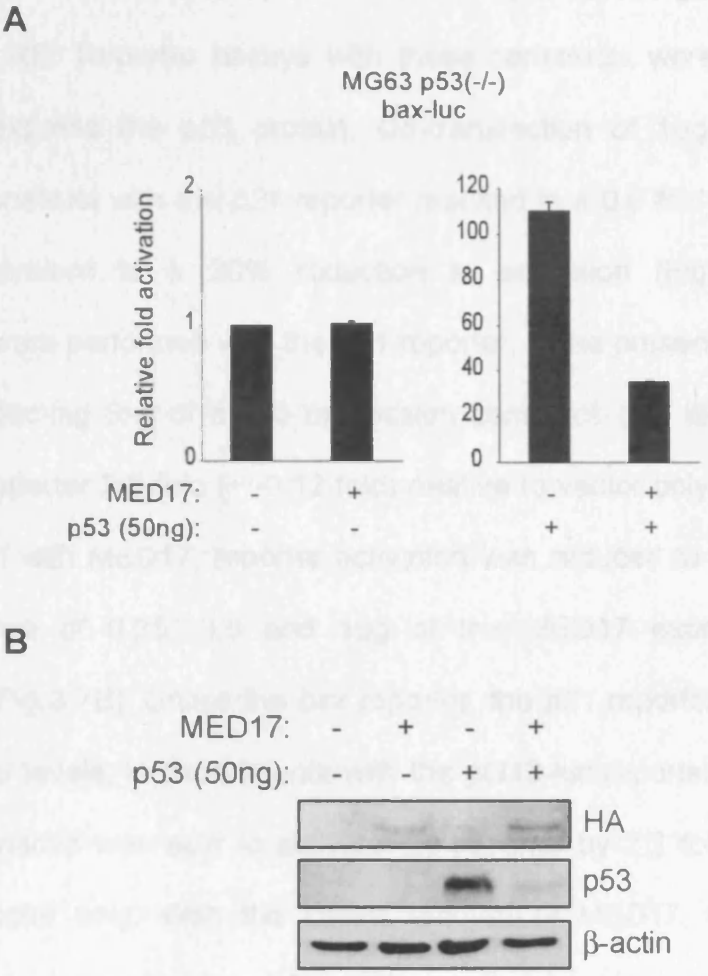


Fig 3.6 MED17 repression is dependent on expression of the p53 protein

To test whether the mechanism of MED17 repression of p53 was dependent on its expresssion bax luciferase reporters were done in p53 null cells, MG63

A. MED17 *bax*-luciferase reporter assays in MG63 cells in the presence or absence of co-transfected p53.
B. Immunoblots on MG63 reporter lysates for HA and p53.

artificial p53 reporter construct. The artificial p53 reporter (pG13-luc) contains 13 tandem p53 DNA binding sites upstream of a firefly luciferase gene while the *p21* contains one RE. Reporter assays with these constructs were done in U2OS cells which express the p53 protein. Co-transfection of 1μg of the MED17 expression construct with the *p21* reporter resulted in a 0.8 fold activation of the reporter, equivalent to a 20% reduction in activation (Fig 3.7A). Further experiments were performed with the *p21* reporter, in the presence of exogenous p53, by transfecting 5ng of a p53 expression construct. p53 alone was able to activate the reporter 2.5 fold (+/-0.12 fold) relative to vector only. When p53 was co-transfected with MED17, reporter activation was reduced to 2.1, 1.7 and 1.6 fold by titration of 0.25, 0.5 and 1μg of the MED17 expression plasmid, respectively (Fig 3.7B). Unlike the *bax* reporter, the *p21* reporter did not repress to below basal levels. In experiments with the pG13-luc reporter, 5ng of the p53 expression plasmid was able to activate the reporter by 2.2 fold (+/- 0.35 fold) relative to vector only. With the lowest amount of MED17, 0.25μg, a slight increase in reporter activation of 0.1 fold was observed on co-transfection with p53 (Fig 3.8A). This was in contrast to transfection of 0.5 and 1μg of MED17 which repressed reporter activation to 1.8 and 1.3 fold. Immunoblots were performed on these cell lysates prepared for the reporter assays to confirm transfection and titration of MED17, as well as p53, in these experiments. Analysis of p53 protein levels showed enhanced expression with transfection of p53 but expression decreased with increasing expression of MED17 (Fig 3.8B). However, p53 expression remained higher than endogenous levels of p53 at the

1µg titration of MED17 suggesting that the reporter repression by MED17 was not entirely attributed to decreased expression of p53 protein. These data suggest that the transcriptional activity of p53-responsive genes is decreased in a dose-dependent manner by MED17 over-expression and are consistent with previous data, that MED17 decreased expression of p53 transcriptional targets, like p21, when over-expressed in U2OS cells.

3.8 Generation of a MED17 constitutively over-expressing cell line

To further investigate the function of MED17 *in vivo*, a cell line constitutively expressing MED17 was generated in non-transformed cells, NIH-3T3. NIH-3T3 cells were isolated as a spontaneously immortalised cells derived from mouse embryonic fibroblasts which do not express *p16INK4A* and *p19ARF*. Both these genes share the same locus but are transcribed by two transcripts, α and ß respectively, which are generated by alternative splicing. Transcription of both genes is inhibited by epigenetic silencing. Using these cells to generate the MED17 cell line is advantageous over a tumour derived cell line as they contain a characterised gene mutation whereas tumour cells may contain a number of mutations that may affect many cellular processes, such as apoptosis. More generally, the NIH-3T3 cells are more representative of a normal proliferating cell making identification of a MED17 phenotype more distinguishable. Though the cell line is derived from mouse fibroblasts, there is a 96% identity between mouse and human MED17 proteins (see Table 1.1). Functionally, murine and human Mediator complexes are similar as indicated studies of transgenic mice

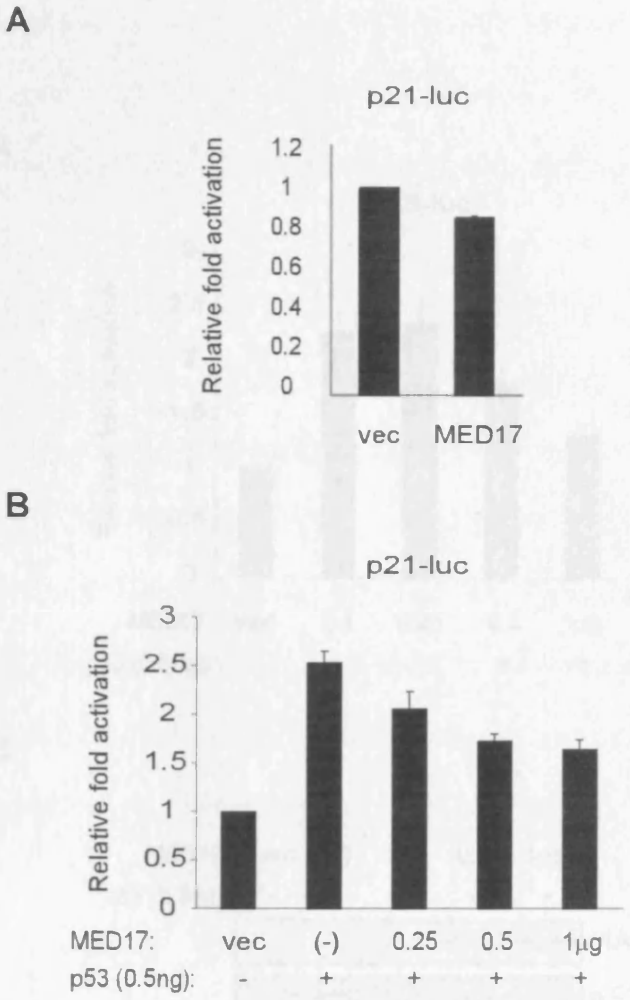


Fig 3.7 MED17 represses activation of a *p21*-luciferase reporter

MED17 effect of p53 mediated transcription was tested on the p53 responsive *p21*-luciferase reporter construct.

A. *p21*-luciferase reporter assays with co-transfected MED17 (1µg).
B. *p21*-luciferase reporter assays co-transfecting 5ng of p53 and increasing amounts of MED17.

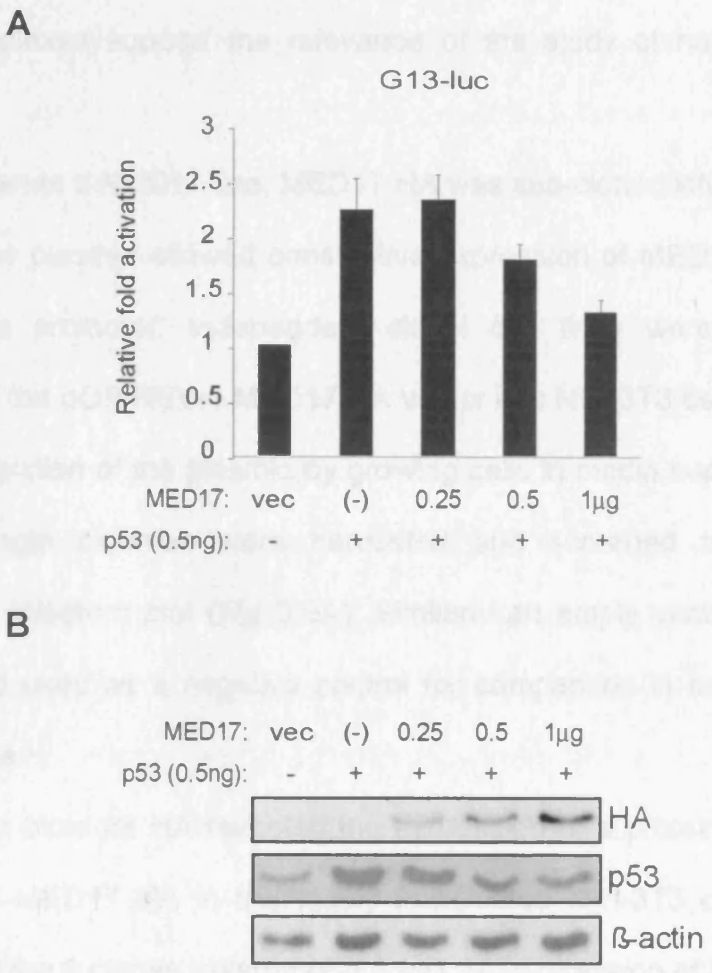


Fig 3.8 MED17 represses activation of a synthetic p53 responsive gene reporter, G13-luc

p53 reporter, pG13, contains 13 tandem p53 response elements and was used to assay MED17 effects on a p53 responsive promoter.

A. G13-luciferase reporter assay in U2OS cells with co-transfection of p53 and increasing amounts of MED17.
B. Western blot of G13 reporter lysates blotting for HA and p53.

null for Mediator subunit MED1 (TRAP220), which is deficient in thyroid hormone receptor signalling (Ito et al., 2000). The high identity between human and mouse MED17 proteins as well as the functional similarity between the respective Mediator complexes support the relevance of the study of human MED17 in murine cells.

To generate a MED17 line, MED17 HA was sub-cloned into the pOP RSV-1 plasmid. This plasmid allowed constitutive expression of MED17 from a Rous sarcoma virus promoter. Independent clonal cell lines were generated by transfection of the pOP RSV-1 MED17 HA vector into NIH-3T3 cells and selected for stable integration of the plasmid by growing cells in media supplemented with neomycin. Single colonies were harvested and screened for MED17 HA expression by Western blot (Fig 3.9A). Similarly an empty vector cell line was generated and used as a negative control for comparison in assays using the MED17 cell line.

Western blots for HA revealed the expression of a protein corresponding to the size of MED17 HA in the stably transfected NIH-3T3 cells which was present in 4 of the 6 clones isolated (Fig 3.9A). No expression of MED17 HA was seen in the vector only cell line (denoted (-)), and as a positive control for MED17 expression, lysate from NIH-3T3 cells transiently transfected with MED17 HA were Western blotted in parallel (denoted (+)). Comparison of MED17 expression between stably and transiently transfected cells showed expression in the cell line was significantly lower. Of the MED17 over-expressing clonal lines, clone number 13, hereafter referred to as MED17(C13), was chosen as the highest

expressing MED17 cell and was subsequently used in assays to test the p53 transcriptional response in this cell line.

3.9 MED17(C13) line represses p53 responsive gene reporters

The transcriptional response of p53 regulated genes in the MED17(C13) cell line was tested using p53 responsive gene reporters, as similarly used in the U2OS cells. Using the MED17(C13) line would allow investigation of MED17 function at lower levels of overexpression compared to transient transfection of a MED17 expression vector. Both the vector and MED17(C13) lines were transfected with p53 responsive luciferase reporters: bax, Mdm2 and p21 as well as a Lac Z expression vector at the same amounts as used in previous reporter assays. Luciferase assays were conducted as before, and values were determined relative to the vector control cell line. MED17(C13) cells showed repression of both the bax-luc (0.42 fold) and Mdm2-luc (0.35 fold) reporters, which was comparable to the levels of repression seen when MED17 was transiently transfected (Fig 3.9B and Fig 3.9C). The p21-luc reporter, however, failed to demonstrate significant repression as seen for the bax-luc and Mdm2-luc reporters.

These data show that MED17(C13) represses the p53 responsive gene luciferase reporters, bax and MDM2 but not p21, relative to the vector line, suggesting that the MED17 line represses transcription of some but not all p53 responsive genes (Fig 3.9D). Repression of p53 responsive genes in the MED17(C13) line was further investigated.

3.10 Expression of Mdm2 in the MED17(C13) line is decreased

Reporter assays in the MED17(C13) line showed that bax and MDM2 luciferase gene reporters were repressed by up to 42% compared to the vector control line. To test whether the repression of p53 transactivation correlated with a decrease in expression of the endogenous proteins, Western blot analysis for the expression of p53, MDM2 and p21 in cell lysates prepared from the vector and MED17(C13) lines were performed. Previous experiments involving transient over-expression of MED17 showed that expression of all three of these proteins decreased. Immunoblots of cell lysates prepared from both cell lines show decreased expression of Mdm2 but slightly elevated levels of p53 and p21 in the MED17(C13) line compared to the vector control cell line (Fig 3.9E).

These data show that the effects of transient over-expression of MED17 did not correlate with those observed in the MED17(C13) cell line. This is demonstrated by the increased expression of p21, alongside the reporter assay data with p21 reporter in MED17(C13) cells, suggesting that there is specificity in the p53 transcriptional targets repressed by MED17. Transcription of the Mdm2 gene however seems to be repressed in the MED17(C13) cell line as suggested by the reporter assays and its decreased expression in the immunoblots. Furthermore, the decreased expression of Mdm2 may account for the elevated expression of p53. With the relative decrease of Mdm2 expression, the turnover of p53 by ubiquitin mediated degradation is decreased and may elevate p53 expression. Due to the enhanced expression of p53 but decreased

transactivation of some p53 regulated genes, we next investigated the response of MED17 cells to apoptotic stimuli.

3.11 MED17(C13) cells resistant to apoptosis

Data from the reporter assays with transient and stable over-expression of MED17 suggested that it repressed p53 transcription and decreased expression of some proteins regulated by p53. To study the effect of MED17 on p53 function *in vivo*, the MED17(C13) cell line was used to test the ability of these cells to activate apoptotic pathways in response to cellular insults.

Apoptosis was measured by annexin V staining of cells induced into apoptosis by cytotoxic chemicals. Annexin V conjugated to FITC was used to detect cells with externalised phosphatidylserine on the cell membrane, a feature observed in early apoptotic cells. Staining with propidium iodide (PI) was also used to distinguish early apoptotic cells from late apoptotic and/or necrotic cells which will stain positively for both annexin V and PI. When cell membrane integrity is lost, cells become permeable to PI which intercalates to DNA. Annexin V-FITC staining cells were scored as apoptotic cells whereas doubly stained annexin/PI cells may represent cell death by necrosis or cells in late apoptosis.

The cytotoxic chemicals, etoposide and staurosporine were used to induce apoptosis. Etoposide induces DNA damage through its inhibition of topisomerase II, to create double stranded DNA breaks which activates the transcriptional activity of p53 (Baldwin and Osheroff, 2005). In contrast, staurosporine is a protein kinase C inhibitor which induces p53 transactivation.

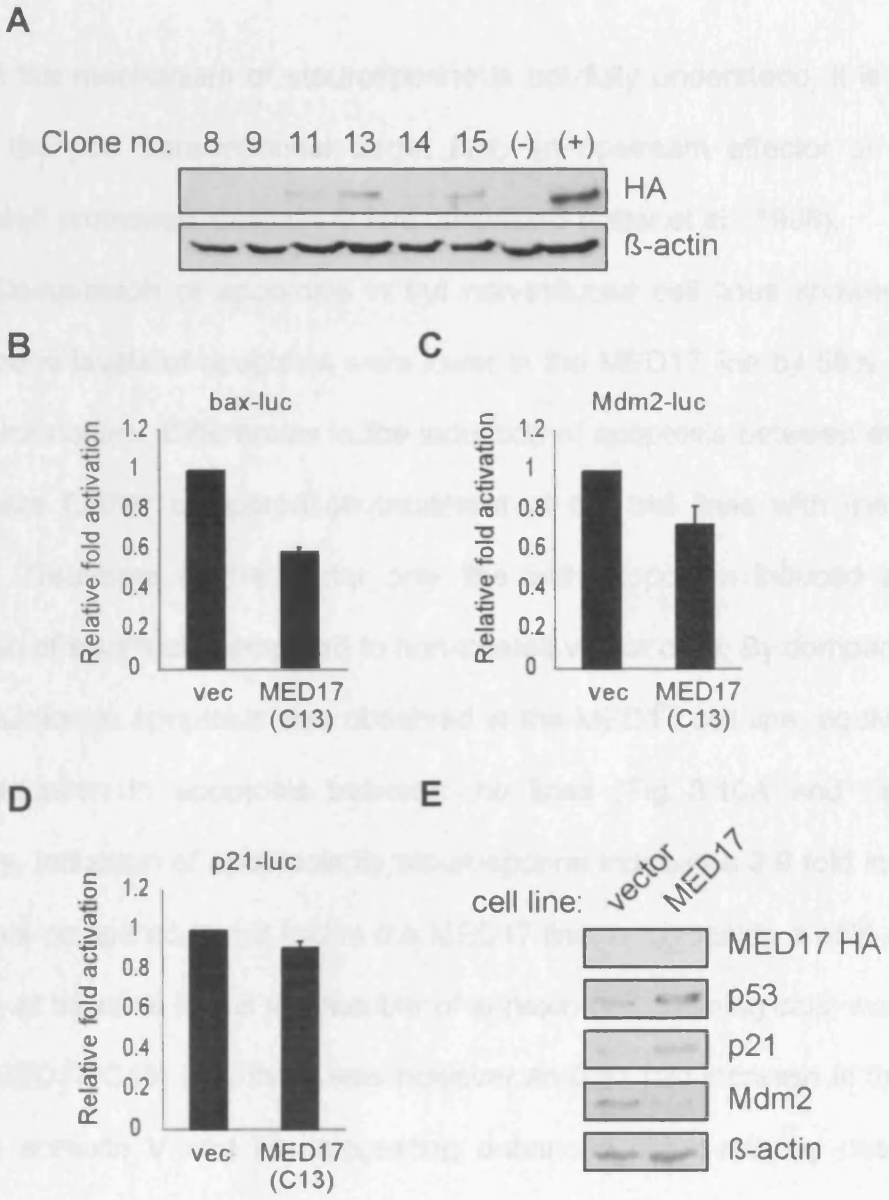


Fig 3.9 Generation of a constitutively over-expressing MED17 cell line

A MED17 HA cell line was generated in NIH-3T3 cells by transfection with pOP-RSV-1 MED17 HA vector and selection of clones for stable integration of the plasmid by culturing in G418. Clones were screened for HA expression by immunoblot. Reporter assays were done on a selected clone alongside Immunoblots for p53 regulated genes to investigate p53 function in this cell line.

A. Immunoblots for screening of clones expressing MED17 HA.
B. *bax*-luciferase reporter assay with MED17(C13) and the vector control cellline.
C. *Mdm2*-luciferase reporter assay in vector and MED17(C13).
D. *p21*-luciferase reporter assay in vector and MED17(C13).
E. Immunoblots of lysates from the vector cell line and MED17(C13) for p53 regulated proteins.

Though the mechanism of staurosporine is not fully understood, it is known to induce the p53 transcriptional target *bax,* an upstream effector of apoptosis associated proteases, caspase-9 and caspase-3 (Otter et al., 1998).

Comparison of apoptosis in the non-induced cell lines showed that the background levels of apoptosis were lower in the MED17 line by 68% relative to the vector control. Differences in the induction of apoptosis between the two cell lines were further compared on treatment of the cell lines with the cytotoxic agents. Treatment of the vector only line with etoposide induced a 4.2 fold induction of apoptosis compared to non-treated vector cells. By comparison a 2.2 fold induction in apoptosis was observed in the MED17 cell line, equivalent to a 52% reduction in apoptosis between the lines (Fig 3.10A and Fig 3.10B). Similarly, induction of apoptosis by staurosporine induced a 2.9 fold induction of apoptosis compared to 1.3 fold in the MED17 line, equivocal to a 45% reduction. Though at baseline levels the number of annexin only staining cells was reduced in the MED17(C13) line, there was however an 0.51 fold increase in the number of cells annexin V and PI, suggesting enhanced cell death by necrosis (Fig 3.10C). Treatment with etoposide and staurosporine enhanced the annexin V/PI staining cells by 3.8 and 4.5 fold respectively in the vector only cell line. In the MED17(C13) cell line, etoposide induced a 2.6 fold increase whereas staurosporine induced a 3.6 fold increase in doubly staining cells, representing a 32% and 20% decrease compared to the treated vector only cells. These data suggest an overall decrease in cell death is observed in the MED17(C13) cells

compared to the vector only cells which is most likely to be attributed to repression of apoptotic pathways.

3.12 MED17(C13) cells inhibit cleavage of caspase-9 when treated with staurosporine

To confirm the induction of apoptosis by staurosporine, lysates from the vector and MED17 cells were prepared after treatment with staurosporine. The lysates were immunoblotted for cleaved forms of caspase-9 to investigate expression of these proteins between the cell lines. Staurosporine treatment induced cleavage of pro-caspase-9 into its active form, which was observed in the vector line but not the MED17 cell line (Fig 3.10D). This result suggests that over-expression of MED17 blocked the cleavage of caspase-9 to repress apoptosis. This may result from repression of *bax* transcription. As an upstream regulator of caspase-9 activation, the reduced expression of bax protein could supress induction of apoptosis. The mechanism of staurosporine induced apoptosis involves the activation of *bax* transcription. The decrease in staurosporine induced apoptosis as well as the repression seen in *bax* reporter assays in the MED17(C13) cell line support the hypothesis that the decrease in apoptosis in this cell line may in part result from the reduced response of *bax* to apoptotic stimuli.

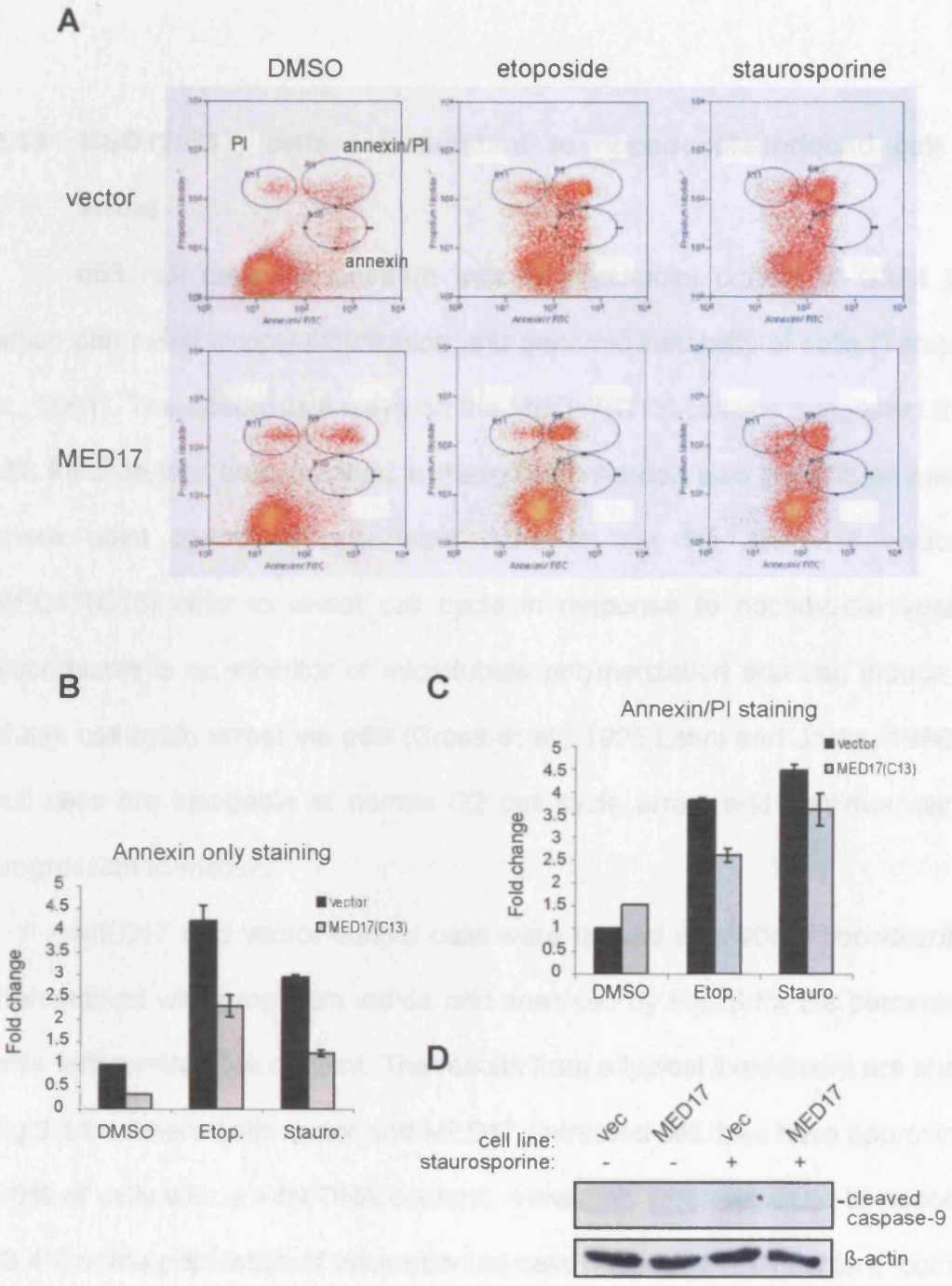


Fig 3.10 MED17(C13) cells have enhanced resistance to etoposide and staurosporine induced apoptosis

A. Annexin V-FITC/PI staining and FACS analysis of vector and MED17 (C13) cells treated with DMSO, etoposide (etop.) or staurosporine (stauro.) Annexin V-FITC only staining cells were quantified as a marker of early apoptosis.
B. Fold change in apoptotic cells (annexin V only staining) normalised to DMSO treated vector cells.
C. Fold change in annexin V/PI staining cells normalised to DMSO treated vector cells.
D. Western blot of cell lysates from prepared from vector and MED17(C13) cells treated with staurosporine, blotting for cleaved caspase-9 expression.

3.13 MED17(C13) cells are resistant to nocodozole-induced cell cycle arrest

p53 null cells demonstrate loss of checkpoint control in G2/M phase, which can result in poly-ploidization and genomic instability of cells (Tarapore et al., 2001). The apoptosis assays on the MED17(C13) cell line suggested that the p53 function was compromised in these cells. As p53 also impacts on cell cycle check point control, assays were done to test the ability of vector and MED17(C13) cells to arrest cell cycle in response to nocodazole treatment. Nocodazole is an inhibitor of microtubule polymerization and can induce G2/M phase cell cycle arrest via p53 (Cross et al., 1995;Lanni and Jacks, 1998). p53 null cells are incapable of normal G2 cell cycle arrest and continue cell cycle progression to mitosis.

MED17 and vector control cells were treated with 40nM nocodozole and then stained with propidium iodide and analysed by FACS for the percentage of cells with a >4N DNA content. The results from a typical experiment are shown in Fig 3.11A, where both vector and MED17 untreated cell lines have approximately 1.5% of cells with a >4N DNA content. However, when exposed to nocodozole 13.4% of the population of vector control cells have >4N DNA content, compared to 23.1% of MED17(C13) cells. This result indicated that approximately 1.7 fold more MED17 cells bypassed a nocodazole-induced arrest than vector cells. Furthermore, these data suggest that MED17 may have a negative regulatory effect on other p53 transcriptional targets involved in cell cycle arrest. The previous data presented, however, suggests that *p21* may not be one of the

genes negatively regulated by MED17 as its expression is enhanced in the MED17(C13) cell line compared to the vector cells.

3.14 MED17(C13) cells have increased numbers of centrosomes

p53 null cells often demonstrate excessive numbers of centrosomes per cell indicating either centrosomes reduplication or mis-segregation. To quantify centrosome number in the vector and MED17(C13) cell lines, immunofluorescence assays were performed to detect γ-tubulin, a component of the centrosomes. Centrosomes observed within nuclei of the cells were counted using confocal microscopy, and a minimum of 100 nuclei were scored per experiment, which was repeated in triplicate. Nuclei were counter-stained with Hoechst dye. Visualisation of centrosomes in both cell lines revealed differences in their size, number and structure between the two cell lines: MED17(C13) cells frequently had multiple centrosomes with enlarged and disorganised morphologies. This was not seen in the vector cell line (Fig 3.11B). Although the vector control cells demonstrated a low, but reproducible number of cells with multiple nuclear centrosomes (≥3 per nucleus), most cells (72%) had the normal number of centrosomes (1 or 2 centrosomes per nucleus). However, in MED17-expressing cells, there was approximately a 25% decrease in the number of cells with 1 or 2 centrosomes per nucleus with a concomitant rise in those exhibiting abnormal, multiple centrosomes (Fig. 3.11C).

These analyses of apoptotic induction, DNA content after nocodazole treatment, and centrosome number indicate MED17-expressing cells phenocopy

cells which have a compromised p53 function, consistent with the hypothesis that MED17 functions as a repressor of p53.

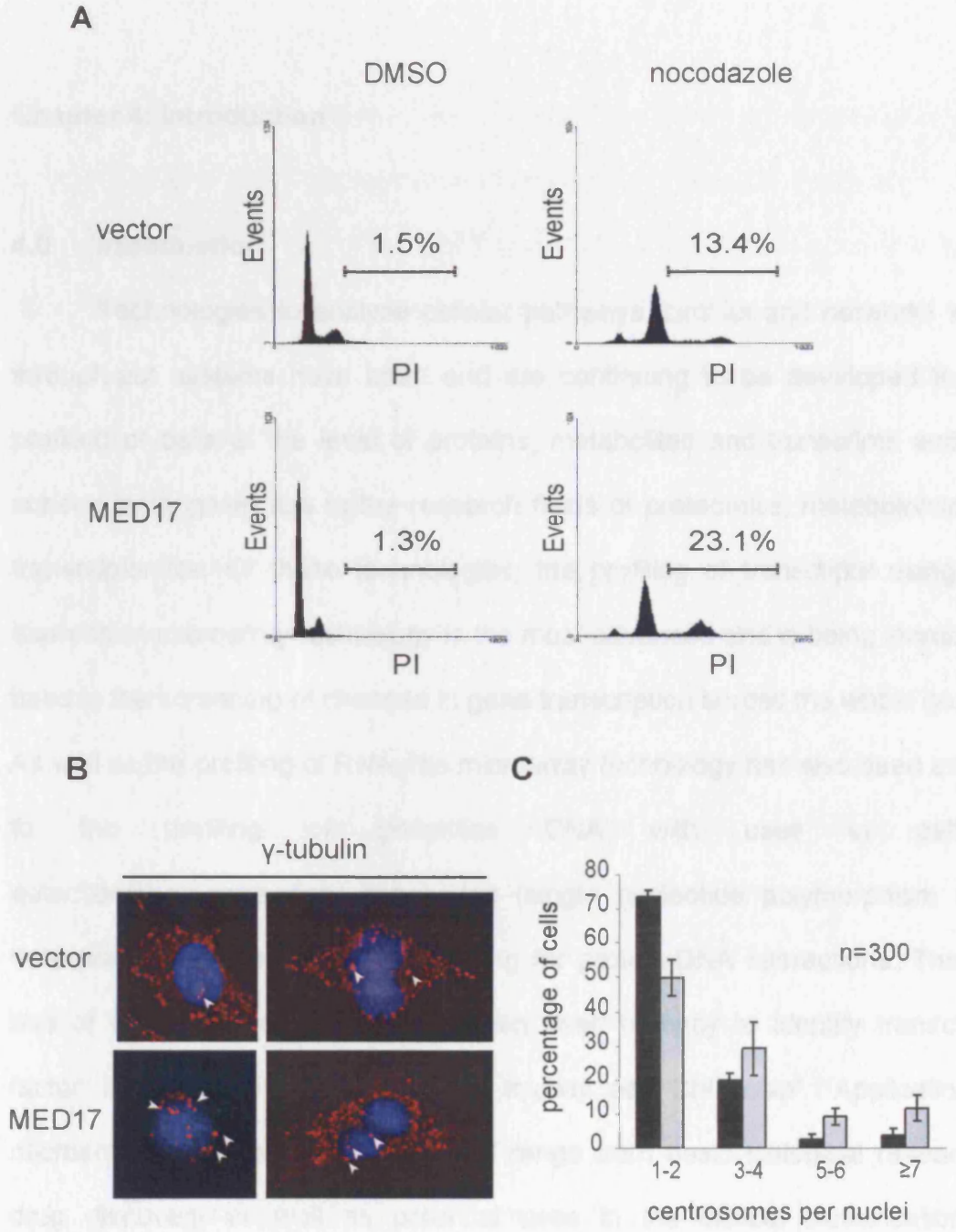


Fig 3.11 MED17 cell line displays phenotypes of p53 deregulated function

A. Propidium iodide staining for cell cycle analysis.Asynchronous vector and MED17(C13) cells were treated with nocodazole for 48 hours to induce G2 phase arrest.DMSO treated cells were used as a control. 8N cells were quantified and expressed as a fraction of total population.
B. Immunofluoresence detection of γ-tubulin (red) to visualise centrsomes in the vector and MED17(C13) cell lines. Counter staining with Hoescht (blue) was done to visualise nuclei by confocal microscopy. Centrsomes per nuclei were quantified.
C. Quantification of nuclear centrosome numbers between the vector and MED17(C13) lines.

Chapter 4: Introduction

4.0 Introduction

Technologies to analyse cellular pathways, circuits and networks in high through-put systems have been and are continuing to be developed to allow profiling of cells at the level of proteins, metabolites and transcripts and have subsequently given rise to the research fields of proteomics, metabolomics and transcriptomics. Of these technologies, the profiling of transcripts using gene expression microarray technology is the most advanced and is being increasingly used in the screening of changes in gene transcription across the whole genome. As well as the profiling of RNA, the microarray technology has also been adapted to the profiling of genomics DNA with uses in pathogen detection/characterisation, genotyping (single nucleotide polymorphism (SNP) detection), resequencing and screening for protein-DNA interactions. This later use of DNA chip technology has been used recently to identify transcription factor binding sites by a method known as "ChIP-chip". Applications of microarray technology are broad and range from basic biological research, to drug discovery as well as potential uses in the clinical classification and prognosis of disease.

In use are two types of microarrays which differ generally in their hybridisation of either cDNA or cRNA (known as the "target") to the chip. More commonly used than the cDNA microarrays are the oligonuceotide arrays for which cRNA is hybridised to "probes". Affymetrix genechip probes are

synthesised on the genechips themselves by a process involving photolithography and span genes across the whole genome. Each probe has its own corresponding mismatch probe which serves as an internal control for non-specific binding of the target RNA. For each gene there are a number of probes which span the 5', middle and 3' regions of the transcript allowing for greater specificity of detection. In comparison to probes on the cDNA microarrays, oligonucleotide array probes are smaller at 25bp long. Labelling of the target DNA also differs with as cRNA is biotinylated during the *in vitro* transcription of the cDNA and is only stained with streptavidin-phycoerythrin once hybridised to the probes. Cells are then laser confocal scanned and computational analysis done to determine gene expression which is usually denoted as a ratio of abundance, also known as a reference biological sample, and can be expressed as a fold change between the control and experiment arrays. This methodology differs from cDNA microarrays where differently labelled cDNAs from control and experimental samples are hybridised to the same chip thus expression of a specific transcript in the experimental sample is represented as relative to the control.

Microarrays generate large data sets that require computational analysis to determine significant changes in gene expression patterns. Investigation of these data sets us usually done in an unsupervised manner by clustering of genes, as represented by the probe-sets, whose expression varies significantly between the control and experiment samples. Annotation of the probe-sets allows functional interpretation of genes and facilitates clustering of functionally

similar genes that may be co-regulated. Such methods for uncovering gene networks are commonly used and are sometimes referred to as "guilt by association" where it is assumed that similar expression patterns between genes means that they are functionally related. In contrast, supervised learning is used in the generation of classifications whereby algorithms are used to identify predictive gene markers that discriminate between classes.

In this thesis I have used oligonucleotide arrays to analyse gene expression in a cell line constitutively over-expressing MED17 compared to a vector only control. Significantly regulated probe-sets were identified and clustering done using two web based applications to determine biological functions attributed to these differentially regulated genes. MED17 has been shown to interact with a number transcription factors suggesting a role in regulating diverse biological functions. Gene expression microarrays have been used to identify genes regulated by specific transcription factors. Similarly, this chapter aims to identify potential transcriptional networks and biological functions that may be directly or indirectly regulated by MED17.

Chapter 4: Results

4.1 Microarray analysis of the MED17 cell line

MED17 has been shown to interact with a number of transcription factors thus may be able to affect several different transcriptional networks. To investigate the transcriptional networks regulated by MED17, the MED17(C13) cell line was used in a gene expression microarray experiment to produce a global transcription profile upon over-expression. mRNA was harvested from the two cell lines and used to make cDNA, which was hybridised to Affymetrix MOE430A Genechip© microarrays. The grouped microarray gene expression scores were analysed for differential expression. In the MED17 cell line, 1858 probe-sets were found to be significant (corrected q<0.001) of which 1124 were up-regulated (representing 886 genes) and 734 were down-regulated (representing 646 genes). These marker probe-sets were analysed further to infer the biological functions of the genes they represented.

4.2 Genes involved in DNA metabolism and cell proliferation are significantly up-regulated in the MED17 cell line

Using the *GOBrowser* tool, available on the Affymetrix *NetAffx* website, the significantly up-regulated probe-sets were annotated with their biological function and categorised into processes to analyse the global effects of MED17 over-expression (see Chapter 2, section 2.30). Amongst the marker probe-sets, 92 appeared in a category annotated "DNA metabolism". Of these, a further sub-

category annotated 16 (17.4%) with an involvement in "chromatin assembly/disassembly" (Fig 4.1A). This sub-category was found to be statistically over-represented ($p=5.15^{-23}$) when compared to expectation using a chi-squared test of independence. Of note in these up-regulated probe-sets are those of Nap1l1 (nucleosome assembly protein 1-like-1), which is represented by 5 probe-sets. Probe-sets for Smarca5 (**S**wi/Snf related, **m**atrix-associated, **a**ctin dependent **r**egulator of **c**hromatin, subfamily **A**, member **5**) and cbx5 (**c**hromo**b**o**x** homolog 5, also known as heterochromatin protein 1) were also up-regulated. The up-regulation of these genes may be required for putative co-repressor and co-activator functions of MED17 in transcription.

In addition to the DNA metabolism category, 128 probe-sets annotated as "cell proliferation" were also over-represented. Within the cell proliferation category, two sub-categories, "cell cycle" and "DNA replication", were found to be highly represented ($p=6.3^{-39}$ and $p=1.86^{-36}$ respectively) amongst the cell proliferation probe-sets (Fig 4.1B and Fig 4.1C). These data suggest that MED17 global transcriptional function may promote cell proliferation. The MED17 markers were analysed further using Ingenuity Pathway Analysis, to determine specific gene networks that were changed by MED17 over-expression and which might contribute to global changes in the expression of genes involved in DNA metabolism and cell proliferation.

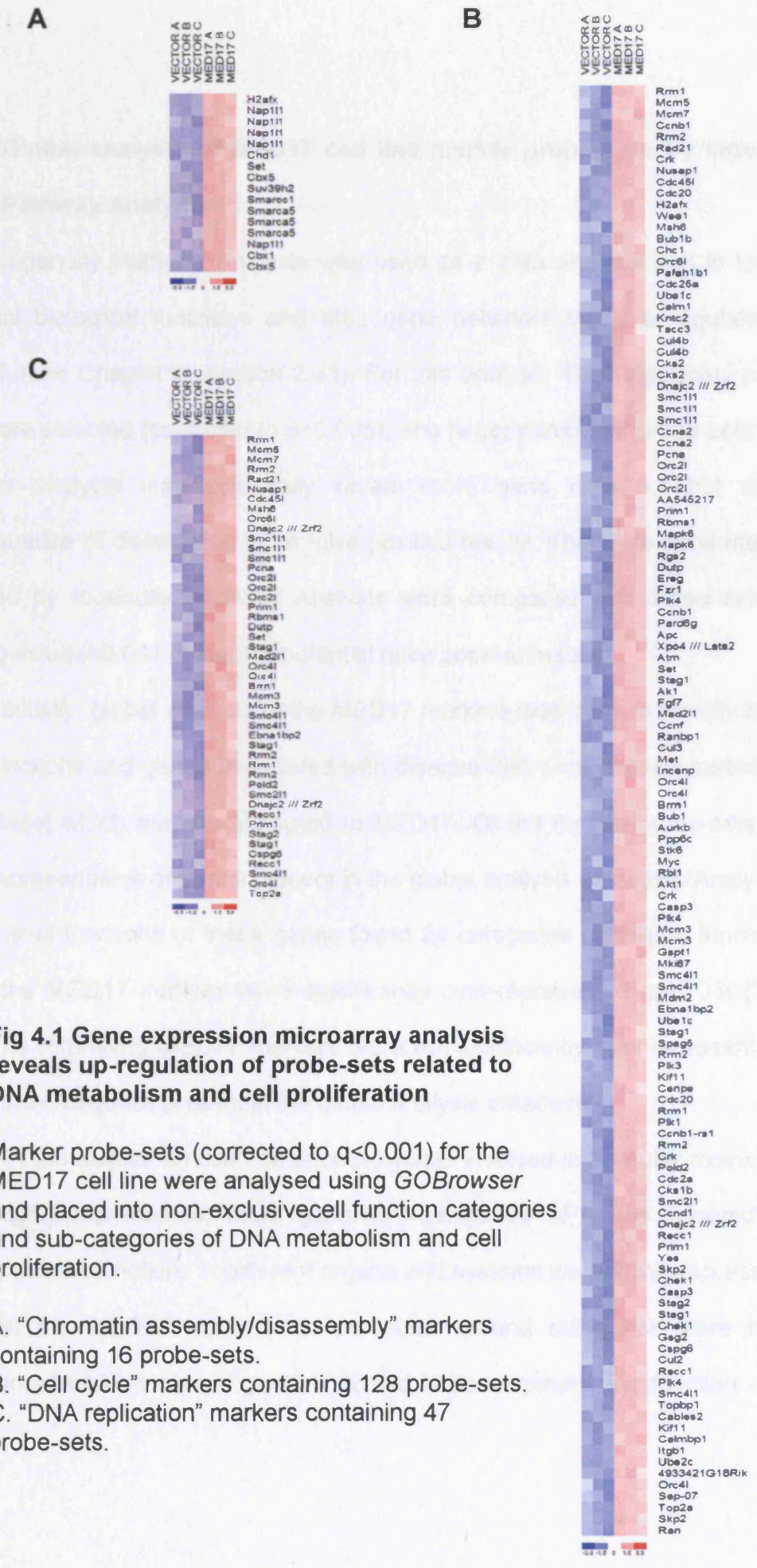


Fig 4.1 Gene expression microarray analysis reveals up-regulation of probe-sets related to DNA metabolism and cell proliferation

Marker probe-sets (corrected to q<0.001) for the MED17 cell line were analysed using *GOBrowser* and placed into non-exclusivecell function categories and sub-categories of DNA metabolism and cell proliferation.

A. "Chromatin assembly/disassembly" markers containing 16 probe-sets.
B. "Cell cycle" markers containing 128 probe-sets.
C. "DNA replication" markers containing 47 probe-sets.

4.3 Global analysis of MED17 cell line marker probe–sets by Ingenuity Pathway Analysis

Ingenuity Pathway Analysis was used as a data analysis tool to identify potential biological functions and their gene networks that are regulated by MED17 (see Chapter 2, section 2.31). For this analysis 4730 significant probe-sets were selected (corrected to $p<0.005$). The larger number of probe-sets used for this analysis may potentially reveal more gene networks but at the consequence of discovering more false positive results. Therefore gene markers selected by Ingenuity Pathway Analysis were compared with those selected using q-values<0.001 to identify potential false positive results.

Initially, global analysis of the MED17 markers was done to identify higher level functions and genes associated with disease that were over-represented in the dataset which may be attributed to MED17. Of the marker probe-sets, 830 were representative of genes present in the global analysis database. Analysis of higher level functions of these genes found 24 categories of cellular function in which the MED17 markers were significantly over-represented ($p<0.05$) (Table 4.1). The remaining MED17 markers were not significantly over-represented in any of the categories present in the global analysis database.

Of the cellular function categories, genes involved in "Cellular movement" were highly represented. More generally, categories of genes involved with developmental functions in different organs and systems were highly represented amongst the MED17 markers. In particular several categories were highly represented which included genes involved in development and function of the

haematological, immune and lymphatic systems, as well as immune response and haematological disease, suggesting a function for MED17 in the haematological system. Also represented was a functional category labelled "Cell death", however, none of the seven genes present in this category had functions related to p53. A category termed "Cellular growth and proliferation" was similarly identified by the Ingenuity Pathway Analysis. The genes present in this category contained a number of interleukins and chemokines which were not identified in the "cell proliferation" category previously identified using *GOBrowser*. Simliar to the *GOBrowser*, the Ingenuity Pathway analysis identified statistically significant categories of genes associated with particular cellular function. However, new functional categories were identified that were not present in the *GOBrowser* analysis. To investigate pathways that may be involved in these general cellular functions, further analysis was performed to ascertain functional relationships between the MED17 markers.

4.4 Analysis of MED17 cell line probe-set markers by Ingenuity Pathway Analysis reveals activation and repression of p53 regulated genes

Network analysis of the MED17 markers allows the identification of groups of genes that may be directly or indirectly regulated by MED17. From the total number of MED17 markers, 742 were placed into 105 networks that contained at least one of the markers. Each network was scored on the number of MED17 markers present. Seven networks scored 35 markers as present, the highest score for any of the MED17 networks. One of these networks, termed "cellular

Function	Number of markers present	*p*-value
Cellular movement	47	4.51E-3 – 4.29E-2
Haematological System Development and Function	62	4.51E-3 – 4.29E-2
Immune Response	57	4.51E-3 – 4.29E-2
Nucleic Acid Metabolism	16	4.51E-3 – 4.29E-2
Cell Morphology	19	6.35E-3
Cellular Growth and Proliferation	25	7.08E-3 – 4.29E-2
Immune and Lymphatic System Development and Function	26	7.08E-3 – 4.29E-2
Tissue Development	16	7.08E-3 – 4.29E-2
Embryonic Development	14	1.11E-2 – 1.64E-2
Organ Development	14	1.11E-2 – 1.64E-2
Organismal Development	14	1.11E-2 – 1.64E-2
Cell-To-Cell Signalling and Interaction	39	1.74E-2 – 4.29E-2
Reproductive System Development and Function	16	1.74E-2 – 4.29E-2
Amino Acid Metabolism	8	2.73E-2
Haematological Disease	8	2.73E-2
Cardiovascular System Development and Function	21	3.65E-2 – 4.29E-2
Cell Death	7	4.29E-2
Cellular Development	13	4.29E-2
Endocrine System Development and Function	11	4.29E-2
Gastrointestinal Disease	7	4.29E-2
Inflammatory Disease	7	4.29E-2
Lipid Metabolism	14	4.29E-2
Renal and Urological System Development and Function	7	4.29E-2
Skeletal and Muscle Disorders	7	4.29E-2

Table 4.1 Global functions of MED17 cell line markers analysed by Ingenuity Pathway Analysis

The Ingenuity Pathway application allocated probe-set markers (corrected to $p<0.005$) in to categories of cellular function and disease. All statistically significant categories ($p<0.05$) over-represented by the MED17 gene markers are given in the table above with total numbers of gene markers for each category.

compromise, DNA replication, recombination and repair, cancer", contained a p53 node, indicating high expression of the p53 transcript. These data concur with previous Western blot analysis of the MED17 cell line, which showed enhanced expression of p53 compared to the vector line (Fig 4.2). The transcription of many genes in this network is regulated by p53. Of the down-regulated genes, the transcription of gluthatione S-transferase M1 (GSTM1), GSTM5, thrombospondin 2 (THBS2) and interferon alpha-inducible protein (G1P2) is associated with the activation of p53, hence the decreased expression of these transcripts may be attributed to MED17 effects on p53 mediated-transcription. Surprisingly, the Mdm2 gene was up-regulated. Previous analysis of Mdm2 promoter activity and Western blot analysis of the Mdm2 protein showed decreased activation and expression of the protein (Fig 3.10C and Fig 3.10E). These data indicate that the expression of the Mdm2 transcript does not correlate with the gene reporter analysis or the expression of the Mdm2 protein in the MED17 cell line.

Other p53 transcriptional targets were found to be up-regulated in the presence of high levels of p53. Nucleostemin (NS), hyaluronan synthase 2 (HAS2), adenylate kinase 1 (AK1), thrombomodulin (THBD), prostaglandin-enteroperoxide sythase-1 (PSTG1) and collagen type IV alpha I (COL4A1) are all genes transcribed by activated p53 and were highly expressed in the MED17 cell line. Other transcriptionally regulated genes in this network are actively repressed by p53 but are activated in the MED17 cell line, with the exception of microsomal glutathione S-transferase (MGST), which suggests deregulation of

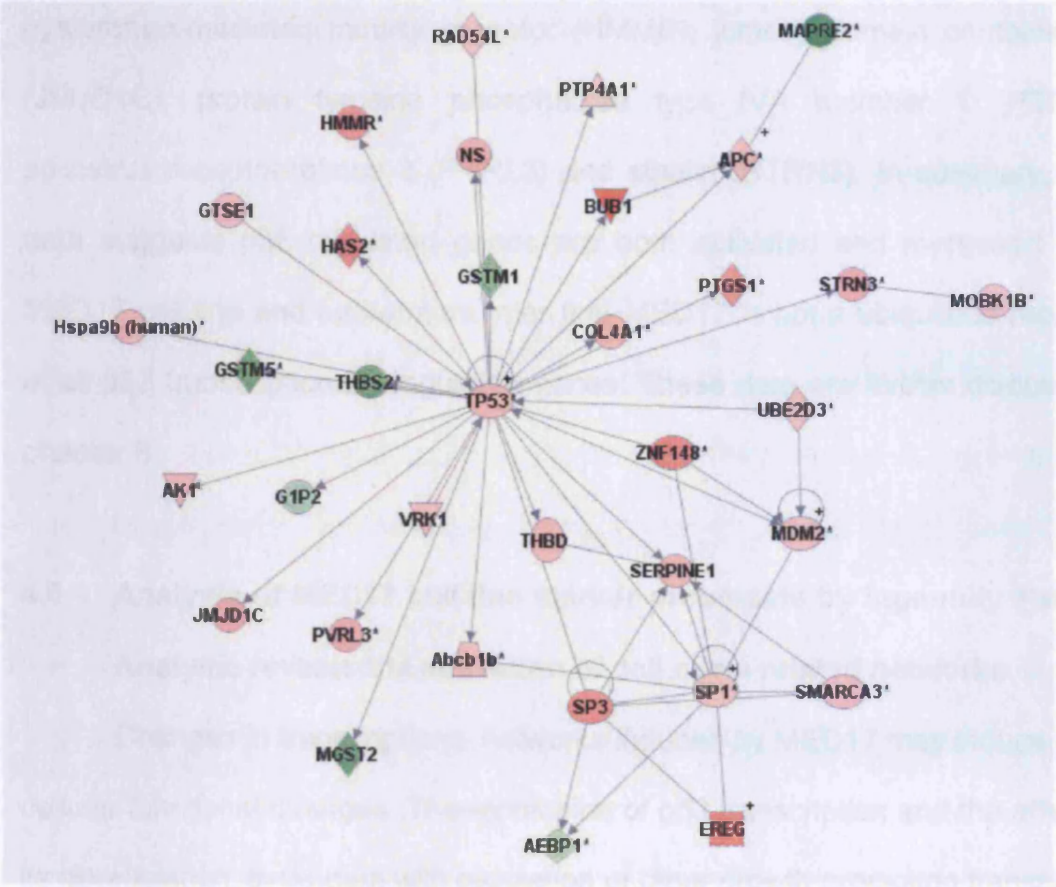


Fig 4.2 MED17 gene markers are present in a network termed "Cellular compromise, DNA Replication, Recombination and repair, cancer"

Ingenuity Pathway Analysis of MED17 gene markers (corrected to $p<0.005$) scored 35 genes as present in this network. Red icons represent upregulated genes whereas green icons are down-regulated genes. Straight lines between genes indicate protein-protein interactions and arrows show transcripton regulatory function between genes. * indicates multiple probe-sets were present in the dataset for the gene.

p53 transcriptional repressor function. These activated genes include multidrug resistance member 1b (Abc1b), adenomatosis polyposis coli (APC), BUB1, hyaluronan-mediated motility receptor (HMMR), jumonji domain containing 1C (JMJD1C), protein tyrosine phosphatase type IVA member 1 (PTP4A1), poliovirus receptor-related 3 (PVRL3) and striatin (STRN3). In summary, these data suggests p53 regulated genes are both activated and repressed in the MED17 cell line and furthermore infer that MED17 is not a ubiquitous repressor of all p53 transcriptionally regulated genes. These data are further discussed in chapter 6.

4.5 Analysis of MED17 cell line marker probe-sets by Ingenuity Pathway Analysis reveals the activation of cell cycle related networks

Changes in transcriptional networks induced by MED17 may induce global cellular functional changes. The repression of p53 transcription and the affects of its deregulation, in tandem with regulation of other growth promoting transcription factors by MED17, may be responsible for the significant representation of "cell proliferation" and "cell cycle" probe-sets in the MED17 dataset, as analysed using *GOBrowser* (see section 4.2). Network analysis using Ingenuity also placed gene marker probe-sets in networks associated with cell cycle.

A total of 35 focus genes from the dataset were placed In a network labelled "cell cycle, DNA replication, recombination and repair, cancer" (Fig 4.3A). Genes placed in this network included cyclin A2 (CCNA2), cyclin B1 (CCNB1) and cdk1 (CDC2), which are regulators of cell cycle phase progression

and transition. Up-regulated marker probe-sets were also present for genes involved in the initiation of DNA replication, such as ORC2L and Mcm3. These data suggest that MED17 induces global transcriptional changes that promote cell proliferation.

Another network, labelled "cell cycle, cellular assembly and organisation, DNA replication and repair", also contained 35 focus genes (Fig 4.3B). Gene markers most notably up-regulated in this network included ATM, BRCA1 and structural maintenance of chromosomes 1-like 1 (SMC1E1) all of which are associated with DNA repair pathways. ATM and BRCA1 are also associated with the regulation of p53 transcription in response to DNA damage and induction of cell cycle check-points. This suggests that pathways involved in damage responses are activated in the MED17 cell line but does not consequently activate apoptosis as shown previously (Fig 4.2). Furthermore these networks implicate cell cycle associated genes in the activation of the damage response.

The identification of these two networks related to cell cycle compares with findings from the *GOBrowser* analysis which identified a "cell cycle" sub-category of genes. Between the analysis applications, 32 genes were common between the cell cycle sub-category and the two cell cycle networks (Table 4.2), thus two independent analyses have suggested the up-regulation of genes involved cell cycle/cell proliferation in the MED17 cell line. These effects may be downstream of cell proliferation promoting transcription factors activated by via MED17. Further analysis was done of the significant gene networks identified by

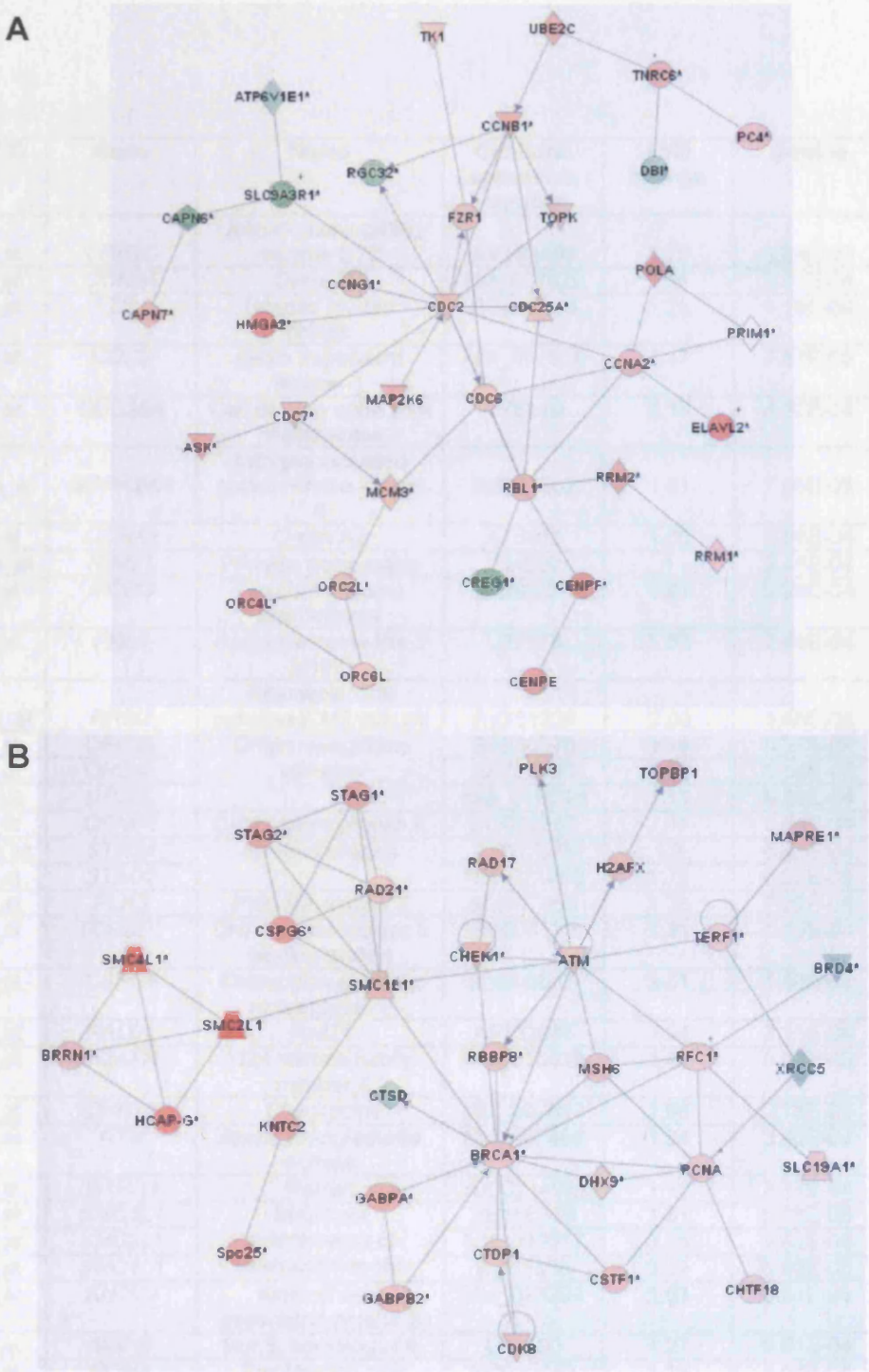


Fig 4.3 Gene marker probe-sets in the MED17 dataset are represented in networks related to cell cycle

Ingenuity Pathway gene markers (corrected to $p<0.005$) were grouped into two networks related to cell cycle.

A. Network termed "cell cycle, DNA replication, recombination and repair, cancer". 35 focus genes were present in the network. B. Network termed "cell cycle, cellular assembly and organistion, DNA replication and repair". 35 focus genes were present in the network.

Probe-set ID	Gene	Name	GenBank accession number	Fold change	*q*-value
1452954_at	*UBE2C*	Ubiquitin conjugating enzyme E2C	AV162459	1.79	1.84E-05
1416076_at	*CCNB1*	Cyclin B1	NM_007629	1.68	2.57E-04
1419451_at	*FZR1*	Frizzled related protein	BC006616	1.25	1.19E-04
1448314_at	*CDC2*	cyclin dependent kinase -1	NM_007659	1.37	7.81E-05
1417131_at	*CDC25A*	Cell division cycle 25A phosphatase	C76119	1.15	4.50E-04
1426850_a_at	*MAPK2K6*	Mitogen activated protein kinase kinase 6	BB261602	1.81	7.96E-05
1417910_at	*CCNA2*	Cyclin A2	X75483	1.55	1.08E-04
1449061_a_at	*PRIM1*	Primase polypeptide	J04620	1	2.48E-04
1426653_at	*MCM3*	Minichromosome maintenance	BI658327	1.41	2.29E-04
1425166_at	*RBL1*	Retinoblastoma-like 1 (p107)	U27178	1.63	2.48E-04
1434437_x_at	*RRM2*	Ribonucleotide reductase, M2 subunit	AV301324	2.03	1.58E-04
1418225_at	*ORC2L*	Origin recognition complex	BB830976	1.19	9.27E-04
1423337_at	*ORC4L*		BB775020	1.89	1.84E-05
1417037_at	*ORC6L*		NM_019716	1.11	6.98E-04
1439040_at	*CENPE*	Centromeric protein E	BG068387	2.11	3.64E-04
1431921_a_at	*STAG1*	Stromal antigen	AK017978	1.9	6.48E-04
1450396_at	*STAG2*		NM_021465	1.75	6.82E-05
1434496_at	*PLK3*	Polo-like kinase 3	BM947855	1.33	7.22E-05
1452241_at	*TOPBP1*	DNA topoisomerase II binding protein	BC007170	1.81	1.15E-04
1450950_at	*CSPG6*	Chondroitin sulphate proteoglycan 6	AK005647	2.11	1.16E-04
1416162_at	*RAD21*	Rad21	AF332085	1.04	2.15E-04
1416746_at	*H2AFX*	H2A histone family member X	NM_010436	1.49	5.02E-05
1450677_at	*CHEK1*	Checkpoint	NM_007691	1.66	2.75E-04
1421205_at	*ATM*	Ataxia telangiectasia mutase	NM_007499	1.34	3.46E-04
1423920_at	*BRRN1*	Barren	BC021499	1.15	3.37E-04
1417830_at	*SMC1L1*	Structural maintenance of chromosomes like	BB156359	1.51	5.16E-05
1448635_at	*SMC2L1*		NM_008017	3.25	9.62E-05
1427275_at	*SMC4L1*		BI665568	3.19	5.93E-05
1417445_at	*KNTC2*	Kinetochore-associated protein 2	NM_023294	1.63	6.91E-05
1416915_at	*MSH6*	Mut S. homolog of 6	U42190	1.27	8.61E-04
1417947_at	*PCNA*	Proliferating cell nuclear antigen	BC010343	1.19	2.04E-04

Table 4.2 Common cell cycle related genes between *GOBrowser* and Ingenuity pathway analyses

The above table gives all the cell cycle related marker genes selected by independent analyses done using *GOBROWSER* and Ingenuity Pathways. *q*-values for marker probe-sets are stated.

Ingenuity application to identify putative transcription targets of MED17 that may mediate the up-regulation of cell cycle genes.

4.6 Activation of ß-catenin/LEF/TCF transcription in the MED17 cell line

The interaction of MED17 with a number of transcription factors in *Drosophila* suggests that it may have a global role in transcriptional regulation. The investigation of transcriptional networks using Ingenuity pathway analysis could reveal transcription factors and their transcriptional targets that are regulated by MED17. Armadillo, a *Drosophila* homolog of ß-catenin, has been shown to interact with MED17 in *Drosophila* embryo soluble nuclear fractions. ß-catenin co-operates with the LEF and TCF transcription factors to regulate transcription of genes involved in proliferation and cell movement. Moreover, it is thought to have a role in oncogenic transformation mediated by inappropriate activation of signalling pathways such as the Wnt signalling pathway. Activation of ß-catenin by MED17 could result in the enhanced expression of genes related to cell cycle and cell movement which were suggested by the analyses of the microarray data using *GOBrowser* and Ingenuity Pathway Analysis.

Identified by Ingenuity Pathway Analysis was one relating to ß-catenin, which scored 35 genes as present among the MED17 markers (Fig 4.4A). This network, termed "cellular movement, cell-to-cell signalling and interaction, tissue development", showed an enhanced fold change expression of ß-catenin, +1.060 (p=4.11^{-05}) for which multiple up-regulated probe-sets were present in the dataset. Significantly, genes transcriptionally regulated by ß-catenin were also

up-regulated in this network. These genes included plasminogen activated receptor, urokinase-type (PLAUR), gap junction protein, alpha 1 (GJA1), kinesin family member 23 (KIF23) and Fos-like antigen 1 (FOSL1), which showed expression fold changes of +1.29, +1.83, +1.23 and +1.84 respectively. In addition to these genes others were sought amongst the MED17 marker probe-sets to support these data suggesting activation of ß-catenin regulated transcription. A further 6 genes were identified including well described transcriptional targets, cyclin D1 (CCND1) and c-myc (MYC) which are both involved in cell cycle and proliferation (Table 4.3). These data suggest that ß-catenin transcription is activated in the MED17 cell line and was subsequently investigated in functional assays.

To test the activation of ß-catenin transcription in the MED17 cell line, as suggested by the analysis of the ß-catenin network, a ß-catenin/LEF/TCF responsive reporter was used in gene reporter assays to compare reporter activation between the vector and MED17 cell lines. Transfection of the LCF/TCF luciferase reporter into the MED17 cell line resulted in a 5-fold activation (+/- 0.82 fold), compared to the vector cell line control (Fig 4.4B). In conjunction with results from the microarray analysis, these data suggest that ß-catenin regulated transcription in the MED17 cell line is enhanced relative to the vector control cell line which may contribute to the enhanced expression of cell cycle genes. In addition, the diversity of transcriptional targets activated by ß-catenin may activate other gene networks, specifically relating to cell motility, which was further investigated.

Fig 4.4 MED17 gene markers are present in a network termed "Cellular movement, cell-to-cell signalling and interaction,tissue development" which contains the ß-catenin transcription factor

A. A schematic of the ß-catenin network, revealed by Ingenuity Pathway Analysis, scored 35 genes as present. Red icons represent up-regulated genes whereas green icons are down-regulated genes. Lines between genes indicate protein-protein interactions and arrows show transcription regulatory function between genes. * indicates multiple probe-sets were present in the dataset for the gene.

B. Gene reporter assays with ß-catenin/LCF/TCF responsive luciferase reporter in the vector and MED17 cell lines. Equal amount of reporter were transfected in to the cell lines with a Lac-Z expression plasmid to normalise luciferase values. Values are expressed as fold change relative to the vector control cell line.

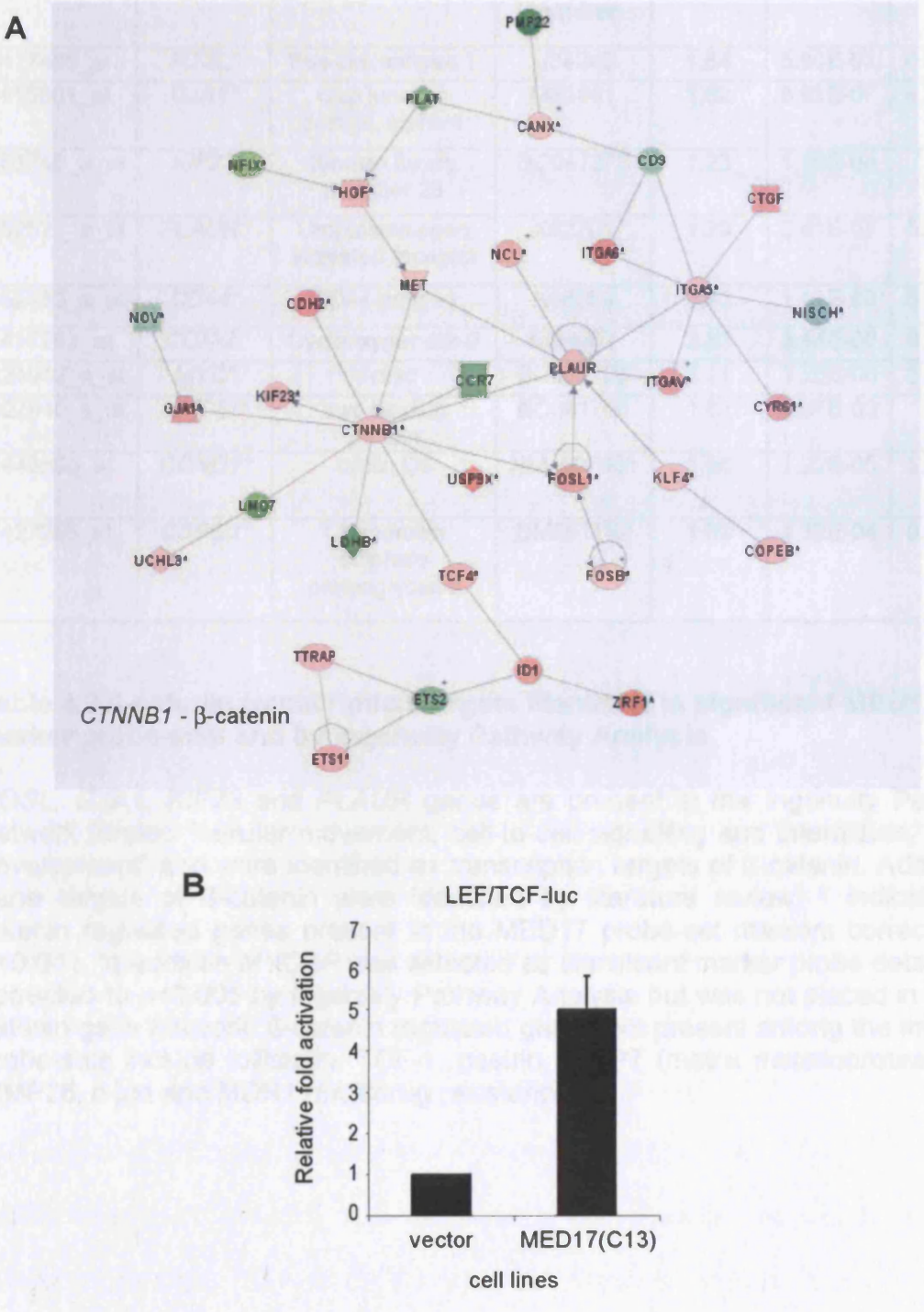


Fig 4.4 MED17 gene markers are present in a network termed "Cellular movement, cell-to-cell signalling and interaction, tissue development" which contains the β-catenin transcription factor

Probe set ID	Gene	Name	Genbank accession number	Fold change	*p*-value	*q*-value
1417488_at	*FOSL**	Fos-like antigen 1	U34245	1.84	5.90E-07	6.36E-05
1415801_at	*GJA1**	Gap junction protein, alpha 1	M63801	1.82	3.93E-07	4.77E-05
1453748_a_at	*KIF23*	Kinesin family member 23	BC047273	1.23	1.18E-04	_
1452521_a_at	*PLAUR**	Uroplasminogen activated receptor	X62701	1.29	3.41E-07	5.89E-05
1452483_a_at	*CD44**	CD44 antigen	X66083	3.43	1.14E-08	6.92E-06
1417263_at	*COX-2**	Cyclooxygenase 2	M94967	2.83	3.14E-09	9.94E-06
1424942_a_at	*MYC**	c-myc	BC006728	1.31	3.20E-06	3.27E-04
1427940_s_at	*MYCBP*	myc binding protein	BC041706	1.02	4.07E-03	_
1448698_at	*CCND1**	cyclin D1	NM_007631	1.06	1.22E-05	1.18E-04
1427256_at	*CSPG2**	Chondroitin sulphate proteoglycan 2	BM251152	1.03	1.32E-04	9.39E-04

Table 4.3 ß-catenin transcription targets identified in significant MED17 marker probe-sets and by Ingenuity Pathway Analysis

FOSL, *GJA1*, *KIF23* and *PLAUR* genes are present in the Ingenuity Pathway network termed "cellular movement, cell-to-cell signalling and interaction, tissue development" and were identified as transcription targets of ß-catenin. Additional gene targets of ß-catenin were identified by literature review. * indicates ß-catenin regulated genes present in the MED17 probe-set markers corrected to $q<0.001$. In addition *MYCBP* was selected as significant marker probe-sets when corrected to $p<0.005$ by Ingenuity Pathway Analysis but was not placed in the ß-catenin gene network. ß-catenin regulated genes not present among the markers probe-sets include follistatin, TCF-1, gastrin, MMP7 (matrix metalloprotease 7), MMP26, c-jun and MDR1 (multidrug resistance 1).

4.7 Genes involved in cellular motility are up-regulated in the MED17 cell line

Global analysis of the MED17 probe-set gene markers showed a significant representation of markers involved in "cellular movement" ($p<0.0429$). Subsequently two networks relating to "cellular motility", in addition to the ß-catenin network, were identified containing 25 and 19 focus genes respectively (Fig 4.5A and Fig 4.5B). In these networks three ß-catenin transcriptional targets, CD44, MYC and CSPG2, were identified. These networks did not contain as many focus genes as identified in the p53 and cell cycle networks, however their significant representation in the global analysis (section 4.3) and potential relationship to the ß-catenin network warranted further investigation.

Genes involved in cell motility as part of the cancer cell invasion process have been investigated using microarray analysis and have identified gene markers indicative of invasive cancer. The presence of such gene markers in the MED17 marker probe-sets was analysed using a list of markers compiled from microarray analysis as well as immunohistochemistry studies of expression, to support the data from the gene networks (Sahai, 2005). From the MED17 markers, 8 of the 10 were found to be markers of genes involved in cell invasion that were not present in the cell motility networks (Table 4.4). *CD44* and radixin (*RDX*) were both present in a network termed "cellular movement, cancer, tumour morphology" as well as the list of cell invasion markers. From the same list of gene markers for cell invasion, *CD44* and *PLAUR* are genes regulated by ß-catenin. These data suggest that a number of genes markers involved in cell

movement are up-regulated in the MED17 cell line which may in part be attributed to the enhanced expression of ß-catenin regulated genes.

Based on the transcriptional profile, functional assays for cell motility were performed using the vector and MED17 cell lines. Morphologically, the MED17 cells contained an increased number of filopodial structures, when observed using phase contrast microscopy (Fig 4.6 upper panels). Filopodia are spike-like structures protruding from the cell membrane and are formed by the restructuring of actin during cell movement. In contrast, the vector cell line contained much fewer such structures. This suggested that MED17 cells morphologically resemble invasive cells. To assess the invasiveness of the MED17 line, cells were tested for their ability to invade an extracellular madtrix (ECM). The assay is modelled on cell invasion through a basement membrane. Invasion chambers consist of a polycarbonate membrane containing 8µm pores covered by an ECM. Invasion occurs when cells migrate through the ECM and through the pores and adhere to the underside of the membrane, which is detected by cell staining. NIH-3T3 cells are non-invasive and likewise, the vector cells were not found to be invasive. However the MED17(C13) cells invaded the ECM indicating that these cell had an invasive capacity (Fig 4.6 bottom panels). This capacity is consistent with the up-regulation of gene markers involved in cell motility, as indicated by the microarray study.

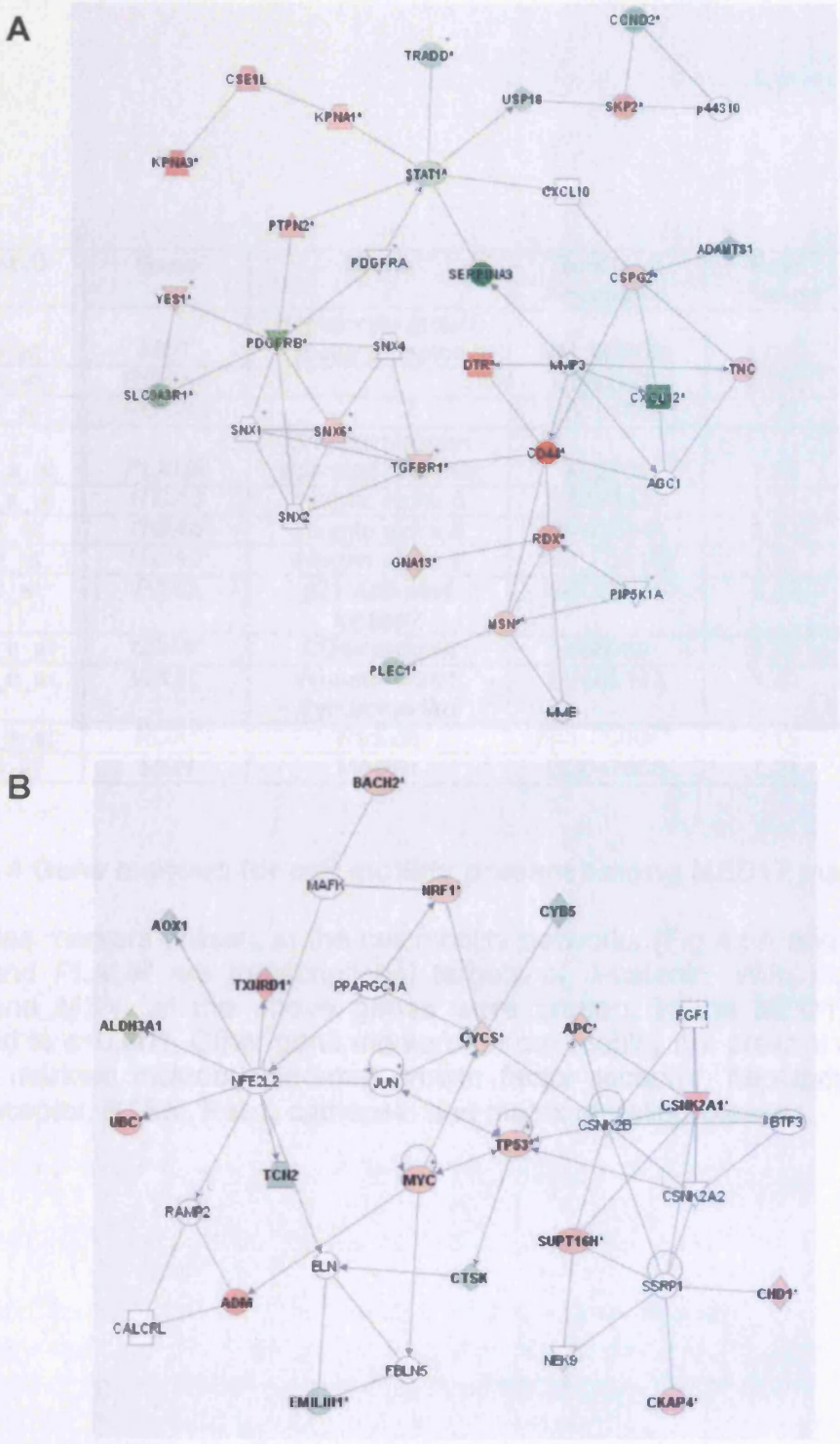


Fig 4.5 Analysis of MED17 cell line markers shows networks involved in cell motility

Analysis of markers using Ingenuity Pathways revealed two pathways relating to cell motility.

A. A network termed "Cellular movement, cancer, tumour morphology". Total of 25 focus genes were present.
B. A network termed "Cellular movement, cellular compromise, DNA replication,recombination and repair". Total of 19 focus genes were present.

Probe-set ID	Gene	Name	GenBank Accession	Fold change	*p*-value
1422990_at	*MET*	Hepatocyte growth factor receptor	NM_008591	1.040	4.95E-05
1423445_at	*ROCK1*		BI662863	2.580	5.82E-06
1451041_at	*ROCK2*		BB761686	1.87	2.41E-07
1452521_a_at	*PLAUR*	Uroplasminogen activated receptor	X62701	1.29	3.41E-07
1423267_s_at	*ITGA5*	Integrin alpha 5	BB493533	1.22	4.97E-03
1422444_at	*ITGA6*	Integrin alpha 6	BM935811	2.33	1.11E-07
1421198_at	*ITGAV*	Integrin alpha V	NM_008402	1.85	1.12E-05
1434250_at	*PAK2*	p21-activated kinase2	BC086650	1.12	5.93E-05
1452483_a_at	*CD44**	CD44 antigen	X66083	3.43	1.14E-08
1426777_a_at	*WASL*	Wiskott-Aldrich Syndrome like	BF466143	1.02	1.60E-05
1416180_a_at	*RDX**	Radixin	NM_009041	2.15	7.87E-08
1450370_at	*MSN*	Moesin	BC047366	1.21	1.42E-05

Table 4.4 Gene markers for cell motility present among MED17 markers

* indicates markers present in the cell motility networks (Fig 4.5A and Fig 4.5B). *CD44* and *PLAUR* are transcriptional targets of ß-catenin. With exception of *PAK2* and *MSN*, all the above genes were present in the MED17 markers corrected to $q<0.001$. Other gene markers for cell motility not present among the MED17 markers include epidermal growth factor receptor, hepatocyte growth factor receptor, PTEN, Rac1, cathepsin and matrix metalloproteases.

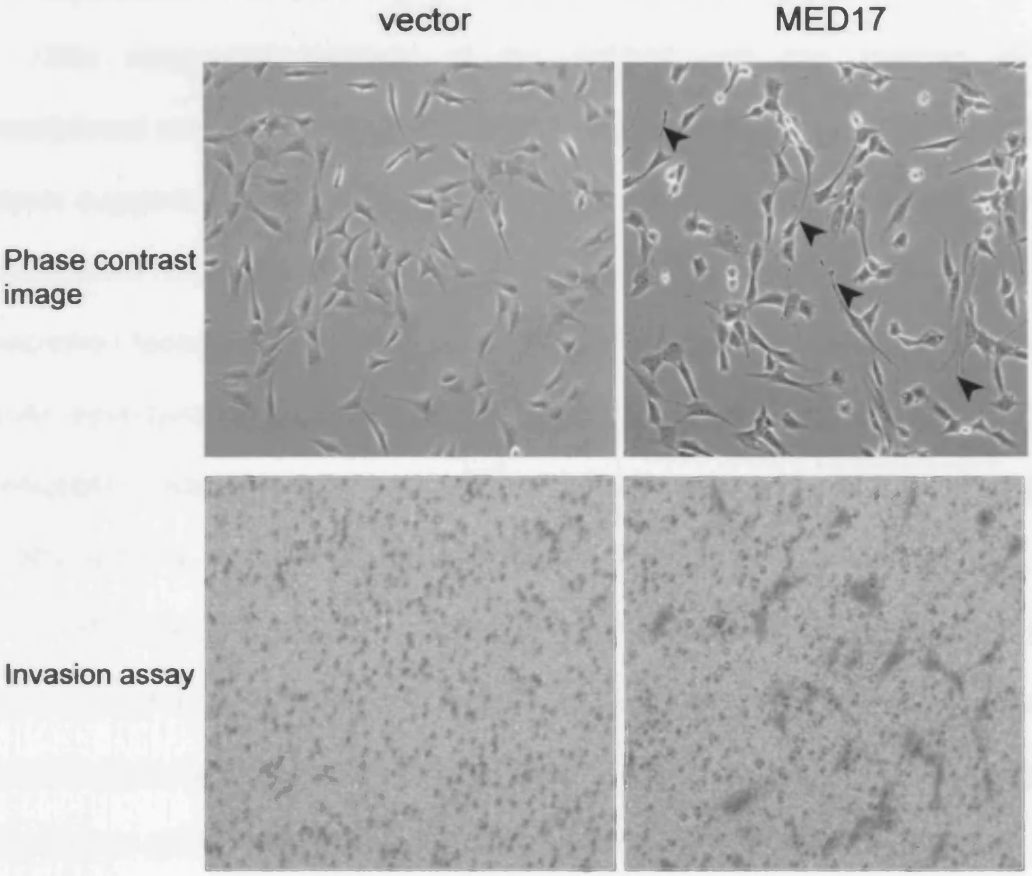


Fig 4.6 MED17 cells are invasive in Matrigel assays

Matrigel assays were used the invasive potential of the MED17 cell line

Top panel: Phase contrast images of the vector and MED17 cell lines observing cell morphology. Arrows indicate filopodial structures.
Bottom panel: Staining of membrane from invasion chambers. Vector and MED17 cells were seeded on the chambers in triplicate and incubated for 48 hours. Images are representative of result for all three chambers.

4.8 Optimisation of MED17 primer concentration for Q-PCR

The microarray analysis of the MED17 cell line showed global transcriptional changes induced by MED17 over-expression (section 4.2). GEM analysis suggests these global changes might be attributed to the activation of transcriptional networks by MED17 interacting with, and regulating, specific transcription factors. These transcriptional networks may be important for tissue specific development. To ascertain whether MED17 has a role in tissue specific development, real time quantitative PCR (Q-PCR) was performed to determine any differences at the level of mRNA of MED17 in a variety of human tissues.

Q-PCR for MED17 was optimised as described in the Material and Methods chapter. Primers for the Q-PCR were designed to amplify an 81-mer sequence from base pairs 1318 to 1398 of MED17 genomic DNA that region transcribes to mRNA. To test whether the selected primers amplified the MED17 81-mer, PCR was done using the primers to amplify the product from a human foetal cDNA library. Foetal cDNA was used as it is representative of a number of developing tissues. The reaction was resolved by agarose gel electrophoresis to visualise the correct sized PCR product (Fig 4.7A). PCR of the GAPDH mRNA transcript (a 78bp amplicon) was also performed to confirm amplification of the template. A reaction containing no template cDNA was also performed. Both MED17 and GAPDH reactions amplified a single product corresponding to the expected sizes of the amplicons suggesting that the primers amplify specifically the MED17 and GAPDH mRNAs. No products were observed in the non-template control. These data show that MED17 primers amplified a product

corresponding to the size of the predicted amplicon, and were used for further optimisation of Q-PCR for MED17.

An important variable of Q-PCR is the concentration of the primers. To obtain the optimal performance of the primers in the amplification, the threshold cycle must be kept low while maintaining a high ΔR_n in the presence of constant template (see Chapter 2, section 2.29). $MgCl_2$ concentration was kept constant at 3.5mM. To optimise the primer concentration both sense and anti-sense MED17 primers, forward and reverse primers respectively, were titrated against each other at 50, 300 and 900nM concentrations in reactions using foetal cDNA as the template. Q-PCR was done with these concentrations and a threshold cycle determined for each reaction at a baseline threshold value of 0.075 (Fig 4.7B and Fig 4.8A). Reactions containing 50nM of the forward or reverse primer correlated with a cycle threshold greater than 23. The maximum the cycle threshold reached was 30.06 for the reaction containing both primers at 50nM. Cycle thresholds were reduced to 19 with the increase in primer concentration (forward and reverse) to 300nM and 900nM. These data suggests that an appropriate primer concentration for MED17 Q-PCR would be between 300 and 900nM for both the forward and reverse primers. A primer concentration of 300F/300R produced a low threshold cycle with a high ΔR_n comparable to 900nM concentrations with low variation between samples as indicated by the error bars. Though results for reactions containing 50nM of primers were also consistent the higher threshold cycle and lower ΔR_n were not as favourable (Fig 4.8A). These

data indicate that the 300F/300R concentration of primers was suitable for Q-PCR.

To investigate whether any of the primer-pair concentrations could be associated with the formation of primer dimers, dissociation curves were generated at the end of reaction (see Chapter 2, section 2.29). The dissociation curves produced for the optimisation of primer concentration showed the presence of one fluorescent peak occurring at 79.2°C for each primer-pair and all primer concentrations tested (Fig 4.8B). The presence of one curve at a high melting temperature suggests the presence of one amplified product with no formation of primer dimers. Non-template controls for the given concentrations of primers were also performed and excluded primer dimer formation (Fig 4.8A). These data indicate that the primer concentrations of 300F/300R selected for use in Q-PCR does not result in primer dimer formation and is therefore suitable for subsequent experiments.

4.9 Validation of MED17 Q-PCR

Expression levels of a gene transcript can be determined within a specific tissue, relative to another ubiquitously expressed house-keeping gene, which can then be compared between tissues. Quantifying expression of such a house-keeping gene allows normalisation of the expression values of the MED17 transcript between tissues, allowing comparison. Q-PCR for the GAPDH transcript (glyceraldehyde-3-phosphate dehydrogenase) was used as a control for normalising MED17 expression between tissues. The optimisation of the

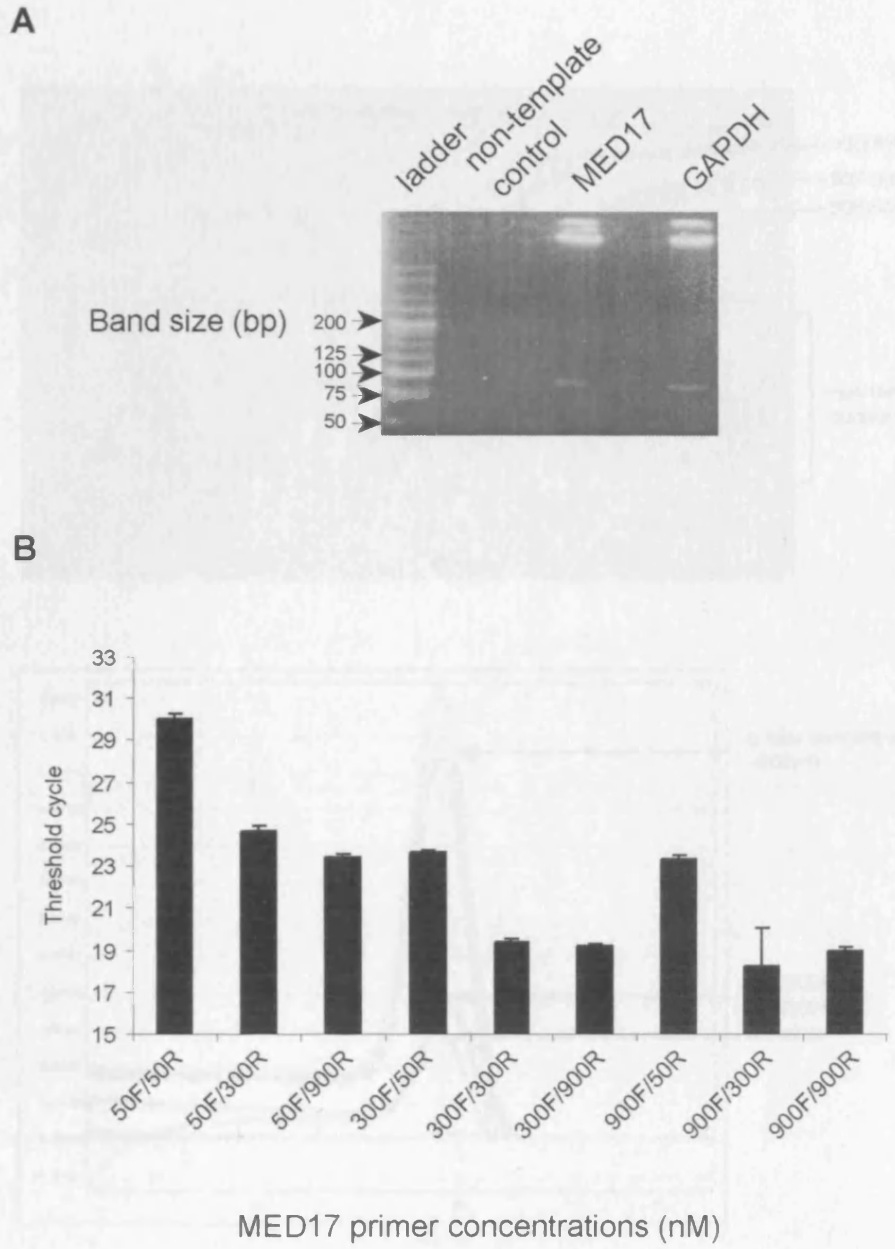


Fig 4.7 Optimisation of primer concentrations for MED17 Q-PCR

A. Agarose gel (1.5%) electrophoresis of PCR for MED17 and GAPDH. Q-PCR primers for MED17and GAPDH were used to amplify respective sequences from foetal cDNA. Also included was a non-template control. B. Threshold cycles for combinations of different MED17 primer concentrations. 50, 300 and 900nM concentrations of the forward (F) and reverse (R) primers were titrated against each other and Q-PCR was done in triplicate for each combination. Mean threshold cycle for each combination is displayed. Error bars show the standard deviation for primer-pair sample.

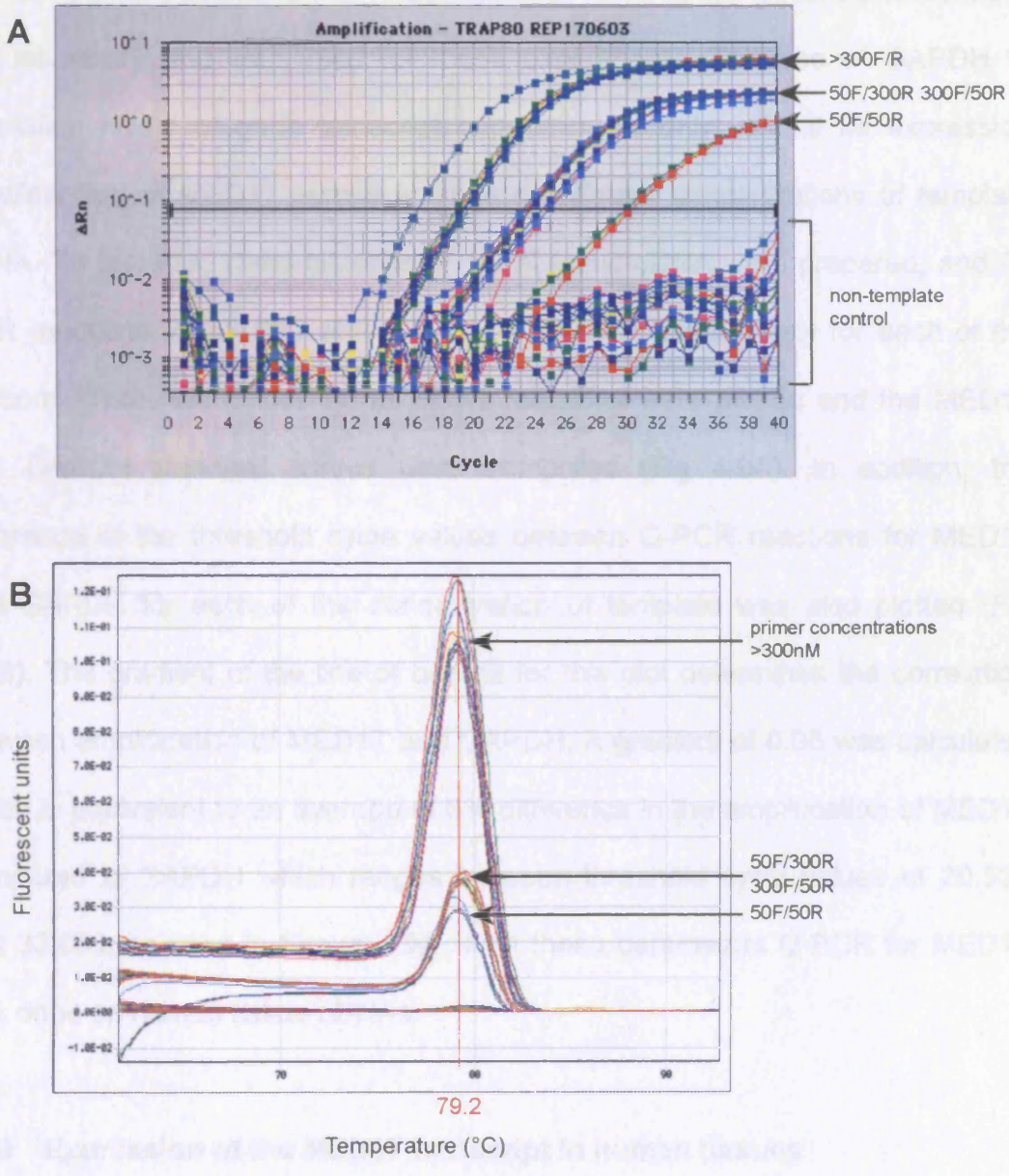


Fig 4.8 Amplification plot and Dissociation curve for MED17 primer optimisation

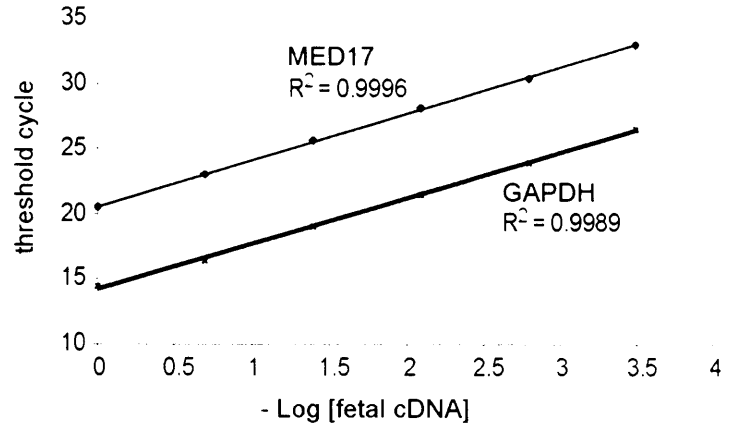
A. Amplification plot for all primer-pair concentrations tested.
B. Dissociation curve was generated for all the concentrations of MED17 primers tested.The dissociation curve is used in the identification of primer dimers which would affect the quantification of MED17 expression.

GAPDH primer-pairs for Q-PCR was previously done by Dr. Dimitra Bourboulia in our laboratory and was used for these experiments. The use of GAPDH to normalise levels of gene transcript expression is only valid if its expression parallels that of MED17 across a range of different concentrations of template cDNA. To test this, serial dilutions of 1:5 of foetal cDNA were prepared, and Q-PCR reactions for MED17 and GAPDH were done concurrently for each of the dilutions. Threshold cycles for all of the reactions were plotted and the MED17 and GAPDH standard curves were compared (Fig 4.9A). In addition, the difference in the threshold cycle values between Q-PCR reactions for MED17 and GAPDH for each of the concentration of template was also plotted (Fig 4.9B). The gradient of the line of best fit for this plot determines the correlation between amplification of MED17 and GAPDH. A gradient of 0.06 was calculated which is equivalent to an average of 6% difference in the amplification of MED17 compared to GAPDH which ranges between threshold cycle values of 20.535 and 33.055, as seen in Figure 4.9A. With these parameters Q-PCR for MED17 was done on human tissue cDNAs.

4.10 Expression of the MED17 transcript in human tissues

Analysis of the networks activated in the MED17 cell line suggested cellular functions that could be attributed to MED17 over-expression. To investigate whether these functions are specific to a given tissue, Q-PCR was done for MED17 to quantify expression of the MED17 transcript and analyse expression among different tissues. Using a panel of cDNAs synthesised from a

A



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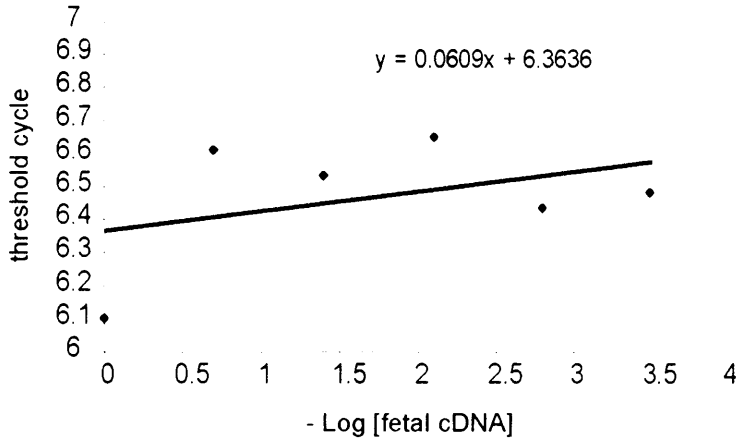


Fig 4.9 Validation of MED17 Q-PCR

Analysis of MED17 amplicatiion against that of GAPDH across a rangeof concentrations of foetal cDNA to ensure their amplification in parallel with one another which allows GAPDH to be used as a control for normalising between different template cDNAs.

A. Standard curve for MED17 and GAPDH Q-PCRs across dilutions of foetal cDNA of 1:5. Correlation co-efficients (R2) are given for each of the lines of best fit.
B. A plot of the difference between threshold cycles for MED17 and GAPDH for the given concentrations of foetal cDNA. The equation for line of best fit is given.

variety of human tissues, Q-PCR reactions were done in parallel for MED17 as well as for GAPDH. The final normalised expression values were then expressed as relative to MED17 expression in small bowel, as the values for threshold cycles for MED17 obtained for this tissue were closest to the mean threshold cycle of 26.64 for the panel of cDNAs. Threshold cycle values for MED17 ranged from 25.05 to 28.675 and were within the acceptable limits for analysis, as determined during the validation experiment (Fig 4.9A).

In the panel of tissue cDNAs expression of MED17 in the pancreas, spleen, testis and ovaries was found to be higher by greater than two fold relative to small bowel (Fig 4.10). High expression was also seen in placenta and lung tissue but only at 1.5 fold. Low expressing tissues included heart, brain and colon, where expression of MED17 was approximately 50% lower than the relative control. In particular, skeletal muscle was the lowest expressing tissue whose expression was only 5% relative to that of small bowel. These data suggest that the expression of the MED17 transcript varies between different human tissues. MED17 may also be important in the development and function of pancreas, spleen, testes and ovaries where its relative expression is high.

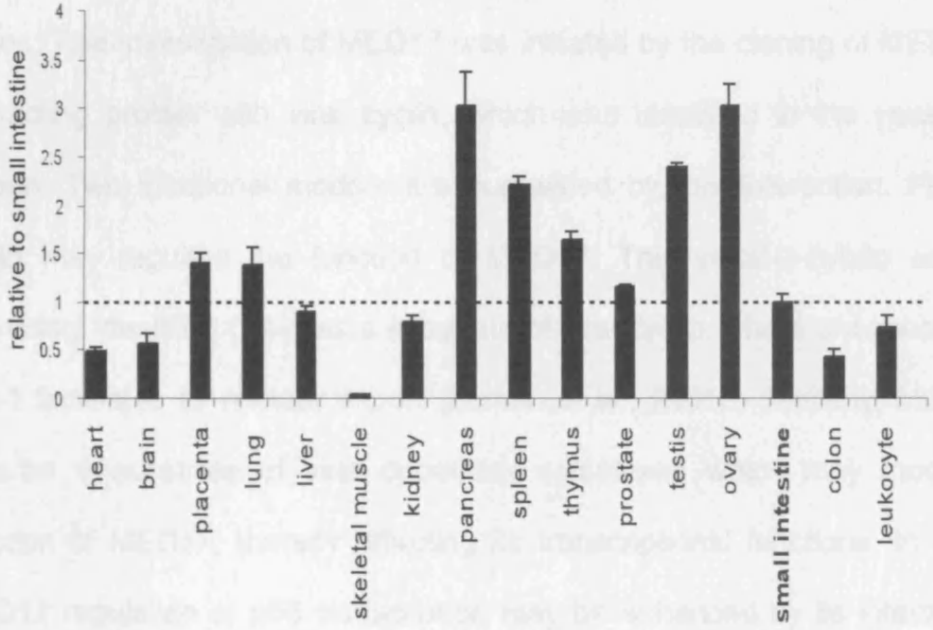


Fig 4.10 Relative expression of the MED17 transcript in a panel of human tissues

Q-PCR for MED17 was done on a panel of cDNAs synthesised from various human tissues. Q-PCR for MED17 and GAPDH was done in triplicate for each tissue cDNA and MED17 expression normalised to that of GAPDH. MED17 expression is displayed as relative to its expression in small intestine.

Chapter 5

5.0 Introduction

My investigation into the role MED17 in the regulation of p53 has indicated that it represses p53's pro-apoptotic activity. The mechanism of repression is most likely to be due to transcriptional inhibition of a subset of pro-apoptotic genes. This investigation of MED17 was initiated by the cloning of MED17 as an interacting protein with viral cyclin, which was identified in the yeast-2-hybrid screen. Two functional models are suggested by this interaction. Firstly, viral cyclin may regulate the function of MED17. This yeast-2-hybrid screen has previously identified Orc-1 as a substrate of viral cyclin, where phosphorylation of Orc-1 facilitates its nuclear export (Laman *et al.*, 2001b) Similarly, MED17 may also be a substrate of viral cyclin/cdk complexes which may modulate the function of MED17, thereby affecting its transcriptional functions. In particular, MED17 regulation of p53 transcription may be enhanced by its interaction with viral cyclin thus contributing to viral cyclin mediated oncogenesis.

An alternative model is that MED17 may affect viral cyclin function. Mediator complexes are associated with cyclin/cdk complexes that regulate of transcription. Cyclin C/cdk8 and cyclin H/cdk7 are involved in the phosphorylation of the CTD of RNA pol II and have been described as both positive and negative regulators its activity, making their exact function unclear (Liu et al., 2004). The Walleye Dermal Sarcoma Virus cyclin (OrfA) also interacts with hyperphosphorylated forms of RNA pol II in complex with cdk8 and can regulate

transcription of viral genes, suggesting that viral cyclins may also be regulators of RNA pol II activity (Rovnak and Quackenbush, 2002). MED17 may recruit viral cyclin to the basal transcription machinery to phosphorylate the CTD and thereby regulate transcription.

This chapter aimed to elucidate the nature of the interaction between viral cyclin and MED17 as well as establishing a function for the interaction.

Chapter 5: Results

5.1 HVS cyclin interacts with MED17 in yeast

The yeast-2-hybrid screen (Y-2-H) is based on the principle of recruitment of an activation domain to a reporter gene by virtue of an interaction with a "bait " protein, which is a fusion between the Gal4 binding domain (GBD) and the protein of interest (Fields and Song, 1989). Gal4 transcription activator consists of two functional domains, a C-terminal activation domain (AD) and an N-terminal sequence specific DNA binding domain (BD). The Y-2-H screen utilises a "bait" protein fused to the BD to select for interacting proteins synthesised from a cDNA library fused to AD. The interaction of library proteins with the bait protein "reconstitutes" the Gal4 transcription activator and allows transcription of reporter genes regulated by upstream activating sequences (UAS) which contain the Gal4 RE, such as *ADE2*, *HIS3* and LacZ. Transformed yeast are selected on amino acid deficient growth medium by auxotrophy for complementation of the bait and library expression plasmids, URA3 and LEU2. Then by virtue of reconstitution of the Gal4 transcription activator, transformants were selected for activation of the reporter genes, *ADE2* and *HIS3*. Screening for interacting proteins by activation of LacZ was done by assaying for ß-galactosidase. The expression of ß-galactosidase can be detected on X-gal supplemented growth plates which induces a blue colour change in the yeast colony or alternatively by liquid assay culture, which I used.

In a Y-2-H screen of a human B and T cell cDNA library fused to pGal4(AD), MED17 was cloned as an interacting protein with the bait protein, V-cyclin of *Herpesvirus saimiri*, which was fused to pGal4(BD). Sequencing of the pGal4(AD)-MED17 clone, showed an in frame fusion of the pGal4(AD) to amino acids 19-651 of MED17. β-galactosidase reporters with the MED17 clone showed a 5.4 fold activation of the reporter relative to vector only, suggesting an interaction between V-cyclin and MED17 (Laman, unpublished data).

To confirm the interaction of MED17 and V-cyclin in the Y-2-H screen, truncations of V-cyclin were used to map the site of interaction of MED17 on HVS cyclin. These truncations of V-cyclin spanning the length of the gene and were fused to the BD of Gal4 (Fig 5.1A). V-cyclin truncations, aa1-215 and aa1-168 contain a region of V-cyclin responsible for interaction with the T-loop of cdk6, located at the extreme N-terminus of V-cyclin via the H3 helix and the loop between helices H3 and H4. At the C-terminus of V-cyclin are a further two helices, H1'and H2', spanning amino acids 154-185, of which V-cyclin aa169-254 only contained the H2' helix. These truncations were used to test the dependency of the MED17 and V-cyclin interaction on the cdk6 subunit.

To test whether the expression of the GBD-V-cyclin truncation were at equal efficiencies, Western blots were conducted on protein lysates from the transformed yeast to detect the Gal4-BD (Fig 5.1B). V-cyclin truncations 1-215 and 1-168 were expressed to an equal efficiency as full length V-cyclin. However, the smaller C-terminus truncations, 169-254 and 216-254 were expressed at much lower efficiencies compared with the full length protein. These expression

efficiencies were taken into consideration when interpreting results from the reporter assays using these truncations.

Yeast were next transformed with DNA encoding the GBD-truncations of V-cyclin and the original pGAD MED17 clone identified in the Y-2-H screen. Transformed yeast were streaked on to synthetic complete media lacking amino acids to enable selection of the transformed yeast. Colonies forming from the transformed yeast were observed for colour changes relating to the activation of *ADE2* gene by Gal4 transcription. Yeast not expressing ADE2 are red in appearance whereas its expression induces a colour change to white from red. A colour change from red to white was observed in the colonies of yeast transformed with the full length V-cyclin and MED17, indicating activation of Gal4 transcription (Fig 5.2A (3)). Yeast transformed with the truncations of V-cyclin (Fig 5.2A (4,5,6,8)) appeared red. pGal4(GBD) V-cyclin (full length) and pGal4(AD) MED17 alone also appeared red, suggesting that the full length cyclin and MED17 were necessary to activate *ADE2* transcription (Fig 5.2A (1,2)).

As an independent test of Gal4 transcriptional activation in the yeast transformed with the truncations of V-cyclin, ß-galactosidase assays were performed on cultures of the transformed yeast and Miller units were calculated (Fig 5.2B). Relative to the GAL vector only, full length V-cyclin induced an increase of 31 fold in the activation of the ß-galactosidase reporter. All the truncations of V-cyclin decreased activation of the reporter compared to the full length cyclin. V-cyclin truncations, aa1-215 and aa1-168 induced 1.4 and 3.1 fold activations relative to GAL whereas aa164-254 and aa215-254 induced 4.3 and

4 fold activations respectively. These data suggest that the first 215 amino acids of V-cyclin are not required for an interaction with MED17. The smaller truncations of the C-terminus of V-cyclin activated the reporter to a higher level than the C-terminal deletions, however their expression to be significantly lower in comparison to full length V-cyclin.

In conclusion, any truncation of V-cyclin significantly reduced the activation of the reporter by at least 10 fold. From these data, no interaction domain on V-cyclin could be mapped however this may suggest that the whole conformational structure of V-cyclin may be important for its interaction with MED17.

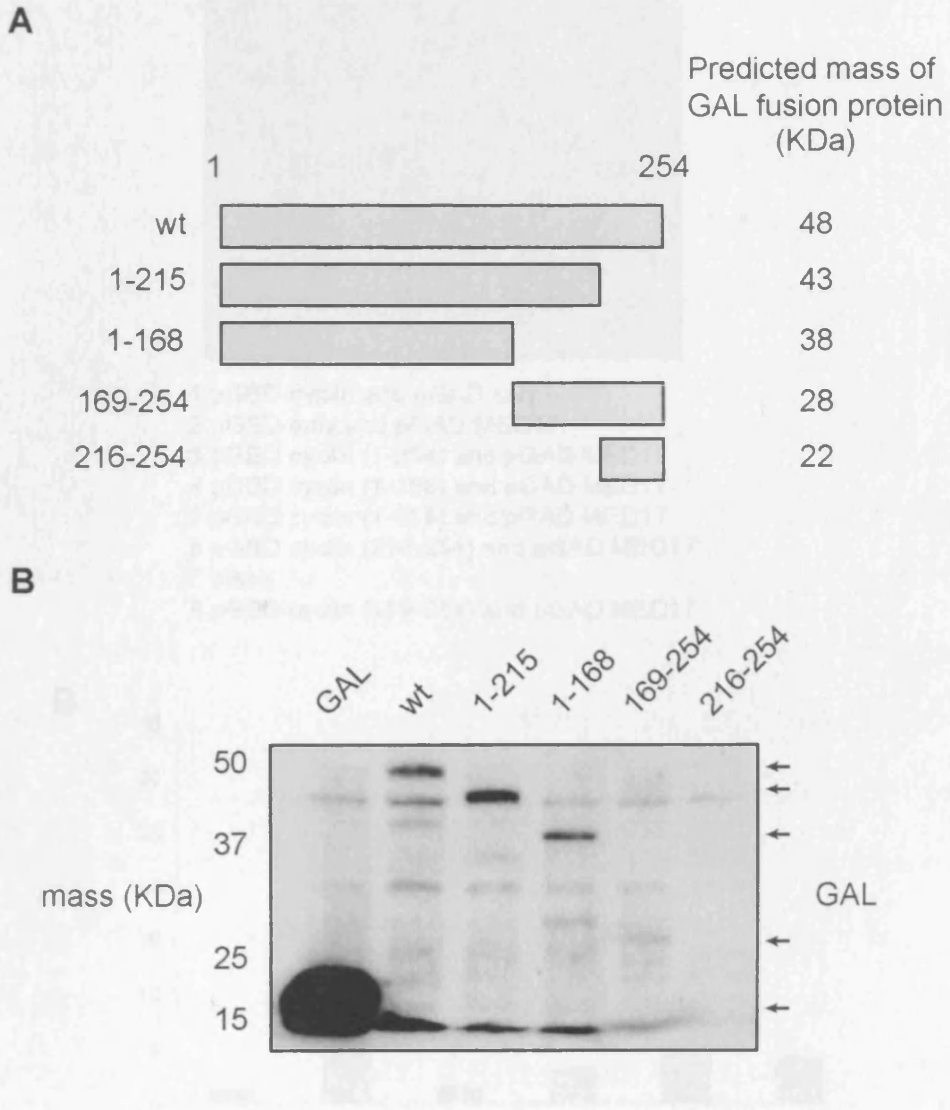
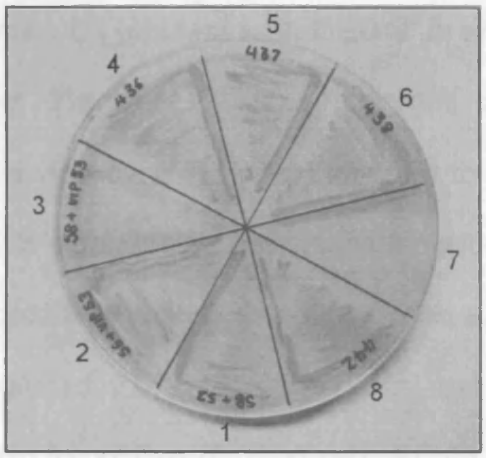


Fig 5.1 Expression of GAL V-cyclin fusion proteins in yeast

A series of truncations spanning the length of V-cyclin and fused to GBD were made to map the MED17 interaction to a region of HVS cyclin in yeast.

A. Schematic of the truncations of GBD V-cyclin fusions cloned with predicted masses of GAL fusion proteins.
B. Western blot of GAL V-cyclin fusion proteins expressed in yeast. Protein were resolved on a 15% polyacrylamide gel.

A



1 pGBD cyclin and pGAD only
2 pGBD only and pGAD MED17
3 pGBD cyclin (1-254) and pGAD MED17
4 pGBD cyclin (1-168) and pGAD MED17
5 pGBD cyclin (1-215) and pGAD MED17
6 pGBD cyclin (216-254) and pGAD MED17
7 blank
8 pGBD cyclin (169-254) and pGAD MED17

B

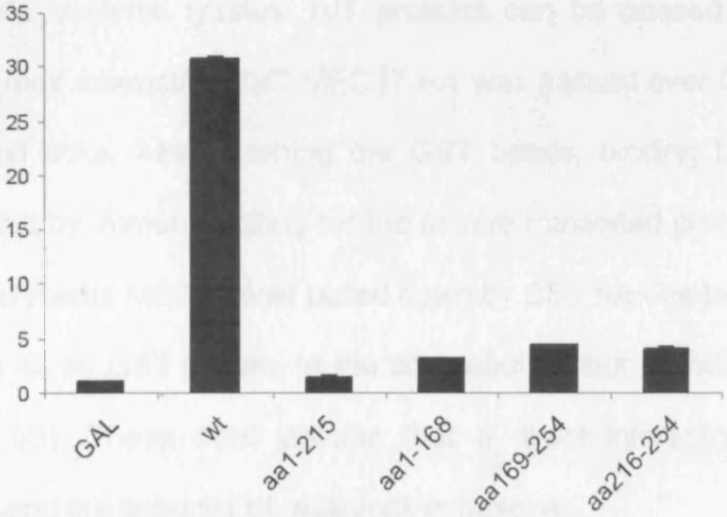


Fig 5.2 MED17 interacts with V-cyclin in yeast

A. Yeast were transformed with the V-cyclin truncations and plated on fully supplemented growth medium to observe colour change from red to white indicating interactions of expressed fusion proteins with MED17.
B. ß-gal reporter assays in yeast with trunctions of pGBD V-cyclin and pGAD MED17.

5.2 vcyclin and cyclin D1 interact with MED17 *in vitro*

The result from the Y-2-H screen indicated an interaction occurred between MED17 and a viral cyclin. To test whether this interaction occurred in an alternative system, *in vitro* binding assays were performed using GST fusions to viral and cellular cyclins and their interacting cdk subunits, cdk4 and cdk6. vcyclin of KSHV, a related γ2-herpesvirus cyclin was used in these assays. MED17 HA was *in vitro* translated (IVT) in reticulocyte lysates and used in the binding assays with the GST fusion proteins to cyclins and cdks.

Binding assays involve the immobilisation of GST fusion protein on glutathione sepharose beads. Homodimerisation of GST portion of the fusion protein with glutathione bound to beads allows isolation and purification of the protein from crude bacterial lysates. IVT proteins can be passed over these beads to test for their interaction. IVT MED17 HA was passed over GST fusions of the cyclins and cdks. After washing the GST beads, binding between the proteins was tested by immunoblotting for the *in vitro* translated protein, MED17 HA. In these experiments MED17 was pulled down by GST fusions to vcyclin and cyclin D1 as well as by GST fusions to the cdk subunits, but not with GST (Fig 5.3A and Fig 5.3B). These data indicate that a direct interaction occurred between MED17 and the subunits of cyclin/cdk complexes.

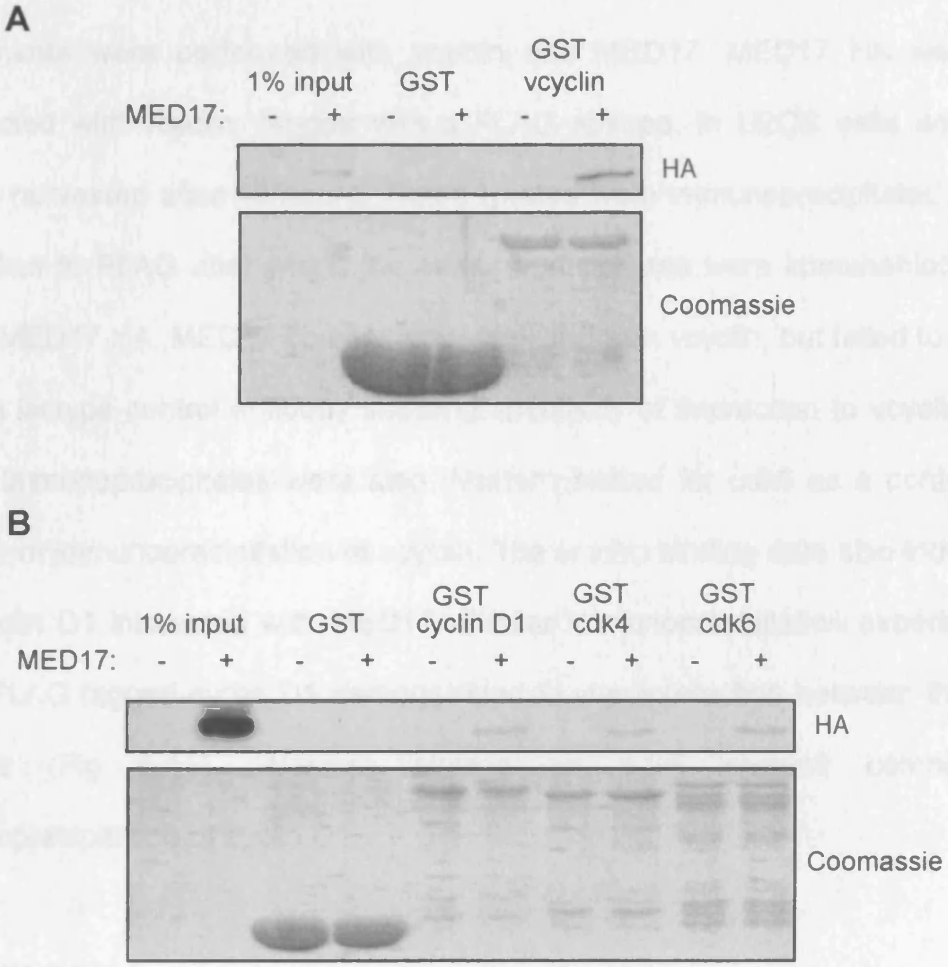


Fig 5.3 MED17 inteacts with vcyclin and cellular cyclins *in vitro*

MED17 binding assays were used to confirm the interaction of vcyclin as suggested by the Y-2-H screen.

A. Western blot for MED17 after pull down with GST and GST-vcyclin. Coomassie staining for GST-protein (lower panel).
B. Western blot for MED17 after pull down with GST and GST-cyclin D1, cdk4 and cdk6.

5.3 vcyclin and cyclin D1 interact with MED17 *in vivo*

To test whether an interaction occurs *in vivo,* co-immunoprecipitation experiments were performed with vcyclin and MED17. MED17 HA was co-transfected with vcyclin, tagged with a FLAG epitope, in U2OS cells and cell lysates harvested after 48 hours. These lysates were immunoprecipitated using antibodies to FLAG after which the immunoprecipitates were immunoblotted to detect MED17 HA. MED17 co-immunoprecipiated with vcyclin, but failed to do so with an isotype control antibody showing specificity of interaction to vcyclin (Fig 5.4A). Immunoprecipitates were also Western blotted for cdk6 as a control for equivalent immunoprecipitation of vcyclin. The *in vitro* binding data also indicated that cyclin D1 interacted with MED17. Similar immunoprecipitation experiments using FLAG tagged cyclin D1 demonstrated *in vivo* interaction between the two proteins (Fig 5.4A). Western blotting for cdk6 showed comparable immunoprecipitation of cyclin D1.

5.4 MED17 interacts *in vivo* with cyclins E and A

Both HVS and KSHV cyclins, in complex with cdk6, can phosphorylate substrates of cyclin E/cdk2 and A/cdk2 complexes. We therefore investigated whether an *in vivo* interaction occurred between MED17 and either cyclins E or A. U2OS cells were transfected with MED17-HA and cell lysates were immunoprecipitated with antibodies to either endogenous cyclin E or A. MED17 was detected in immunoprecipitates to both of these cyclins but not in immunoprecipitates with an isotype control antibody (Fig 5.4B). Western blotting

for cdk2 showed co-immunoprecipitation with cyclin A. These data show that MED17 interacts with cyclin E and cyclin A *in vivo* in addition to vcyclin and cyclin D1. These data imply that MED17 interacts with multiple cyclin/cdk complexes. Furthermore, the interaction of these G1/S phase cyclins with MED17 suggests that the effects of this interaction occur in these phases of the cell cycle. Further investigation of MED17 was done to establish the nature of the MED17/cyclin interaction.

5.5 Synthesis of GST-MED17 fusion proteins

It is possible that the interaction between viral and cellular cyclins/cdks with MED17 mediates its phoshorylation. To test whether MED17 was a substrate of the interacting cyclin/cdks, *in vitro* kinase assays were used. In these assays the substrate were synthesised and purified as a GST fusion protein from bacteria. Two GST MED17 fusion expression constructs were cloned (Fig 5.5A). One of these constructs encodes a N-terminal truncation of the first 147 amino acids of MED17 and was made utilising a convenient internal restriction site within MED17. The other contained the full length coding sequence of MED17. Both MED17 clones are fused to GST at the N-terminus and their expression induced by an IPTG inducible promoter.

GST-MED17 (148-651) expression was performed over a time course to optimise extraction and purification of the protein from bacteria. Lysed bacterial pellets, post-sonication pellets and GST-MED17 purified on glutathione sepharose beads were harvested after 0, 1, 2 and 4 hours of induction of

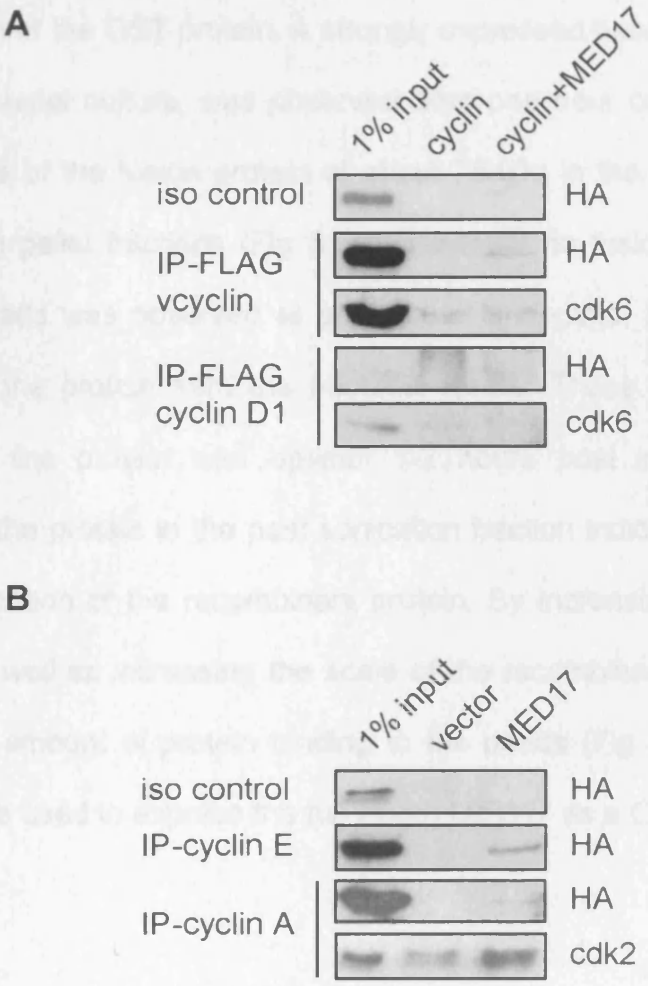


Fig 5.4 MED17 interacts with vcyclin and cellular cyclins *in vivo*

A. Co-immunoprecipitation experiments with FLAG-tagged vcyclin and cyclin D1. Immunoblot for MED17 shown. Immunoblot for cdk6 included as a positive control.
B. Immunoblot MED17 after CO-IP of cyclin E and A. Immunoblot for cdk2 included as a positive control.

bacteria in 1mM IPTG supplemented media. Protein extracts were resolved by SDS-PAGE and the gels stained with Coomassie Blue to observe expression and purification of the GST protein. A strongly expressed band, not present in the uninduced bacterial culture, was observed after one hour corresponding to the predicted mass of the fusion protein of about 75KDa in the bacterial pellet and post-sonication pellet fractions (Fig 5.5B). However no fusion protein bound to gluthatione beads was observed at any of the timepoints, indicating inefficient purification of the protein from the bacterial lysate. These results showed the expression of the protein was optimal 1-2 hours post induction. The high expression of the protein in the post-sonication fraction indicated that there was inefficient extraction of the recombinant protein. By increasing the extraction of the protein as well as increasing the scale of the recombinant protein synthesis enhanced the amount of protein binding to the beads (Fig 5.5C). These same conditions were used to express the full length MED17 as a GST fusion protein.

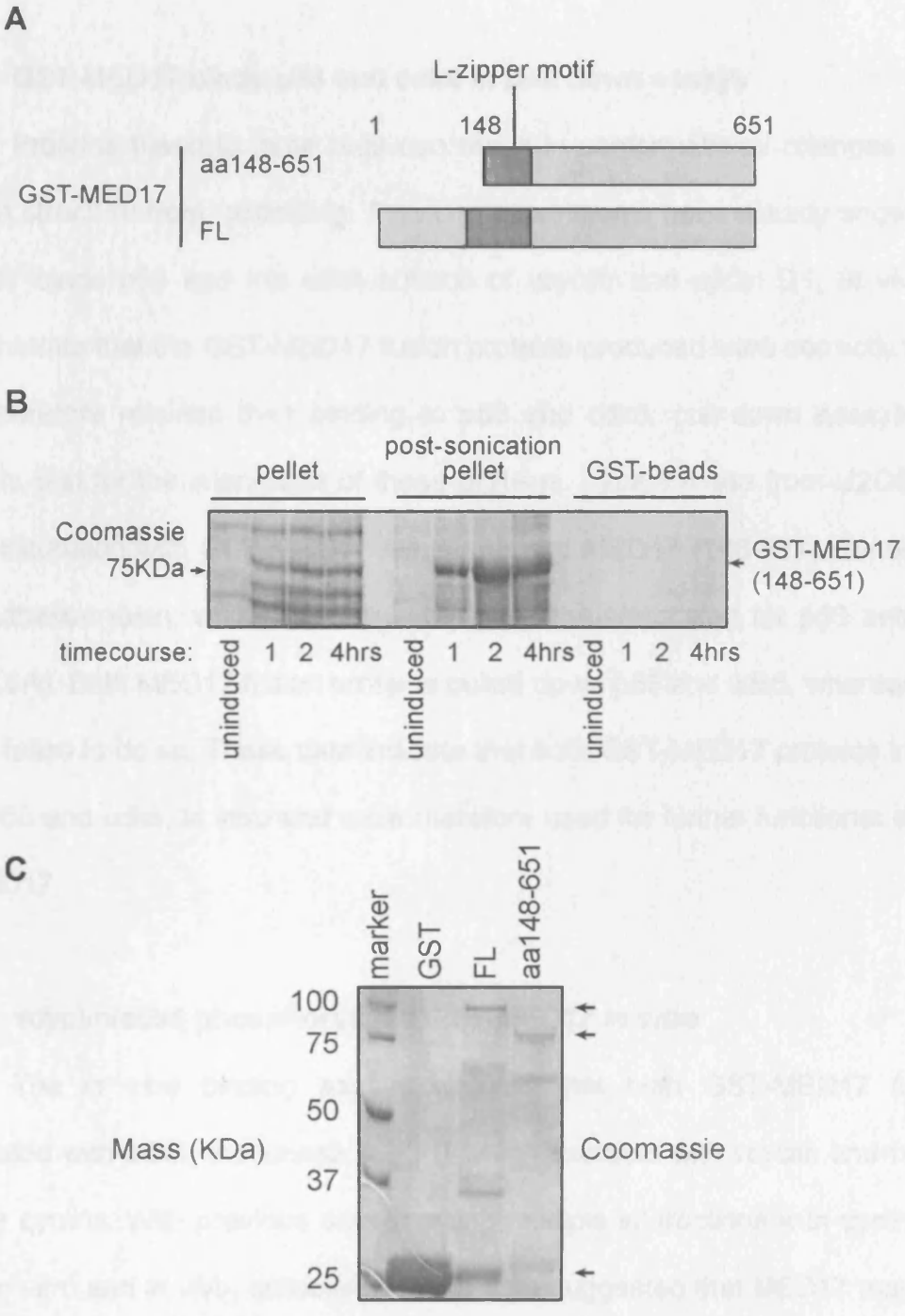


Fig 5.5 Synthesis of a recombinant GST-MED17 full length and N-terminus truncation

MED17 was cloned as a fusion protein with GST to be used in *in vitro* kinase assays with cyclin/cdks.

A. Schematic of the GST fusions of MED17 made.
B. Expression of the N-terminal trunction GST-MED17 in *Escherichia coli* FB810 shown on Coomassie stained 10% polyacrylamide gel.
C. Expression and binding to GST beads of MED17 fusion proteins (FL and aa148-651).

5.6 GST-MED17 binds p53 and cdk6 in pull down assays

Proteins fused to large tags can result in conformational changes to the protein structure from misfolding. Previous experiments have already shown that MED17 binds p53 and the cdk6 subunit of vcyclin and cyclin D1, *in vivo*. To demonstrate that the GST-MED17 fusion proteins produced were correctly folded and therefore retained their binding to p53 and cdk6, pull down assays were used to test for the interaction of these proteins. Lysate made from U2OS cells were incubated with GST-MED17 full length and MED17 (148-651) immobilised on gluthation resin, which were subsequently immunoblotted for p53 and cdk6 (Fig 5.6A). Both MED17 fusion proteins pulled down p53 and cdk6, whereas GST alone failed to do so. These data indicate that both GST-MED17 proteins interact with p53 and cdk6, *in vitro* and were therefore used for further functional studies of MED17.

5.7 vcyclin/cdk6 phosphorylates GST-MED17 *in vitro*

The *in vitro* binding assays showed that both GST-MED17 fusions interacted with cdk6, the kinase subunit which interacts with vcyclin and cellular D-type cyclins. With previous data showing multiple interactions with cyclin/cdks both *in vitro* and *in vivo*, collectively these data suggested that MED17 may be a substrate of cyclin/cdk complexes. *In vitro* kinase assays were therefore conducted with both MED17 GST fusion proteins and vcyclin/cdk6 to test whether MED17 could be phosphorylated. Sf9 cells infected with baculoviruses encoding cdk6 or co-infected with vcyclin and cdk6 were used to produce the

cyclin/cdks for these assays. Infected cells were harvested 48 hours post-infection and their cell lysates used in the kinase reaction, containing the GST bound proteins and radioactively labelled ATP. Both MED17 fusion proteins were phosphorylated by vcyclin/cdk6 and not by cdk6 alone as was GST-Rb (aa792-928) which was used as a positive control (Fig 5.6B). GST alone was not phosphorylated by vcyclin/cdk6. Of the two GST-MED17 proteins, MED17 (148-651) was more robustly and specifically phosphorylated. These results indicate that MED17 both full length MED17 and an N-terminal truncation are phosphorylated and further indicate the presence of phosphorylation sites and other sequences required for phosphorylation were not present in the first 147 amino acids of MED17. Further studies of cyclin/cdk phosphorylation of MED17 were therefore conducted with the GST N-terminus truncation of MED17.

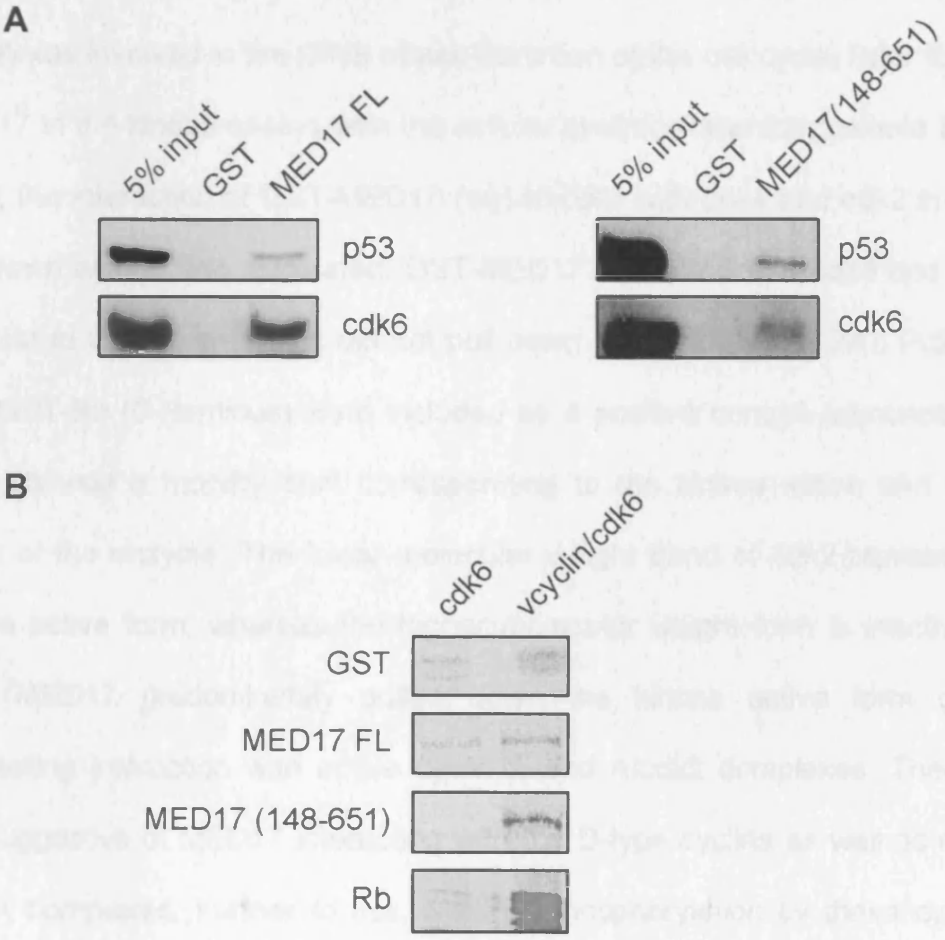


Fig 5.6 GST-MED17 fusion protein interacts with p53 and cdk6 and is phosphorylated by vcyclin/cdk6

A. Western blots for p53 and cdk6 after ex-vivo pull downs from U2OS lysate with GST-MED17 full length (FL) and N-terminus truncation (148-651).
B. *In vitro* kinase assays with cdk6 alone or vcyclin/cdk6 with GST-MED17 (FL and 148-651) as a substrate. GST-Rb was included as a positive control.

5.8 GST-MED17 pulls down cdk4 and cdk2

Data from *in vitro* binding assays, *in vivo* co-immunoprecipitations and *ex vivo* pull down assays have indicated that MED17 interacts with cyclin/cdk complexes involved in the G1/S phase transition of the cell cycle. Prior to testing MED17 in the kinase assays with the cellular cyclin/cdks which promote S-phase entry, the interaction of GST-MED17 (aa148-651) with cdk4 and cdk2 in *ex vivo* pull down assays was evaluated. GST-MED17 interacted with cdk4 and cdk2 in contrast to GST alone which did not pull down either cdk (Fig 5.7A). Pull downs with GST-Rb (C-terminus) were included as a positive control. Immunoblots for cdk2 showed a mobility shift corresponding to the kinase active and inactive forms of the enzyme. The lower molecular weight band of cdk2 represents the kinase active form, whereas the higher molecular weight form is inactive. Like pRb, MED17 predominately pulled down the kinase active form of cdk2 suggesting interaction with active cyclin E and A/cdk2 complexes. These data are suggestive of MED17 interacting with the D-type cyclins as well as cyclin E and A complexes. Further to this, MED17 phosphorylation by these cyclin/cdk complexes was tested by *in vitro* kinase assays.

5.9 MED17 is phosphorylated by cellular cyclin/cdks *in vitro*

vcyclin/cdk6 is functionally homologous to cellular cyclin D1/cdk and cyclin E and cyclin A/cdk2 complexes. The data presented thus far indicates that these cellular cyclin complexes interact with MED17. *In vitro* kinase assays, performed exactly as those for the vcyclin/cdk6 complexes, were used to investigate whether cyclinD1/cdk4, cyclinD1/cdk6, cyclinE/cdk2 and cyclinA/cdk2 complexes phosphorylate MED17. GST-MED17 was phosphorylated by all cyclin/cdks complexes tested, while GST was not (Fig 5.7B). Crude lysates made from cells infected with baculoviruses encoding cdk6 resulted in residual levels of phosphorylation of GST-MED17 and Rb, but was greatly enhanced when the cyclin subunit was present. These results demonstrate that MED17 is a substrate of cellular cyclin/cdk complexes involved in the G1/S phase transition of the cell cycle.

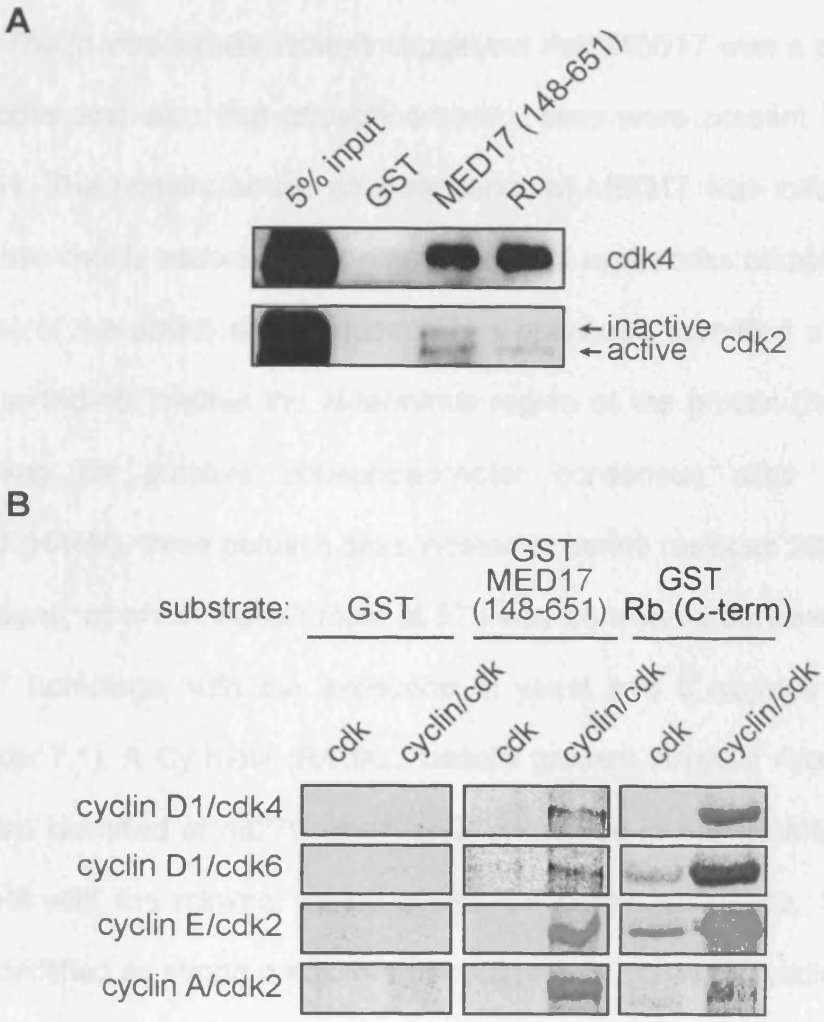


Figure 5.7 GST MED17 is phosphorylated by cyclin/cdks *in vitro*

A. GST-pull downs from U2OS cell lysate with GST alone, GST-MED17 (148-651) and GST-Rb, testing for interactions with cdk4 and cdk2.
B. *In vitro* kinase assays with cellular cyclin/cdks with GST, GST-MED17 and GST-Rb (C-terminus, aa 792-928)

5.10 Identification of phosphoacceptor sites on MED17

The *in vitro* kinase assays suggested that MED17 was a substrate of the cyclin/cdks and also that phosphoacceptor sites were present in amino acids 148-651. The primary amino acid sequence of MED17 was therefore analysed for protein motifs associated with substrates of cyclin/cdks complexes. Previous analysis of the amino acid sequence has previously identified a leucine zipper motif (aa146-167) within the N-terminus region of the protein (Ito *et al.*, 1999). Searching for putative phosphoacceptor consensus sites conforming to (S/T)PX(H/R/K), three putative sites located at serine residues 288, 573 and 647 were found, of which the SP motif at 573 was conserved between all the known MED17 homologs, with the exception of yeast and *C.elegans* (Fig 5.8A and Appendix 7.1). A Cy motif (R/K)XL, usually present in cyclin A/cdk2 substrates, was also identified at aa279 which was conserved in murine MED17 only. The Cy motif with the minimal serine phosphoacceptor sequence, SP, sites have been identified as strong predictors of cyclin A/cdk2 phosphorylation (Stevenson-Lindert et al., 2003).

To investigate which of the three putative phosphoacceptor sites where phosphorylated *in vitro,* point mutations of nucleotides encoding the serine residues within the putative phosphoacceptor sites were engineered to encode alanine residues either singly (S288A, S573A, and S647A) or in combination (573/647A, 288A/573A/647A (ΔSP) within the GST-MED17 (aa148-651) fusion protein (Fig 5.8B). MED17 mutants were then expressed and tested for their ability to be phosphorylated by vcyclin/cdk6 and cyclin A/cdk2 complexes in

kinase assays. Surprisingly, mutation of any serine residue, either singly or in combination, ablated GST-MED17 phosphorylation by vcyclin/cdk6 or cyclin A/cdk2 complexes despite equivalent expression and input of GST-MED17 mutants into the kinase assay, compared to wild type GST-MED17 (Fig 5.8C). These data suggest that each serine residues was important for the conformation and/or phosphorylation of GST-MED17 *in vitro.* Studies of the phosphosite mutants were continued *in vivo* to ascertain a function for MED17 phosphorylation.

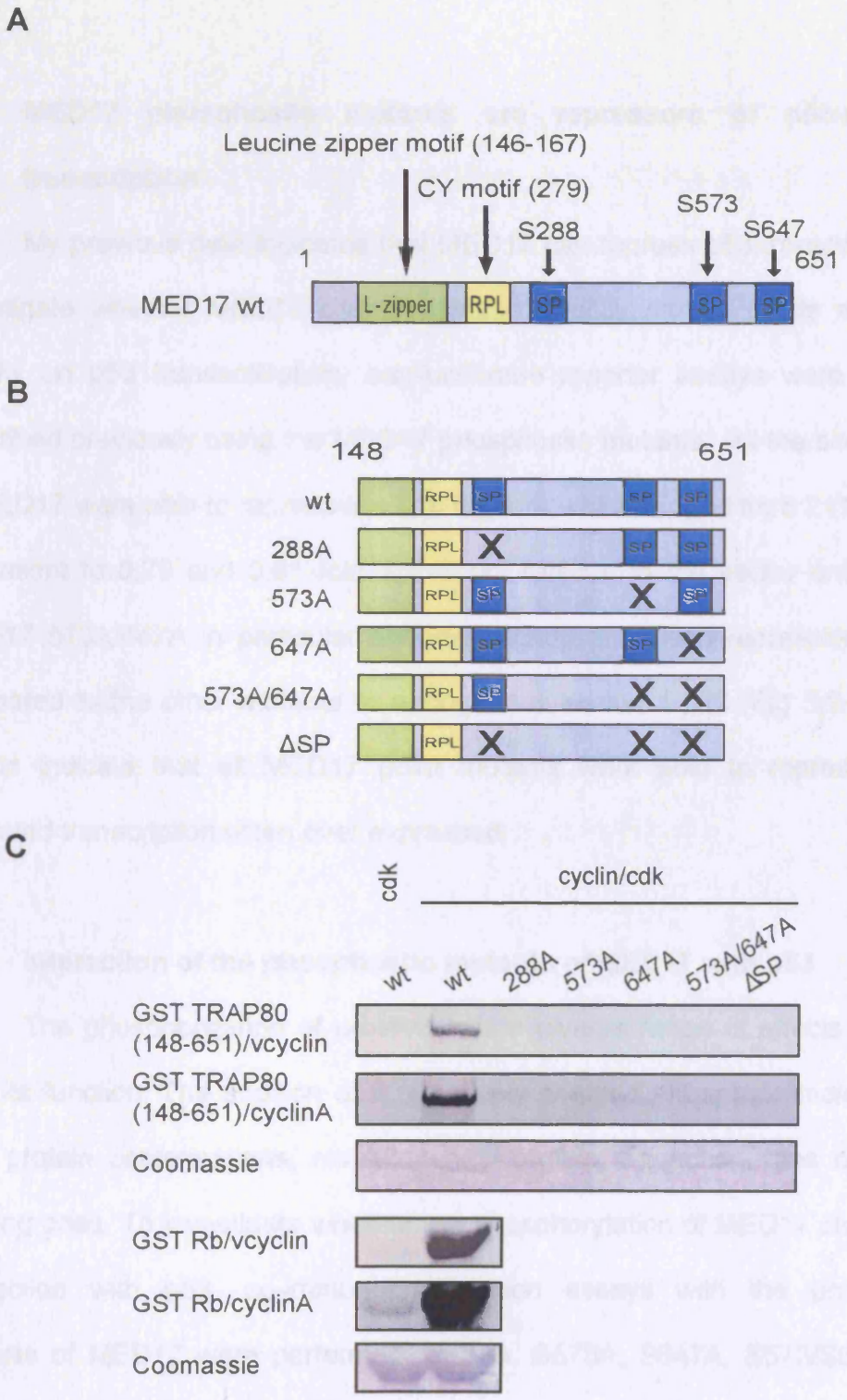


Fig 5.8 Mutation of putative phosphosites ablates MED17 phosphorylation

A. Schematic of MED17 demonstrating location of protein motifs.
B. Schematic of GST-MED17 showing serine to alanine site mutants generated.
C. *In vitro* kinase assays with GST-MED17 site mutants.

5.11 MED17 phosphosite mutants are repressors of p53-mediated transcription

My previous data indicates that MED17 can repress p53 transcription. To investigate whether MED17 phosphorylation directly modulates its repression activity on p53 transactivation, *bax*-luciferase reporter assays were done as described previously using the MED17 phosphosite mutants. All the site mutants of MED17 were able to repress the *bax* reporter which ranged from 21% to 39%, equivalent to 0.79 and 0.61 fold activation, relative to the vector only control. MED17 573A/647A in particular showed slightly enhanced repression of 61% compared to the other mutants at endogenous levels of p53 (Fig 5.9A). These results indicate that all MED17 point mutants were able to repressed p53-mediated transcription when over expressed.

5.12 Interaction of the phosphosite mutants of MED17 with p53

The phosphorylation of proteins has a diverse range of effects that may alter its function. The addition of a negatively charged phosphate molecule can alter protein conformations, revealing new protein interaction sites or altering existing ones. To investigate whether the phosphorylation of MED17 changed its interaction with p53, co-immunoprecipitation assays with the phosphosite mutants of MED17 were performed. S288A, S573A, S647A, S573/S647A and ΔSP mutants were subcloned into a mammalian expression vector, to allow for *in vivo* U2OS cells expression and detection of MED17 HA by immunoblotting. Expression constructs were transfected into the cells and cell lysates were

prepared 48 hours post-transfection. Total cell lysates were then immunoprecipitated with an antibody against p53. All of the MED17 phosphosite mutants tested interacted with p53 by co-immunoprecipitation (Fig 5.9B). However, a difference in expression levels among the mutants was observed in the Western blots of the input lysates. MED17 647A and ΔSP seemed to be less efficiently expressed compared to the other site mutants however both proteins co-immunoprecipitated with p53. Immunoblotting for p300 confirmed the co-immunoprecipitation of another p53 interacting protein. In conclusion, all MED17 point mutants continued to interact with p53 suggesting that these sites did not affect the MED17-p53 interaction. This experiment did however suggest that differences occurred in MED17 expression when the putative phosphosites were mutated to alanine residues, which was further investigated.

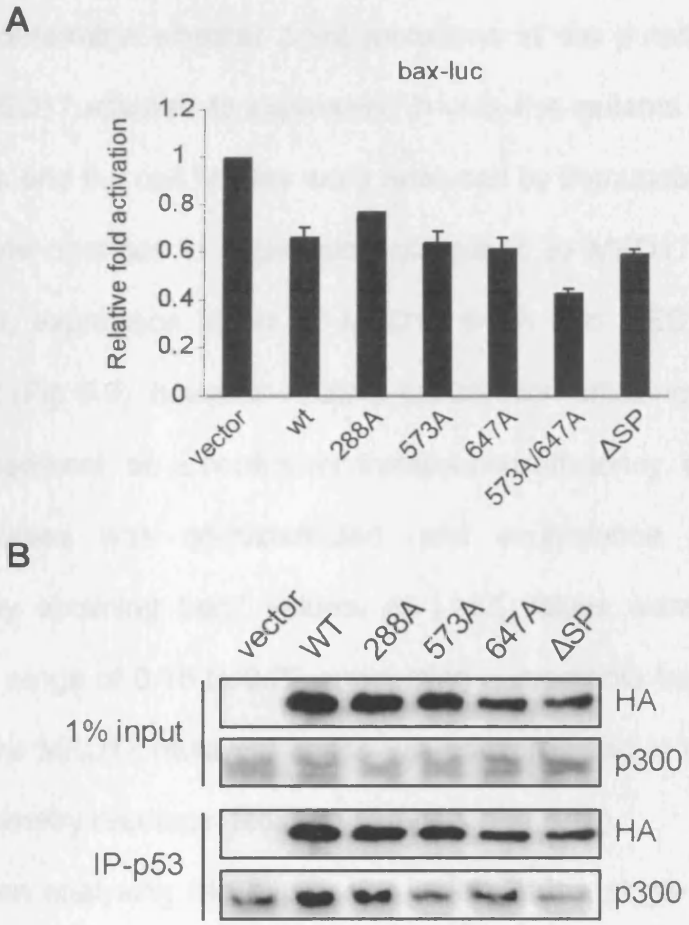


Fig 5.9 MED17 phosphosite mutants interact with p53 and can repress p53-mediated transcription

The interaction and function of the MED17 phosphosite mutants with p53 was tested to determine a phenotype for MED17 phosphorylation.

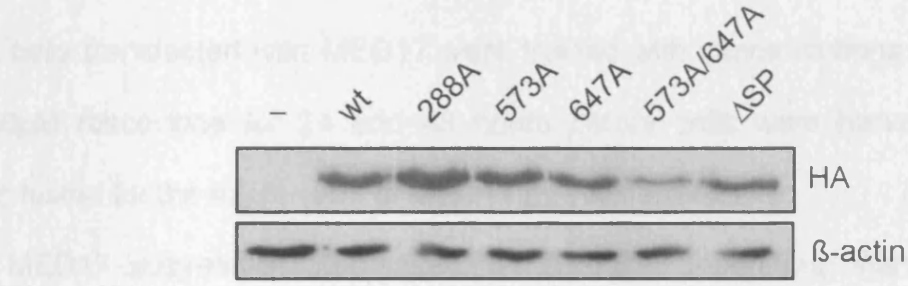
A. *bax*-luciferase reporter assays with MED17 site mutants.
B. Western blots for HA and p300 expression in input lysates and co-immunoprecipitates after depletion with a p53 antibody.

5.13 MED17 mutants are expressed at different efficiencies compared to wild type MED17

To determine whether point mutations at the putative phosphoacceptor sites on MED17 affected its expression *in vivo*, the mutants were transfected into U2OS cells and the cell lysates were analysed by immunoblot and densitiometry to determine changes in expression compared to MED17 wt. In the previous experiment, expression levels of MED17 647A and MED17 ΔSP varied from MED17 wt (Fig 5.9), however relative transfection efficiency was not evaluated. In this experiment, as a control for transfection efficiency, a plasmid expressing ß-galactosidase was co-transfected, and equivalence of transfection was assayed by obtaining LacZ values. All LacZ values were found to be in the equivalent range of 0.15 to 0.22 suggesting comparable transfection efficiencies between the MED17 mutants. These values were used to normalise changes in the densitometry readings between samples (Fig 5.10).

When analysing the expression levels of the single mutants, MED17 wt and MED17 573A and MED17 647A were at similar levels. However the MED17 288A mutant was more highly expressed than the wild type protein by 40% (compare lanes 2 and 3), indicating that mutation of MED17 at S288 decreases its expression. However the double mutant 573A/647A showed a significant decrease in expression to 0.4 fold, representing a 60% decrease in expression (compare lanes 2 and 6). The double mutation of S573 and S647 suggests that both serine residues, which may be phosphorylated *in vivo*, contribute to stabilisation of the MED17 protein. These data show that the point mutants of

MED17 are expressed, however, at different efficiencies compared to the wild type MED17. In conclusion, putative serine phosphoacceptor sites on MED17 contribute differentially to MED17 expression with S288 negatively regulating and S573 and S647 positively regulating MED17 levels.



Fold change in expression:	0	1	1.4	1.1	0.9	0.4	0.8
Lac Z values:	0.18	0.18	0.17	0.19	0.22	0.18	0.15

Fig 5.10 Expression of MED17 is altered by mutation of putative serine phosphoacceptor sites

Immunoblot of MED17 serine site mutants with figures for fold change in expression compared to wild-type MED17 and Lac Z values (below).

5.14 Inhibitors of cdk1/2 and cdk4/6 decrease MED17 expression

Mutation of putative serine phosphoacceptor sites in MED17 resulted in differences in expression at the protein level compared to wild type MED17. These finds suggest phosphorylation of MED17 might be responsible for regulating its expression. To assess the contribution of cdk activity on MED17 wild type expression, roscovitine was used to inhibit endogenous cdk1/2 activity. U2OS cells transfected with MED17 were treated with concentrations of 10, 30 and 50μM roscovitine for 24 and 48 hours before cells were harvested and lysates tested for the expression of MED17 by Western blotting.

MED17 expression decreased, in a dose dependent manner, with increasing concentration of roscovitine as compared to the vehicle control (DMSO) at both 24 and 48 hours of treatment (Fig. 5.11A). A faster migrating form of MED17-HA was also observed, correlating with increasing concentrations of roscovitine treatment. This species was specific to MED17 cells treated with roscovitine at 30 and 50μM, being the only detectable form of MED17-HA in the cells treated at 50μM at 24 hours of treatment. After 48 hours of treatment, with the higher concentrations of roscovitine, MED17 expression was not detectable. Western blots for Rb showed lower molecular weight bands corresponding to dephosphorylated forms of the protein suggesting kinase activity of cdk2, which phosphorylates Rb, was inhibited. These data indicate that inhibition of cdk1/2 activity promotes a mobility shift and reduced levels of MED17.

To observe whether the effect of cdk1/2 inhibition on MED17 was specific to these cdks, an inhibitor of cdk4/6 was used in similar experiments to those

used for roscovitine (Fig 5.11B). At concentrations of 0.125μM, 0.25μM and 0.5μM PD0183812 did not have a significant effect on MED17 expression, though by 48 hours these same concentrations decreased MED17 expression in a dose dependent manner. Western blots for Rb show a molecular weight shift occurring after 48 hours of treatment, which is also phosphorylated by cdk4 and cdk6 cyclin complexes, suggesting that the inhibitory effects of PD0183812 on cdk4 and cdk6 kinase activity occurs only after 48 hours and may explain why the change in MED17 expression levels did not occur at the 24 hour time point. These data suggest that inhibition of the kinase activity of the G1/S phase cyclin/cdks complexes results in decreased MED17 expression, possibly through the inhibiting phosphorylation of residues S573 and S647 of MED17.

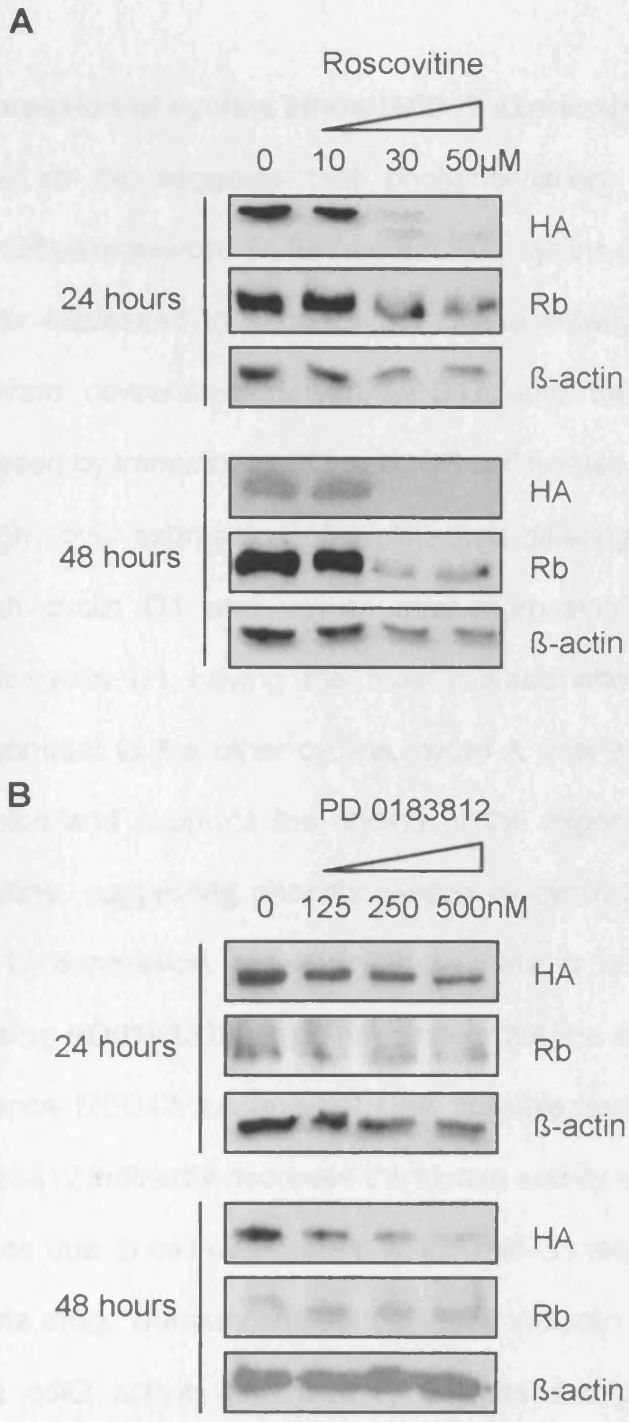


Fig 5.11 MED17 expression decreases in the presence of cdk inhibitor drugs, roscovitine and PD018312

A. Western blot for MED17 expression after treatment with cdk1/2 inhibitor, roscovitine with increasing concentrations and at 24 and 48 hour time points.
B. Western blot for MED17 expression after treatment with cdk4/6 inhibitor, PD0183812 with increasing concentrations and at 24 and 48 hour time points.

5.15 Over-expression of cyclins alters MED17 expression

The data so far suggests that phosphorylation of MED17 by the cyclin/cdks alters its expression. To further test this, cyclins D1 and A, as well as vcyclin were over-expressed to enhance the kinase activity of their interacting cdks. Cyclins were co-transfected with MED17, and the effect on MED17 expression analysed by immunoblot of the U2OS cell lysates (Fig 5.12).

Surprisingly, over-expression of cyclins had differing effects on MED17 expression. Both cyclin D1 and vcyclin over-expression decreased MED17 expression, with cyclin D1 having the most marked effect, ablating MED17 expression. In contrast to the other cyclins, cyclin A over-expression enhanced MED17 expression and supports the finding of the experiments using cdk1/2 inhibitor, roscovitine, suggesting phosphorylation by cyclin A/cdk1/2 complexes enhances MED17 expression. However, these data is not supported by the previous data using PD0183812 which suggested that the activities of cdk4 and cdk6 may enhance MED17 expression. One possible explanation is that the effects of PD0183812 indirectly decrease the kinase activity of cyclin E and cyclin A/cdk2 complexes due to cell cycle arrest at the mid-G1 restriction, which could be induced by the drug. The concomitant decrease in cyclin E and A expression would decrease cdk2 activity and lead to decreased expression of MED17. These data suggest that MED17 expression is differentially regulated by the various cyclin/cdk complexes during the G1/S phase progression through the cell cycle.

Together with the data studying the expression levels of the MED17 site mutants, a model for the differing affects of the cyclins on MED17 expression could be that cyclin D1 phosphorylates MED17 at S288 to negatively regulate its expression whereas cyclin A positively regulates its expression by phosphorylating residues S573 and S647.

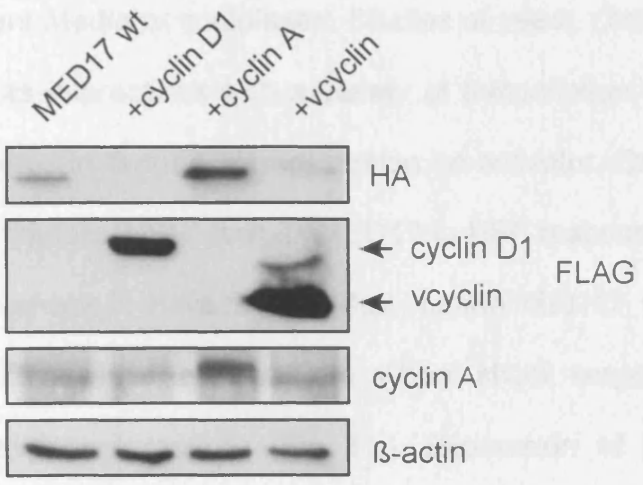


Fig 5.12 MED17 expression is enhanced in the presence of over-expressed cyclin A

Western blot for MED17 expression after co-transfection with cyclin D1, cyclin A and vcyclin.

Chapter 6: Discussion

6.1 Transcriptional co-activation properties of MED17

Previous studies of MED17 function *in vivo* have been mostly limited to studies of the *Drosophila* homolog of MED17 function from which my analysis shows a 46% homology to the human MED17. MED17 is a protein co-purified with a number of different Mediator complexes. Studies of yeast, *Drosophila* and human MED17 shows its interactions with a variety of transcription factors and suggests that MED17 may in fact be a transcription co-activator. One of these interacting transcription factors, HSF, recruits MED17 to HSF response elements upon stimulus of heat shock. I have shown that human MED17 when over-expressed is able to activate transcription from a heat shock responsive gene reporter in a dose dependent manner (Fig 3.2). Expression of heat shock regulated proteins was also tested, Western blotting for HSP60 and HSP40, which showed slight increases in their expression. HSP40 expression is inducible as opposed to HSP60 which is constitutively expressed. These data suggest a role for MED17 in HSF transcription. Though not tested, the stimulus of heat shock may have co-operated with MED17 in the activation of HSF mediated transcription as well as enhance expression of heat shock regulated proteins. In the absence of the stimulus it is possible that expression of transcriptionally active HSF homotrimers is low, therefore MED17 recruitment HSF response elements to activate transcription is limited by the lack of DNA bound HSF. Activation of the heat shock gene reporter by MED17, in the absence of cell

stress, is still significant and suggests that this transcriptional activator function of MED17 is conserved between *Drosophila* and human.

Furthermore, the microarrays of a cell line over-expressing MED17 suggested a role for MED17 in the activation of ß-catenin regulated transcription as indicated by gene network analysis, the presence of ß-catenin transcription targets among lists marker probe-sets and transcriptional assays (Fig 4.4 and Table 4.3). The association between MED17 and ß-catenin is further implied by the interaction of the two proteins in soluble nuclear fractions (Park *et al.*, 2003). These data do not exclude the possible activation of this pathway solely by upstream regulators of the ß-catenin pathway. Further studies are required to establish whether MED17 directly affects ß-catenin regulated transcription.

6.2 MED17 as a repressor of p53

In contrast to its co-activator role in HSF regulated transcription, the results presented in this thesis suggest that MED17 in fact represses p53 activation of some of its target genes, which are mostly likely to be involved in the apoptotic response. The human Mediator complex has been shown to activate p53 transcription *in vitro* possibly via an interaction with MED17, (Ito *et al.*, 1999). However, this interaction does not occur with the *Drosophila* homologues (Park *et al.*, 2003). Using co-immunoprecipitation I have shown that p53 and MED17 interacts in human cells. Upon further characterisation of the MED17 effects on p53 transcription, I found that MED17 was able to repress p53 mediated transcription when over-expressed in mammalian cells, *in vivo*. MED17 over-

expressing cells also display phenotypes associated with p53 loss of function. Mediator has been found to activate p53 *in vitro* these data may not reflect the true function of MED17 effects on p53. Furthermore, my study of the effects of MED17 over-expression on HSE regulated transcription suggest that MED17 does function differently within my experimental systems to what has previously been described *in vivo* for *Drosophila* cells. Typically the *in vitro* transcription assays used to study the affects of Mediator on transcription are reconstituted systems containing highly purified RNA pol II, transcription factors as well as co-activators which are used to study transcription of a naked DNA template. Under such conditions transcriptional activation may be favoured thus repression maybe more difficult to observe. These experiments also assay the gross transcriptional function of the Mediator complex rather than the contribution of individual subunits. Therefore an assumption is made that because Mediator activates p53 transcription these effects must be via the p53 interacting subunit, MED17. How Mediator regulates transcription from different transcription factors is not fully understood. In my studies, I have induced low levels of MED17 over-expression in a cell line and obtained similar results in p53 reporter assays compared to high level over-expression induced by transient transfection, reducing the possibility that repression of p53 is an artefact of MED17 over-expression. MED17 over-expression may in fact promote the assembly of Mediator complexes, containing MED17, that repress p53 transcription as well as positively regulating transcription by other interacting transcription factors. By utilising *in vivo* assays requiring the over-expression of the MED17 protein, these

data presented in this thesis may more accurately define the role of MED17 in regulating p53 mediated transcription.

6.3 Selective repression of p53 transcriptional targets by MED17

Data presented in chapter 3 showed the repression of a number of different p53 responsive reporters, with the exception of a p21 reporter, on over-expression of MED17. MED17 failed to repress activation of the reporter at endogenous levels of p53 but showed dose dependent repression when p53 was over-expressed. These data suggest that MED17 is not a ubiquitous repressor of all p53 transcriptional targets but may specifically repress genes which function in the execution of apoptosis rather than those involved in cell cycle arrest, such as p21. A similar programme of p53 transcriptional repression has also been observed with a related protein, the thyroid hormone receptor (TR). Human TR ß1, via an interaction with p53, can repress p53 transcription by a mechanism enhancing p53 binding to the p53 RE in the proximal promoter region (Barrera-Hernandez et al., 1998). Interestingly, TR ß1 mediated repression of p53 is able to repress induction of two p53 responsive genes, bax and Gadd45, but not p21. Conversely, p53 has been also shown to repress TR ß1 mediated transcription by inhibiting the TR interaction with TREs suggesting cross-talk between these two transcription networks (Yap et al., 1996;Bhat et al., 1997). The isolation of Mediator as a TR interacting complex potentially indicates that MED17 has a synergistic role with TR in the repression of p53 transcription.

The microarray analysis of the MED17 cell line also suggests that MED17 may selectively repress or activate p53 transcription targets. The Ingenuity Pathway Analysis revealed a gene network relating to p53 showing up-regulation and down regulation of MED17 markers representing p53 transcriptional targets. In fact, the expression of the p53 protein is increased while expression of its ubiquitin ligase, Mdm2, is decreased in the MED17 over-expressing cell line. This decrease in Mdm2 expression may cause a decrease in the turnover of p53 resulting in increased p53 expression levels and enhanced expression of some p53 transcriptional targets that are resistant to MED17 repression of p53 transctivation. Alternatively, the enhanced expression of the p53 at the level of mRNA and protein may also be attributed to the activation of DNA damage pathways that are co-regulated by cell cycle gene networks, which were also shown to be up-regulated in the MED17 cell line by two independent analyses. Addressing the low number of down-regulated MED17 markers representing p53 regulated genes, in "unstressed" cell lines activation of the p53 pathway is low therefore establishing significantly repressed genes in the relative absence of p53 transcription activation becomes more difficult. Further GEM analysis of the MED17 cell line using apoptotic insults to activate the p53 pathway may reveal more p53 regulated genes that are significantly repressed by MED17.

6.4 A mechanism for MED17 regulation of transcription

The absence of a mechanistic insight into how Mediator functions makes speculating a function for MED17 during transcription more difficult. The

mechanisms put forward in this section of the discussion therefore assume that MED17 is in complex with other Mediator subunits and the mechanisms proposed are established from studies of large Mediator complexes. Additional complexity is added to these mechanisms as co-activator and co-repressor functions of MED17 are considered.

Studies of the function of the Mediator complex have largely focused on describing its co-activator properties and have often related these properties to the overall structure of the Mediator complex. Mediator is potentially organised into modules and sub-modules of subunits as suggested by the absence of some groups of subunits between the various isolated forms of Mediator. MED17 seems to be one member of a group of core subunits that is conserved between PC2, CRSP and TRAP Mediator complexes (Sato et al., 2004). Modules may in fact be recruited to the core complex dependent on the interacting transcription factor or perhaps the requirement for transcriptional activation or repression. Notably is the presence of a module of Mediator consisting of MED12, MED13, CycC (cyclin C) and CDK8 is associated with a form of Mediator that represses transcription. In this arrangement the MED13 subunit interacts with MED17 (Guglielmi et al., 2004). A mechanism of transcription repression proposed for this module may involve the phosphorylation of the cyclin H component of TFIIH preventing phosphorylation of the RNA pol II CTD and its subsequent activation (Akoulitchev *et al.*, 2000). Alternatively, this module may exclude RNA pol II interaction with the Mediator complex or inactivate transcription activators by phosphorylation, as suggested by studies in yeast (Chi et al., 2001;Nelson et al.,

2003). Recruitment of Mediator complexes containing this repressor module may even be facilitated by gene specific repressors, as has been shown for Tup-1 (Zaman et al., 2001). Mediator complexes devoid of the MED12/MED13/CycC/cdk8 module may be activators. Therefore this offers a mechanism for how Mediator can switch between activator and repressor functions, which can be similarly be applied to the transcription targets of MED17 (Fig 6.1). Other Mediator associated proteins which interact with p53, including TR ß1 (described previously) and MED1 (TRAP220) (Ito *et al.*, 1999) may also feature in MED17 mediated repression.

More general studies of the Mediator complex have revealed other potential regulatory mechanisms which may apply to MED17 mediated repression. Investigation of the *Drosophila* Mediator complex has suggested the further arrangement of the subunits in to three distinct sub-complexes, termed C1, C2 and C3, which were isolated from nuclear extracts by column chromatography (Gu et al., 2002). Two of the sub-complexes, C1 and C2 vary both in size and transcriptional properties, with C2 able to activate transcription whereas C1 is not. The largest complex C3 is of similar size to previously identified Mediator complexes and has properties of a transcriptional activator. Identification of the C1 and C2 complexes, both of which contain the MED17 subunit, suggests added complexity to the regulation of transcription through their differentially recruitment to transcription factors and effects transcriptional activation or repression. Similar studies of human Mediator complexes have also revealed smaller sub-complex, present in a 150KDa fraction, able to repress

transcription *in vitro*. This sub-complex also contains the MED12, MED13 and CycC and cdk8 subunits, suggesting that transcriptional repressor sub-complexes exist alongside the larger 2MDa co-activator complex (Wang et al., 2001). These subunits have also been identified in a 550KDa complex in association with pol II but in the absence of GTFs, hinting that this potential smaller repressor complex acts via an interaction with pol II (Liu et al., 2001). In addition, structural studies of the CRSP complex suggest that Mediator complexes may adopt specific conformations dependent on the interacting transcription factor, which may further regulate the transcriptional properties of Mediator (Naar et al., 2002).

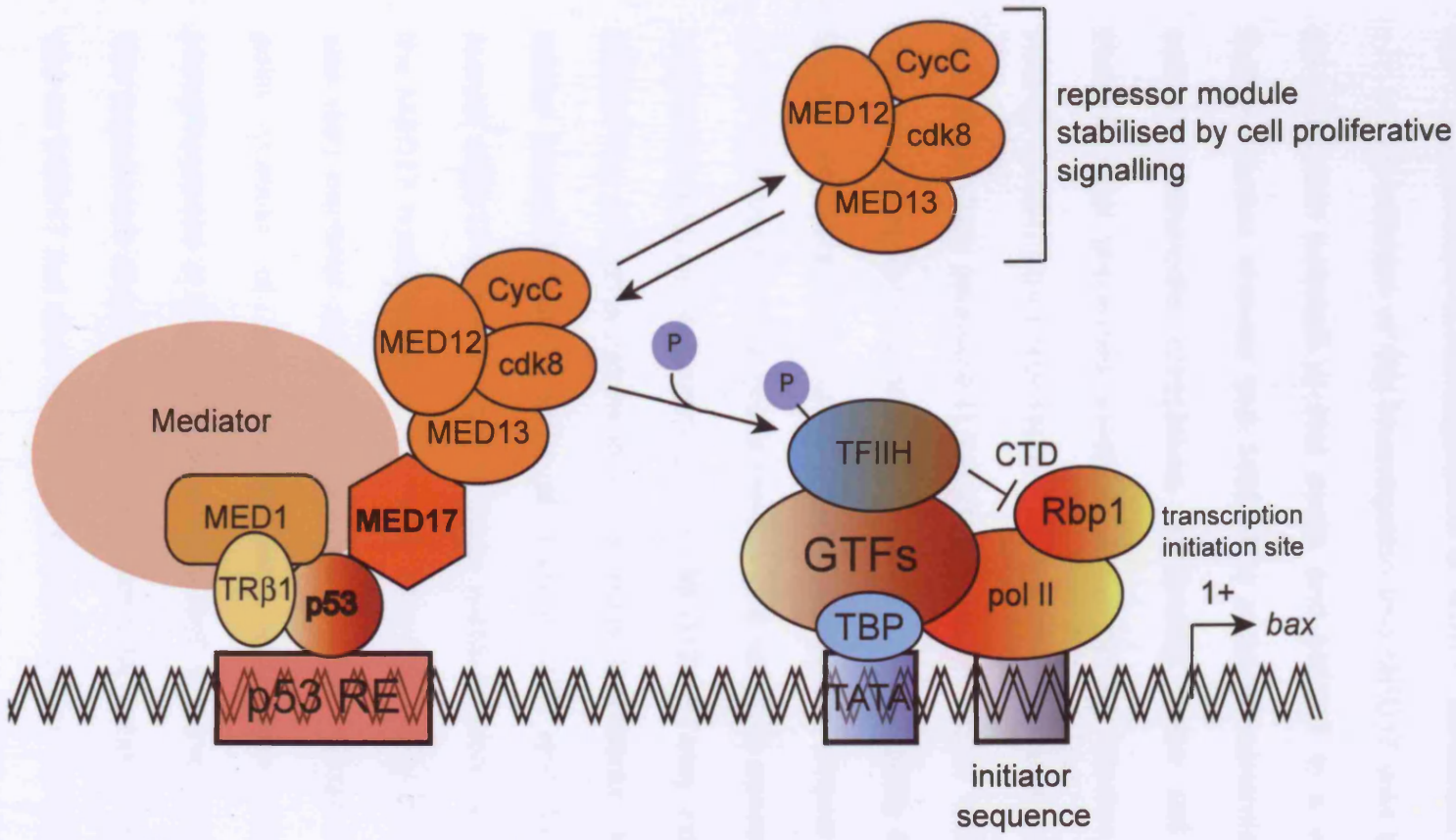


Fig 6.1 Model for MED17/Mediator repression of p53 regulated transcription

Recruitment of the MED12/MED13/CycC/cdk8 module to Mediator is required for transcriptional repression whereby CycC/cdk8 phosphorylate the cyclin H component of TFIIH thus inhibiting its kinase activity with cdk7. Inhibtion of TFIIH kinase prevents phosphoryation of the CTD of RNA pol II subunit, Rbp1, preventing initiation of transcription. TRβ1 and MED1 are proteins shown to interact with p53 and may facilitate recruiment of Mediator, alongside MED17, to sites of p53 transcription.

6.5 Cyclin/cdks in the regulation of MED17 mediated transcription

The initiation of this investigation into MED17 was by the identification of an interaction between a viral cyclin and MED17 in a yeast-2-hybrid screen. Further studies showed that MED17 is a novel substrate of both vcyclin and cellular cyclins/cdks complexes, implicating these cell cycle regulators in transcriptional processes involving Mediator. My studies with roscovitine, an inhibitor of cdk2, the predominant cdk subunit of cyclin A complex also showed a dose dependent decrease in the expression of MED17 with the appearance of a faster migrating form of MED17, possibly corresponding to non-phosphorylated MED17. Analysis of the human MED17 amino acid sequence showed a putative serine phosphoacceptor site at residue 573, which is conserved between human, rodent, chicken and fly homologues of MED17 and may indicate regulation of the MED17 by phosphorylation in all of these organisms. A further two putative serine phosphoacceptor sites, at residues 288 and 647, were identified on human MED17 however both of these residues were not conserved among all the MED17 homologues. A Cy motif, found on many cyclin A/cdk2 substrates, was also identified and was conserved between human and mouse MED17. A point mutation of any one of these sites was able to ablate MED17 phosphorylation *in vitro* by both vcyclin/cdk6 and cyclin A/cdk2. The result from this experiment does not identify any particular putative serine phosphoacceptor site on MED17 but does show that phosphorylation of MED17 can be disrupted. A possible reason for why mutation of just one of the serine residues ablated MED17 phosphorylation maybe the misfolding of the recombinant proteins

preventing phosphorylation. Alternatively, if MED17 is phosphorylated at all three serine residues, this result may indicate co-operation between the serine phosphoacceptor sites whereby disruption of one site inhibits phosphorylation at the other two sites due to conformational changes in the protein.

The experiments looking at the expression of the MED17 point mutants, both singly and in combination with one another, does however suggest that all three putative serine phosphoacceptor sites function in the regulation of MED17 expression. Furthermore, cyclin/cdk mediated phosphorylation of MED17 may differentially regulate MED17 expression, as suggested by the data over-expressing the cyclins with MED17 wt. In contrast to cyclin A, over-expression of cyclin D1 and vcyclin with MED17 resulted in its decreased expression, suggesting they are negative regulators of MED17/Mediator associated transcription. It is possible that cyclin D1 and vcyclin mediated decrease in MED17 expression could involve phosphorylation of S288, as indicated by the mutation of this residue which in fact enhances MED17 expression compared to the wild type. The S288 residue may also be too close to the Cy motif to be a target for phosphorylation by the cyclin A/cdk2 complex, as has been suggested by studies of substrates of this cyclin complex (Stevenson-Lindert et al., 2003). More likely is that S573 and S647 of MED17 maybe targeted for phosphorylation by the cyclin/cdk2 complexes. Mutation of both these serine residues resulted in decreased expression of MED17 compared to the wild type protein, suggesting that cyclin A/cdk2 mediated phosphorylation of MED17 enhances its expression. These data are further supported by the previous data, over-expressing cyclin A

with wild-type MED17 as well as experiments with cdk2 inhibitor, roscovtine, where changes in MED17 wt expression were observed, consistent with the hypothesis that cyclin A/cdk2 phosphorylation enhances MED17 expression.

With differential expression of the MED17 serine phosphosite mutants it would therefore be expected that repression of p53 transcription is decreased, however, all mutants were able to repress a *bax*-luciferase reporter at endogenous levels of p53 (Fig 5.9). Only slight variations occurred between the mutants for repression of the reporter suggesting that MED17 phosphorylation does not directly influence MED17 mediated repression of p53 transcription. These studies were limited by the requirement for transient, high level over-expression of MED17 HA which would make differences in p53 repression more difficult to observe. These data do not exclude the possibility that MED17 phosphorylation affects its transcriptional transactivation properties.

The targeting of the MED17 subunit for phosphorylation by cyclin/cdks would seem to directly associate cell cycle phase transition with transcriptional processes. As MED17 has been shown to be required for global transcriptional regulation, it would seem that cyclin/cdks are upstream regulators of multiple transcription factors regulated by MED17 and Mediator. In a model of cell cycle cyclin/cdk regulation of Mediator it could be envisaged that MED17 expression and therefore transcriptional activity would be highest during late G1/S-phase of the cell cycle when the activity cyclins E and A, in complex with cdk2, will be at its highest. During this phase, complex formation could be enhanced leading to quantitative changes in transcriptional activation or repression.

6.6 Mediator and the integration of signalling pathways in to transcription

Mediator has often been viewed as an end-point to signal transduction pathways, that integrates signalling pathways and regulates global transcription accordingly (Myers and Kornberg, 2000;Levine *et al.*, 2003). A direct role for cyclin/cdks in transcription regulation has previously been found by the discovery of cyclin C/cdk8 and cyclin T/cdk9 complexes as both activators and repressors of transcription. More closely linked to cell cycle regulation, cyclin H/cdk7 has also been attributed with a role in transcription, as a part of the TFIIH complex, as well as functioning as subunits of CAK responsible for activation of the kinase activity of cdks. The kinase activity of these cyclin/cdk complexes is associated with phosphorylation of components of the pre-initiation complex, which include the phosphorylation RNA pol II CTD during transcription initiation and elongation. However these kinase activities have also been extended to the phosphorylation of Mediator subunits.

The post-translational modification of Mediator subunits has been suggested by mobility shifts during SDS-PAGE, in particular MED4 has been noted to have changed migration pattern after phosphatase treatment of purified Mediator complex. Subsequently MED4 and MED16 have been demonstrated to be novel substrates of Kin28, the yeast homolog of cdk7 (Liu *et al.*, 2004). A single threonine residue at Thr237 was identified as the phosphoacceptor site on MED4 (Guidi et al., 2004). The function of MED4 phosphorylation remains unknown however it is thought that the unphosphorylated protein preferentially

interacts with other Mediator subunits, suggesting that phosphorylation alters formation of the complex (Balciunas et al., 2003). MED2 has also been proposed as a novel substrate of cdk8 where mutational studies of serine 208 phosphoacceptor repressed transcriptional activation from a 2μ yeast expression plasmid (Hallberg et al., 2004). Other kinases may also target Mediator subunits for phosphorylation. MED2 phosphorylation also repress transcription of genes regulated by the Rcs1/Aft1 transcription factor that are involved in responses to low iron (van de et al., 2005). Protein kinase A (PKA) as a part of the Ras-PKA proliferation signalling pathway in yeast was found to phosphorylate serine 608 of MED13 to repress expression of a subset of genes (Chang et al., 2004). These signalling pathways link Mediator to cell proliferation as similarly seen for MED17 phosphorylation by cell cycle associated cyclin/cdks. Interestingly, MED12, MED13, CycC and cdk8 expression in yeast is dependent on nutritional status, with expression of these subunits decreasing in response to nutrient limitation (Hengartner *et al.*, 1998;Nelson *et al.*, 2003). Whether the Ras-PKA pathway has a role in stabilising the expression of these subunits is yet to be determined. Further studies may yet reveal more Mediator subunits and different post-translational modification that add to the complexity of Mediator regulation.

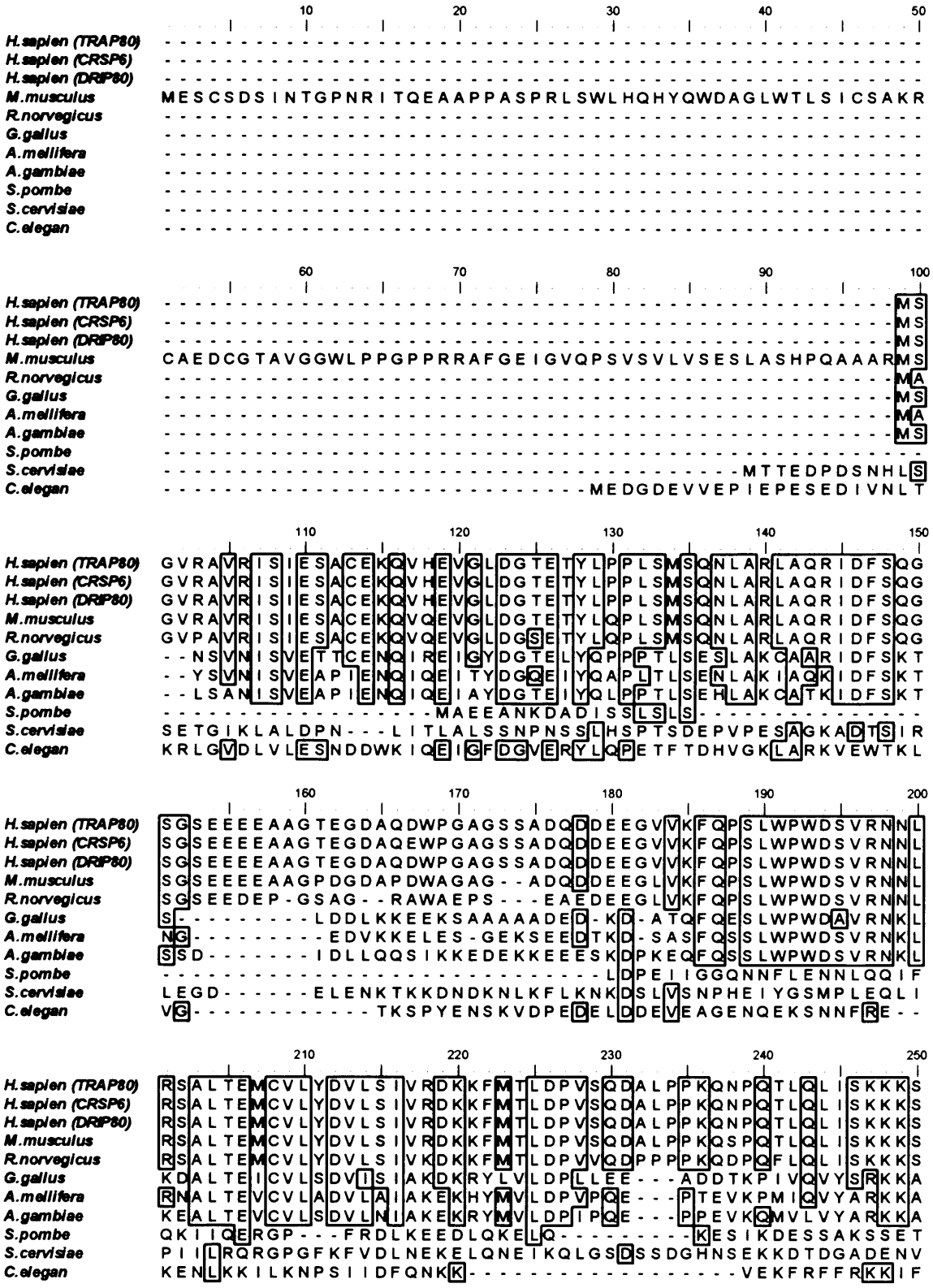
6.7 Conclusion

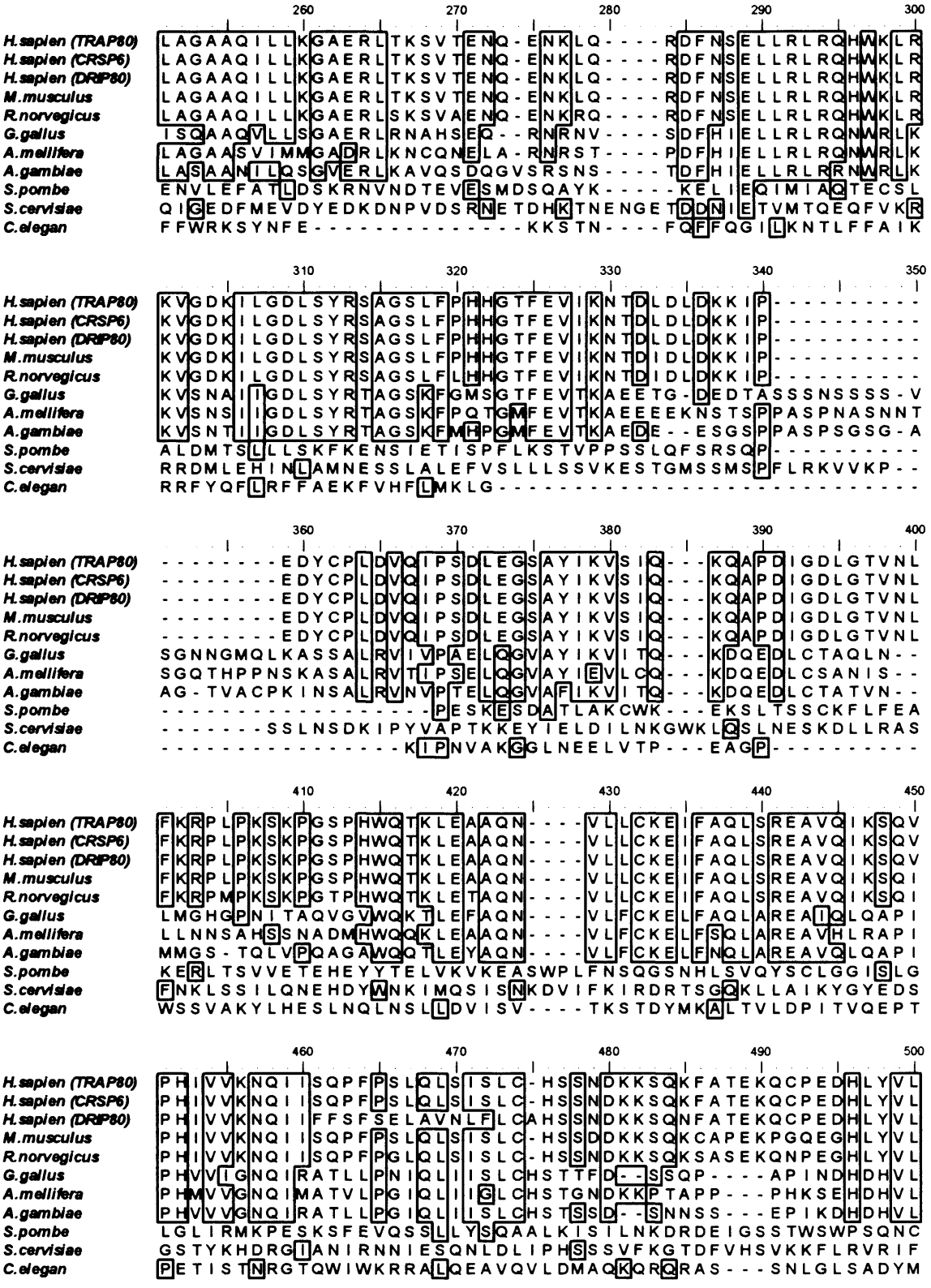
The data presented in this thesis has suggested novel transcriptional functions and regulators for MED17 that can be more broadly implicated in the functioning of the Mediator complex. I have shown a novel repressor function for MED17 in the regulation of p53 transcription in addition to confirming its more established role as a transcriptional activator. Also presented is data revealing MED17 is a novel substrate of viral and cellular cyclin/cdk complexes, implicating these cell cycle proteins as regulators of the Mediator complex. My work has contributed to the knowledge of the Mediator complex however, continuing studies of this complex are required to understand the complexities of this global transcriptional regulator.

Unified nomenclature	*s.cerevisiae*	*D.melanogaster*	*H.sapien* TRAP/SMCC
MED1	Med1	Trap220	TRAP220
MED1L			
MED2	Med2		
MED3	Pgd1/Hrs1/Med3		
MED4	Med4	Trap36	TRAP36
MED5	Nut1		
MED6	Med6	Med6	hMed6
MED7	Med7	Med7	hMed7
MED8	Med8	Arc32	
MED9	Cse2/Med9	CG5134	
MED10	Nut2/Med10	Nut2	hNut2
MED11	Med11	Med21	
MED12	Srb8	Kto	TRAP230
MED12L			
MED13	Ssn2/Srb9	Skd/Pap/Bli	TRAP240
MED13L			
MED14	Rgr1	Trap170	TRAP170
MED15	Gal11	Arc105	
MED16	Sin4	Trap95	TRAP95
MED17	Srb4	Trap80	TRAP80
MED18	Srb5	P28/CG14802	
MED19	Rox3	CG5546	
MED20	Srb2	Trfp	hTRFP
MED21	Srb7	Trap19	hSrb7
MED22	Srb6	Med24	
MED23		Trap150ß	TRAP150ß
MED24		Trap100	TRAP100
MED25		Arc92	
MED26		Arc70	
MED27		Trap37	TRAP37
MED28		Med23	
MED29		Intersex	
MED30		Trap25	TRAP25
MED31	Soh1	Trap18	hSoh1
CDK8	Srb10/Ssn3/Ume5	Cdk8	hSrb10
CycC	Srb11/Ssn8/Ume3	CycC	hSrb11

Table 7.1 Unified nomenclature for Mediator Subunits

The new nomenclature for the Mediator subunits is stated with Mediator subunits identified in yeast, *Drosophila* and Human Mediator complex TRAP/SMCC (Bourbon *et al.*, 2004).


H.sapien (TRAP80)
H.sapien (CRSP6)
H.sapien (DRIP80)
M.musculus
R.norvegicus
G.gallus
A.mellifera
A.gambiae
S.pombe
S.cervisiae
C.elegan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H.sapien (CRSP6) LAGAAQILLKGAERLTKSVTENQ-ENKLQ----RDFNSELLRLRQHWKLR
H.sapien (DRIP80) LAGAAQILLKGAERLTKSVTENQ-ENKLQ----RDFNSELLRLRQHWKLR
M.musculus LAGAAQILLKGAERLTKSVTENQ-ENKLQ----RDFNSELLRLRQHWKLR
R.norvegicus LAGAAQILLKGAERLSKSVAENQ-ENKRQ----RDFNSELLRLRQHWKLR
G.gallus ISQAAQVLLSGAERLRNAHSEQ--RNRRNV----SDFHIELLRLRQNWRLK
A.mellifera LAGAASVIMMGADRLKNCQNELA-RNRST----PDFHIELLRLRQNWRLK
A.gambiae LASAANILQSGVERLKAVQSDQGVSRSNS----TDFHIELLRLRRNWRLK
S.pombe ENVLEFATLDSKRNVNDTEVESMDSQAYK-----KELIEQIMIAQTECSL
S.cervisiae QIGEDFMEVDYEDKDNPVDSRNETDHKTNENGETDDDNIETVMTQEQFVKR
C.elegan FFWRKSYNFE--------------KKSTN----FQFFQGILKNTLFFAIK
310 320 330 340 350
H.sapien (TRAP80) KVGDKILGDLSYRSAGSLFPHHGTFEVIKNTDLDLDKKIP----------
H.sapien (CRSP6) KVGDKILGDLSYRSAGSLFPHHGTFEVIKNTDLDLDKKIP----------
H.sapien (DRIP80) KVGDKILGDLSYRSAGSLFPHHGTFEVIKNTDLDLDKKIP----------
M.musculus KVGDKILGDLSYRSAGSLFPHHGTFEVIKNTDIDLDKKIP----------
R.norvegicus KVGDKILGDLSYRSAGSLFLHHGTFEVIKNTDIDLDKKIP----------
G.gallus KVSNAIIGDLSYRTAGSKFGMSGTFEVTKAEETG-DEDTASSSNSSSS-V
A.mellifera KVSNSIIGDLSYRTAGSKFPQTGMFEVTKAEEEEKNSTSPPASPNASNNT
A.gambiae KVSNTIIGDLSYRTAGSKFMHPGMFEVTKAEDE--ESGSPPASPSGSG-A
S.pombe ALDMTSLLLSKFKENSIETISPFLKSTVPPSSLQFSRSQP----------
S.cervisiae RRDMLEHINLAMNESSLALEFVSLLLSSVKESTGMSSMSPFLRKVVKP--
C.elegan RRFYQFLRFFAEKFVHFLMKLG----------
360 370 380 390 400
H.sapien (TRAP80) --------EDYCPLDVQIPSDLEGSAYIKVSIQ---KQAPDIGDLGTVNL
H.sapien (CRSP6) --------EDYCPLDVQIPSDLEGSAYIKVSIQ---KQAPDIGDLGTVNL
H.sapien (DRIP80) --------EDYCPLDVQIPSDLEGSAYIKVSIQ---KQAPDIGDLGTVNL
M.musculus --------EDYCPLDVQIPSDLEGSAYIKVSIQ---KQAPDIGDLGTVNL
R.norvegicus --------EDYCPLDVQIPSDLEGSAYIKVSIQ---KQAPDIGDLGTVNL
G.gallus SGNNGMQLKASSALRVIVPAELQGVAYIKVITQ---KDQEDLCTAQLN--
A.mellifera SGQTHPPNSKASALRVTIPSELQGVAYIEVLCQ---KDQEDLCSANIS--
A.gambiae AG-TVACPKINSALRVNVPTELQGVAFIKVITQ---KDQEDLCTATVN--
S.pombe ----------------PESKESDATLAKCWK---EKSLTSSCKFLFEA
S.cervisiae -------SSLNSDKIPYVAPTKKEYIELDILNKGWKLQSLNESKDLLRAS
C.elegan ----------------KIPNVAKGGLNEELVTP---EAGP----------
410 420 430 440 450
H.sapien (TRAP80) FKRPLPKSKPGSPHWQTKLEAAQN----VLLCKEIFAQLSREAVQIKSQV
H.sapien (CRSP6) FKRPLPKSKPGSPHWQTKLEAAQN----VLLCKEIFAQLSREAVQIKSQV
H.sapien (DRIP80) FKRPLPKSKPGSPHWQTKLEAAQN----VLLCKEIFAQLSREAVQIKSQV
M.musculus FKRPLPKSKPGSPHWQTKLEAAQN----VLLCKEIFAQLSREAVQIKSQI
R.norvegicus FKRPMPKSKPGTPHWQTKLETAQN----VLLCKEIFAQLSREAVQIKSQI
G.gallus LMGHGPNITAQVGVWQKTLEFAQN----VLFCKELFAQLAREAIQLQAPI
A.mellifera LLNNSAHSSNADMHWQQKLEAAQN----VLFCKELFSQLAREAVHLRAPI
A.gambiae MMGS-TQLVPQAGAWQQTLEYAQN----VLFCKELFNQLAREAVQLQAPI
S.pombe KERLTSVVETEHEYYTELVKVKEASWPLFNSQGSNHLSVQYSCLGGISLG
S.cervisiae FNKLSSILQNEHDYWNKIMQSISNKDVIFKIRDRTSGQKLLAIKYGYEDS
C.elegan WSSVAKYLHESLNQLNSLLDVISV----TKSTDYMKALTVLDPITVQEPT
460 470 480 490 500
H.sapien (TRAP80) PHIVVKNQIISQPFPSLQLSISLC-HSSNDKKSQKFATEKQCPEDHLYVL
H.sapien (CRSP6) PHIVVKNQIISQPFPSLQLSISLC-HSSNDKKSQKFATEKQCPEDHLYVL
H.sapien (DRIP80) PHIVVKNQIIFFSFSELAVNLFLCAHSSNDKKSQNFATEKQCPEDHLYVL
M.musculus PHIVVKNQIISQPFPSLQLSISLC-HSSDDKKSQKCAPEKPGQEGHLYVL
R.norvegicus PHIVVKNQIISQPFPGLQLSISLC-HSSNDKKSQKSASEKQNPEGHLYVL
G.gallus PHVVIGNQIRATLLPNIQLIISLCHSTTFD--SSQP----APINDHDHVL
A.mellifera PHMVVGNQIMATVLPGIQLIIGLCHSTGNDKKPTAPP---PHKSEHDHVL
A.gambiae PHVVVGNQIRATLLPGIQLIISLCHSTSSD--SNNSS---EPIKDHDHVL
S.pombe LGLIRMKPESKSFEVQSSLLYSQAALKISILNKDRDEIGSSTWSWPSQNC
S.cervisiae GSTYKHDRGIANIRNNIESQNLDLIPHSSSVFKGTDFVHSVKKFLRVRIF
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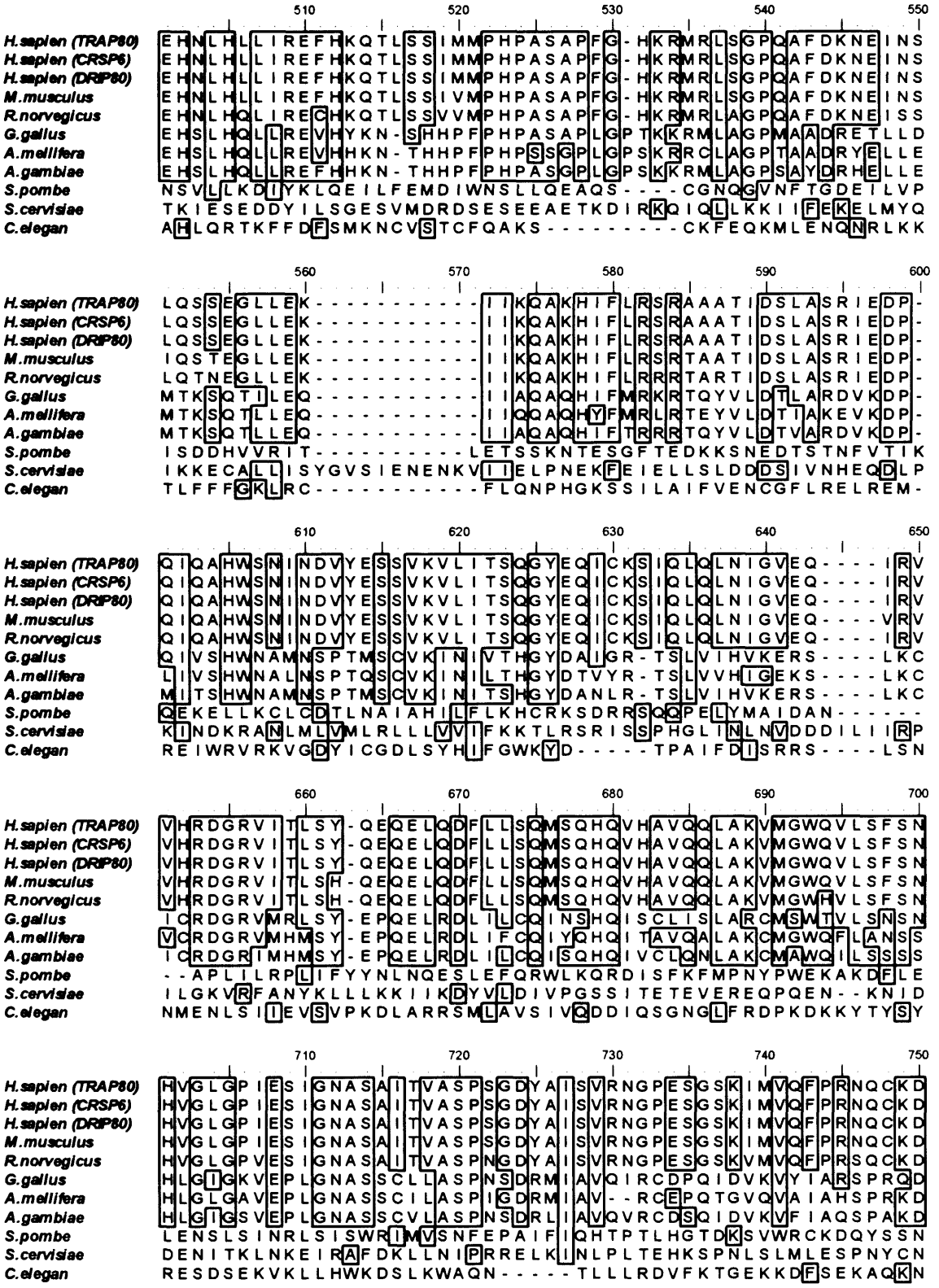
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H.sapien (TRAP80) EHNLHLLIREFHKQTLSSIMMPHPASAPFG-HKRMRLSGPQAFDKNEINS
H.sapien (CRSP6) EHNLHLLIREFHKQTLSSIMMPHPASAPFG-HKRMRLSGPQAFDKNEINS
H.sapien (DRIP80) EHNLHLLIREFHKQTLSSIMMPHPASAPFG-HKRMRLSGPQAFDKNEINS
M.musculus EHNLHLLIREFHKQTLSSIVMPHPASAPFG-HKRMRLSGPQAFDKNEINS
R.norvegicus EHNLHQLIRECHKQTLSSVVMPHPASAPFG-HKRMRLAGPQAFDKNEISS
G.gallus EHSLHQLLREVHYKN-SHHPFPHPASAPLGPTKKRMLAGPMAADRETLLD
A.mellifera EHSLHQLLREVHHKN-THHPFPHPSSGPLGPSKRRCLAGPTAADRYELLE
A.gambiae EHSLHQLLREFHHKN-THHPFPHPASGPLGPSKKRMLAGPSAYDRHELLE
S.pombe NSVLLKDIYKLQEILFEMDIWNSLLQEAQS----CGNQGVNFTGDEILVP
S.cervisiae TKIESEDDYILSGESVMDRDSESEEAETKDIRKQIQLLKKIIFEKELMYQ
C.elegan AHLQRTKFFDFSMKNCVSTCFQAKS---------CKFEQKMLENQNRLKK
560 570 580 590 600
H.sapien (TRAP80) LQSSEGLLEK----------IIKQAKHIFLRSRAAATIDSLASRIEDP-
H.sapien (CRSP6) LQSSEGLLEK----------IIKQAKHIFLRSRAAATIDSLASRIEDP-
H.sapien (DRIP80) LQSSEGLLEK----------IIKQAKHIFLRSRAAATIDSLASRIEDP-
M.musculus IQSTEGLLEK----------IIKQAKHIFLRSRTAATIDSLASRIEDP-
R.norvegicus LQTNEGLLEK----------IIKQAKHIFLRRRTARTIDSLASRIEDP-
G.gallus MTKSQTILEQ----------IIAQAQHIFMRKRTQYVLDTLARDVKDP-
A.mellifera MTKSQTLLEQ----------IIQQAQHYFMRLRTEYVLDTIAKEVKDP-
A.gambiae MTKSQTLLEQ----------IIAQAQHIFTRRRTQYVLDTVARDVKDP-
S.pombe ISDDHVVRIT----------LETSSKNTESGFTEDKKSNEDTSTNFVTIK
S.cervisiae IKKECALLISYGVSIENENKVIIELPNEKFEIELLSLDDDSIVNHEQDLP
C.elegan TLFFFGKLRC----------FLQNPHGKSSILAIFVENCGFLRELREM-
610 620 630 640 650
H.sapien (TRAP80) QIQAHWSNINDVYESSVKVLITSQGYEQICKSIQLQLNIGVEQ----IRV
H.sapien (CRSP6) QIQAHWSNINDVYESSVKVLITSQGYEQICKSIQLQLNIGVEQ----IRV
H.sapien (DRIP80) QIQAHWSNINDVYESSVKVLITSQGYEQICKSIQLQLNIGVEQ----IRV
M.musculus QIQAHWSNINDVYESSVKVLITSQGYEQICKSIQLQLNIGVEQ----VRV
R.norvegicus QIQAHWSNINDVYESSVKVLITSQGYEQICKSIQLQLNIGVEQ----IRV
G.gallus QIVSHWNAMNSPTMSCVKINIVTHGYDAIGR-TSLVIHVKERS----LKC
A.mellifera LIVSHWNALNSPTQSCVKINILTHGYDTVYR-TSLVVHIGEKS----LKC
A.gambiae MITSHWNAMNSPTMSCVKINITSHGYDANLR-TSLVIHVKERS----LKC
S.pombe QEKELLKCLCDTLNAIAHILFLKHCRKSDRRSQQPELYMAIDAN------
S.cervisiae KINDKRANLMLVMLRLLLVVIFKKTLRSRISSPHGLINLNVDDDILIIRP
C.elegan REIWRVRKVGDYICGDLSYHIFGWKYD-----TPAIFDISRRS----LSN
660 670 680 690 700
H.sapien (TRAP80) VHRDGRVITLSY-QEQELQDFLLSQMSQHQVHAVQQLAKVMGWQVLSFSN
H.sapien (CRSP6) VHRDGRVITLSY-QEQELQDFLLSQMSQHQVHAVQQLAKVMGWQVLSFSN
H.sapien (DRIP80) VHRDGRVITLSY-QEQELQDFLLSQMSQHQVHAVQQLAKVMGWQVLSFSN
M.musculus VHRDGRVITLSH-QEQELQDFLLSQMSQHQVHAVQQLAKVMGWQVLSFSN
R.norvegicus VHRDGRVITLSH-QEQELQDFLLSQMSQHQVHAVQQLAKVMGWHVLSFSN
G.gallus ICRDGRVMRLSY-EPQELRDLILCQINSHQISCLISLARCMSMTVLSNSN
A.mellifera VCRDGRVMHMSY-EPQELRDLIFCQIYQHQITAVQALAKCMGWQFLANSS
A.gambiae ICRDGRTMHMSY-EPQELRDLILCQISQHQIVCLQNLAKCMAWQILSSSS
S.pombe --APLILRPLIFYYNLNQESLEFQRWLKQRDISFKFMPNYPWEKAKDFLE
S.cervisiae ILGKVRFANYKLLLKKIIKDYVLDIVPGSSITETEVEREQPQEN--KNID
C.elegan NMENLSIIEVSVPKDLARRSMLAVSIVQDDIQSGNGLFRDPKDKKYTYSY
710 720 730 740 750
H.sapien (TRAP80) HVGLGPIESIGNASAITVASPSGDYAISVRNGPESGSKIMVQFPRNQCKD
H.sapien (CRSP6) HVGLGPIESIGNASAITVASPSGDYAISVRNGPESGSKIMVQFPRNQCKD
H.sapien (DRIP80) HVGLGPIESIGNASAITVASPSGDYAISVRNGPESGSKIMVQFPRNQCKD
M.musculus HVGLGPIESIGNASAITVASPSGDYAISVRNGPESGSKIMVQFPRNQCKD
R.norvegicus HVGLGPVESIGNASAITVASPNGDYAISVRNGPESGSKVMVQFPRSQCKD
G.gallus HLGIGKVEPLGNASSCLLASPNSDRMIAVQIRCDPQIDVKVYIARSPRQD
A.mellifera HLGLGAVEPLGNASSCILASPIGDRMIAV--RCEPQTGVQVAIAHSPRKD
A.gambiae HLGIGSVEPLGNASSCVLASPNSDRLIAVQVRCDSQIDVKVFIAQSPAKD
S.pombe LENSLSINRLSISWRIMVSNFEPAIFIQHTPTLHGTDKSVWRCKDQYSSN
S.cervisiae DENITKLNKEIRAFDKLLNIPRRELKINLPLTEHKSPNLSLMLESPNYCN
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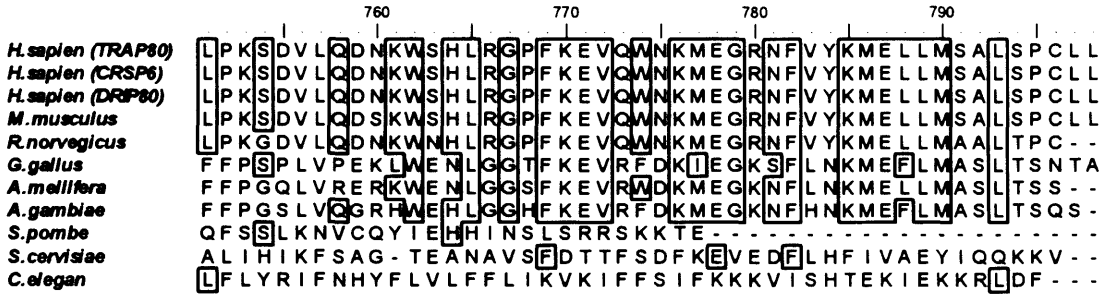

Fig 7.1 Alignment of MED17 homologues

Alignment was done using the Bio Edit sequence alignment programme (www.mbio.ncsu.edu/BioEdit/bioedit.html). The protein sequences used for this alignment correspond to the Genbank accession numbers given in Table 1.1. Bordered sequences indicate amino acid identity between all homologues.

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