# Identification of TopBP1 Chromatin Modification Domains and Transcriptional Targets

Roni H G Wright

A thesis submitted to the University of Glasgow, Faculty of Veterinary Medicine
for the degree of Doctor of Philosophy


**UNIVERSITY**
***of***
**GLASGOW**

Division of Pathological Sciences
Faculty of Veterinary Medicine

September 2007



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

For my Mum and Dad

# Acknowledgements

I would like to thank to my supervisor Iain Morgan for all his support, guidance and patience over the last three years. Thanks to everyone in the PV lab especially Mary Donaldson for their interest in the project and help with experimental protocols, but above all thank you for your friendship. A special thanks to Edward Dornan for all the laughs, and perhaps somewhat less scientific conversations over the years.

And last but by no means least thanks to Allan for all his love and unwavering support

-This thesis is as much yours as it is mine.

# Table of Contents

Wright RH, Dornan ES, Donaldson MM, Morgan IM. "TopBP1 contains a transcriptional activation domain suppressed by two adjacent BRCT domains." Biochem J. (2006), Dec 15;400(3):573-82.

# List of Figures

*Figure Number* *Page*

# List of Tables

# Abbreviations

| | |
|---|---|
| AD | Activation domain |
| ATP | Adenosine triphosphate |
| ATR | A-T Rad3-related protein |
| AT | Ataxia telangiectasia |
| ATM | Ataxia telangiectasia mutated |
| ATRIP | ATR interacting protein |
| BER | Base excision repair |
| bp | Base pairs |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| Brm | Brahma |
| Brg1 | Brahma related gene 1 |
| BRCT | BRCA1 carboxyl-terminal |
| BRCA1 | Breast cancer susceptibility Gene 1 |
| CMV | Cytomegalovirus |
| DAVID | Database for Annotation Visualization and Integrated Discovery |
| $^{\circ}$C | Degree centigrade |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | Deoxyribonucleic acid |
| DMSO | Dimethyl sulphoxide |
| DBD | DNA binding domain |
| DNA-PK | DNA dependent protein kinase |
| $ddH_2O$ | Double distilled water |
| DSB | Double strand breaks |

| | |
|---|---|
| dsDNA | Double stranded DNA |
| DMEM | Dulbecco's modified eagle's medium |
| ECL | Enhanced chemiluminescence |
| FACS | Fluorescence activated cell sorting |
| FCS | Foetal calf serum |
| g | Gram |
| HAT | Histone Acetyl Transferase |
| HR | Homologous Recombination |
| HEK293T | Human Embryonic Kidney Cells |
| HPV | Human papillomavirus |
| IP | Immunoprecipitation |
| IVT | *In vitro* transcription |
| Kb | Kilobase pairs |
| kDa | Kilodaltons |
| L | Litre |
| mRNA | Messenger ribonucleic acid |
| µg | Microgram |
| µl | Microlitre |
| mg | Milligram |
| ml | Millilitre |
| mM | Millimolar |
| MMR | Mismatch Repair |
| M | Molar |
| MW | Molecular weight |
| nm | Nanometres |
| NBS | Nijmegen breakage syndrome |
| NHEJ | Non-homologous end joining |
| NER | Nucleotide excision repair |

| | |
|---|---|
| OD | Optical Density |
| PBS | Phosphate buffered saline |
| pmol | Picomole |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PML | Promyleocytic leukemia |
| ROS | Reactive oxygen species |
| RPA | Replication protein A |
| RNase | Ribonuclease |
| RNA | Ribonucleic acid |
| SV40 | Simian Virus 40 |
| ssDNA | Single stranded DNA |
| siRNA | Small interfering RNA |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard Error of Mean |
| SWISNF | Sucrose non-fermenting, switch mating type |
| SC | Synthetic complement |
| TBP | TATA binding protein 1 |
| tk | Thymidine kinase |
| TopBP1 | Topoisomerase binding protein 1 |
| TCR | Transcription coupled repair |
| UV | Ultraviolet |
| UVB | Ultraviolet wave length B (312nm) |
| v/v | Volume per unit volume |
| w/v | Weight per unit volume |
| WB | Western blot |
| wt | Wild type |

# Abstract

Topoisomerase II β-binding protein 1 (TopBP1) is an essential BRCT domain containing protein involved in DNA damage signalling, replication and repair. BRCT domains have the ability to bind single and double stranded DNA as well as phosphopeptides and are found within a large group of proteins involved in the DNA damage response. These domains give the proteins the ability to bind damaged DNA and change their interacting partners following DNA damage, making their role in the DNA damage response essential for efficient signal transduction and the activation of DNA damage checkpoint and repair. TopBP1 binds single and double stranded DNA and co-localises to sites of DNA damage with other sensory proteins including BRCA1, NBS1 and Rad9 and is an essential protein in the activation of the downstream checkpoints and the initiation of cell cycle arrest. No other protein contains as many as eight BRCT domains indicating that TopBP1 plays an essential role in these responses. TopBP1 has been implicated as a possible transcription factor activating transcription of the HPV16 transcription/replication protein E2 and repressing the activation of E2F1 and Miz1. The modification of chromatin is essential for all functions of TopBP1; this thesis has describes the identification of three chromatin modification domains within three distinct BRCT domains of TopBP1. The function of one of these domains is mediated by a direct interaction with the chromatin remodeling enzyme Brg1 and the transcriptional regulation by this protein-protein interaction is regulated by DNA damage. The

identification of these domains and their regulation by DNA damage provides further insight into the mechanism of DNA damage signalling, repair, transcription and replication by TopBP1 via the modification of chromatin.

In addition to the identification of chromatin modification domains, gene targets of TopBP1 were also identified. This was carried out using MCF7 cells with endogenous TopBP1 down-regulated by siRNA treatment. These studies identified several pathways regulated by TopBP1 expression, all of which play a role in breast carcinogenesis. These results provide more evidence supporting a role for TopBP1 in the development of breast cancer; previous studies have shown aberrant expression of TopBP1 in breast cancers as well as polymorphisms pre-disposing towards this disease.

## Authors Declaration

I declare that the work described in this thesis was carried out by personally unless otherwise stated, and has not been submitted in full or in part for consideration for any other degree or qualification.

Roni H.G. Wright

October 2007

# Chapter 1 - Introduction

## 1.1. The DNA Damage Response

Cells are continually bombarded with endogenous and exogenous DNA damaging agents which produce as many as 1,000,000 lesions in DNA per cell per day (Lodish *et al.* 2004). The type of damage to DNA which results depends upon the source of the damage, but irrespective of the type of damage the DNA damage signal transduction pathway is the same, consisting of sensors, transducers and effectors (Figure 1). The responses activated during this pathway include 1) DNA repair, 2) the activation of cell cycle checkpoints 3) Transcription and 4) Apoptosis. The roles of these distinct set of proteins within the cascade are as follows 1) Sensors; identify the damage and initiate the DNA damage cascade, 2) Transducers; phosphorylate downstream targets and 3) Effectors induce cell cycle arrest, repair and apoptosis.

**Figure 1**

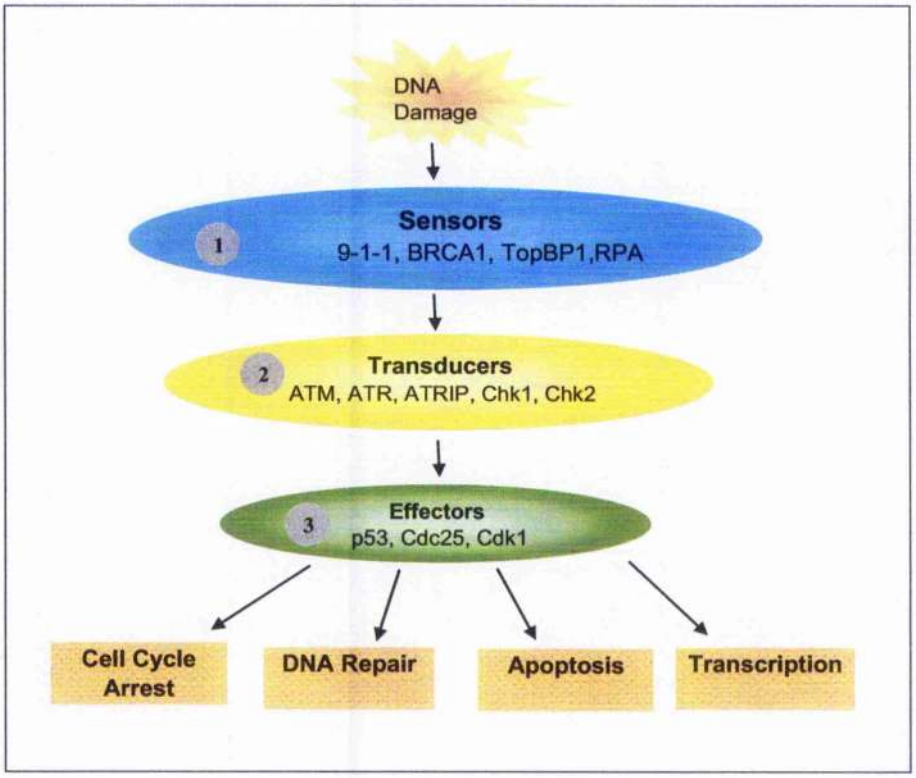



**Figure 1: Diagrammatic Representation of the DNA Damage Signalling Cascade.** This diagram represents the DNA damage response signalling cascade, showing the position of the sensors, transducers and effectors within the cascade. The end results being the activation of DNA repair, cell cycle arrest apoptosis and transcription.

**Table 1**

| | | Species | | | |
|---|---|---|---|---|---|
| **Function** | | **Mammals** | ***S.pombe*** | ***S.cerevisiae*** | ***D.melanogaster*** |
| **Sensor** | | | | | |
| | RFC1-like | Rad17 | Rad17 | Rad24 | Rad24 |
| | PCNA-like | Rad9<br>Rad1<br>Hus1 | Rad9<br>Rad1<br>Hus1 | Ddc1<br>Rad17<br>Mec3 | |
| | ssDNA binding | RPA | | RFA | |
| | DSB recognition/nuclease | Mre11<br>RAD50<br>NBS1 | Rad32<br>Rad50 | Mre11<br>Rad50<br>Xrs2 | Mre1<br>Rad50<br>Nbs1 |
| | | Claspin | Mrc1 | Mrc1 | |
| | BRCT containing | TopBP1<br>BRCA1<br>MDC1 | Cut5<br>Crb2 | Dpb11<br>Rad9 | Mus101 |
| **Transducers** | | | | | |
| | PI3 Kinases | ATM<br>ATR | Tel1<br>Rad3 | Tel1<br>Mec1 | ATM<br>Mei-41 |
| | PI3 kinase binding partners | ATRIP | Rad26 | Ddc2 | Mus304 |
| | Ser/Thr Kinase | Chk1<br>Chk2 | Chk1<br>Cds1 | Chk1<br>Rad53 | Grapes<br>Chk2 |
| **Effector** | | | | | |
| | Transcription Factor | p53 | | | Dp53 |
| | Phosphatase | Cdc25A<br>Cdc25B<br>Cdc25C | | | String |
| | Kinase | Cdk1 | Cdc2 | Cdc28 | Cdk1 |

**Table 1: Sensor, Transducer and Effector proteins involved in the DNA Damage Response.** This table list the sensors, transducers and effectors in the DNA damage response, in mammals, *Saccharomyces cerevisiae*, *Schizosaccaromyces pombe* and *Drosophila melanogaster*. The grey box indicates proteins which are sensors but are also involved in the activation of the cell cycle checkpoints (1.2).

### 1.1.1. Sensors

Sensors are proteins which either directly bind DNA or indirectly bind DNA via a DNA-protein-protein interaction. The interactions of these proteins recognise the lesion and initiate the cascade of events which lead to the activation of the cell cycle checkpoints, repair transcription or apoptosis. These proteins and the homologs in S. *cervisiae*, D. *melanogaster* and S. *pombe* as listed in Table 1. However although the proteins described here play a sensory role following DNA damage several also function during the initiation of both the cell cycle checkpoints and repair (Discussed later 1.2.).

**RFC and PCNA like sensors** are recruited to the sites of DNA damage and include the Rad9-Rad1-Hus1 (9-1-1) complex, Rad17 and the replication factor complex proteins (2-5). Under "normal" conditions the RFC subunits 1-5 form a complex and act as a "clamp loader" loading the PCNA "sliding clamp" onto the site of replication. However following DNA damage the RFC1 subunit is replaced with Rad17, and again this complex acts as a clamp loader this time loading the 9-1-1 complex (Venclovas *et al.* 2000). The loading of the 9-1-1 complex then facilitates the recruitment of repair proteins onto the scaffold.

**ssDNA binding** occurs during the repair of double strand breaks when intermediate single strand breaks occur. These sections of DNA are recognised and coated by the

replication protein A (RPA), this loading of RPA is dependent on the Rad17-RFC2-5 and the 9-1-1 complex and in turn this coating of RPA recruits the DNA damage transducer complex ATR-ATRIP (1.1.2.) continuing the damage signal (Manke *et al.* 2003).

**DSB recognition** occurs via protein-DNA, protein-protein interactions described here but DSB are also recognised by the modification of the surrounding chromatin (Discussed later 1.5.) One such DSB recognition complex is the Mre11-RAD50-NBS-1 (MRN) complex which binds DNA (Mirzoeva *et al.* 2006) and localises to the site of double strand breaks, with other sensory and repair proteins; BRCA1, RAD51, PCNA, and proteins involved in mismatch repair. Although MRN is involved in the sensing of DNA damage, this complex also functions in the activation of the cell cycle checkpoints (1.2.) experiments in ATM null AT cell lines show a failure to phosphorylate NSB1 in response to gamma irradiation and ultra violet light. In addition Mre-11 NBS1 deficient cells continue to replicate following IR showing a defective checkpoint response (Shiloh *et al.* 1997) demonstrating the role of MRN in both sensing DNA damage and activating the cell cycle checkpoints (Gatei *et al.* 2000, Wu *et al.* 2000). Claspin is a mediator of signalling of DNA damage and binds the sites of DNA damage via Rad9 of the 9-1-1 complex (Lee *et al.* 2003) and is essential for Chk1 phosphorylation by ATR (1.1.2.).

**BRCT containing sensory proteins** include BRCA1, TopBP1, 53BP1 (53 binding protein 1) and MDC1 (Mediator of DNA damage checkpoint 1). The BRCT

domains contained within this group of proteins have the ability to bind ssDNA, dsDNA, and phosphopeptides providing a mechanism for these proteins to bind at the sites of DNA damage and recruit repair proteins by phospho-protein interactions (Yu *et al.* 2003). Breast Cancer Susceptibility Gene 1 (BRCA1) directly binds DNA (Paull *et al.* 2001) and co-localises with other sensor proteins, TopBP1 (Yamane *et al.* 2002, Yamane *et al.* 1999), the MRN complex and PCNA (Wang *et al.* 2002) to the sites of DNA damage. In doing so BRCA1 recruits substrates for the downstream kinase transducers (Figure 2) and acts as a link between the two phases of the response. TopBP1 interacts directly with NBS1 of the MRN complex (Morishima *et al.* 2007), and the localisation of TopBP1 to damage foci is dependent on this interaction, as NBS BRCT domain point mutations abolish TopBP1 localisation. In addition TopBP1 localisation to the sites of DNA damage is also dependent on a direct interaction with the 9-1-1 subunit Rad9 (Greer *et al.* 2003).

TopBP1 is involved in the activation of the transducers, Chk1 and the protein kinases ATR and ATM as following DNA damage TopBP1 interacts directly with the phosphorylated S345 of Rad9 via BRCT 1 and 2 (Delacroix *et al.* 2007). As described following replication stalling the exposed ssDNA is coated with RPA (Zou *et al.* 2003), which facilitates the loading of the 9-1-1 complex. TopBP1 has recently been shown to provide a link between the recognition of the damage and the activation of Chk1. The binding of Rad9 to TopBP1 is essential for Rad9 to interact with ATR-ATRIP and consequently activate the cell cycle checkpoints (see below).

### 1.1.2. Transducers

Transducers are proteins which amplify the signal from the sensor proteins and phosphorylate effector proteins to continue the DNA damage cascade.

**The Phosphoinositide-3 kinase related kinases (PIKKs)** are two DNA damage transducers, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad3 related (ATR). ATM and ATR are both large proteins 350kDa and 303kDa respectively which contain a catalytic kinase domain which phosphorylate downstream effector proteins serine and threonine residues to amplify the DNA damage signal. ATM exists as an inactive homodimer which is autophosphorylated at S1981 following DNA damage releasing the active monomers (Bakkenist *et al.* 2003). Active ATM then phosphorylates BRCA1 (Cortez *et al.* 1999), TopBP1 (Yamane *et al.* 2002), NBS1 (Lim *et al.* 2000) and the downstream transducer Chk2 mediating repair and cell cycle arrest. ATM activity is linked to chromatin modifications (1.5.) and the sensor complex MRN, as binding of ATM to Mre-11 increases the ability of ATM to phosphorylate its targets (Lee *et al.* 2004).

Unlike ATM, ATR does not exist as a homodimer but forms a heterodimer with ATRIP (ATP Interacting Protein) both budding yeast and fission yeast homologues of ATR, Mec1 and Ded2 have also shown similar binding with the ATRIP homologs Ded2 and Rad26 (Edwards *et al.* 1999). The ATR-ATRIP complex is recruited by

the sensory protein RPA and can only bind ssDNA coated with RPA (Zou *et al.* 2003). ATR then phosphorylates Chk1 (see below) to continue the DNA damage signal pathway.

Whether ATR or ATM is activated depends on the type of DNA damage which occurs (Discussed later). Mutations within both ATM and ATR are associated with human disease; Ataxia Telangiectasia (Shiloh *et al.* 1997) and Seckel syndrome respectively (O'Driscoll *et al.* 2007) resulting in an increase in cancer predisposition for disease sufferers.

**Serine/Threonine Kinases,** Chk1 and Chk2 (Checkpoint kinase) are also transducers (Figure 2) which are activated via phosphorylation by ATM or ATR (Walworth *et al.* 1996). As discussed following DNA damage ATR phosphorylates S317 and S345 of Chk1 (Zhao *et al.* 2001). This phosphorylation of Chk1 then activates the effectors Cdc25A, B and C mediating cell cycle arrest. Following ATM phosphorylation at T68 Chk2 phosphorylates p53, Cdc25A and C mediating cell cycle arrest, and BRCA1 facilitating DNA repair (Bartek *et al.* 2003).

### 1.1.3. Effectors

The effector proteins are discussed in the context of the effect they produce; activation of the DNA damage checkpoints (1.2.), DNA repair (1.3.) or apoptosis (1.4.).

## 1.2. DNA Damage Checkpoints

Cell cycle arrest by the induction of cell cycle checkpoints is essential to allow time for the DNA damage to be repaired and prevent damaged DNA from being replicated. These checkpoints occur via different mechanisms depending upon what point in the cell cycle the damage occurs; G1, S or G2. A diagram of these checkpoints is shown in Figure 2 and each checkpoint is described below.

### 1.2.1. G1 Checkpoint

The G1 cell cycle checkpoint allows time for repair and prevents damaged DNA from being replicated. Cell cycle control is controlled by p53 levels which are "normally" kept low as it is continually targeted for degradation by the proteosome by MDM2 (Vargas *et al.* 2003). However to activate the checkpoint following DNA damage, p53 is stabilised and the levels increased. In response to DSB, ATM

phosphorylates Chk2 at T68 (Matsuoka *et al.* 2000). Phosphorylated Chk2 then phosphorylates S20 of p53, which prevents the interaction between MDM2 and p53, resulting in an increase in p53 levels. The phosphorylation of S20 of p53 is essential for its stabilisation, but during the activation of the G1 arrest S15 of p53 is also phosphorylated, by both ATR following UV and ATM following IR. The phosphorylation of S15 activates p53 and increases transcription of target genes; MDM2, GADD45a and p21 (Banin *et al.* 1998, Canman *et al.* 1998). The increase in the cyclin dependent kinases inhibitor p21 levels decreased Cyclin E/Cdk2 kinase activity and promotes a G1 arrest (Bartek *et al.* 2001). p21 also binds Cdk4-Cyclin D and prevents p53 phosphorylation of Rb; it is the phosphorylation of Rb which results in the transcription of S-phase genes (Bartek *et al.* 2001).

### 1.2.2. S phase

The S-phase checkpoint decreases the amount of DNA synthesis following DNA damage. Following IR ATM phosphorylates Chk2 at T62 (Falck *et al.* 2001). Chk2 targets Cdc25A for degradation by phosphorylating S123, meaning that it cannot remove inhibitory phosphorylation on Cdk2 (T14 and Y15). Therefore Cdk2/CyclinE and Cdk2/Cyclin A remain inactive and prevent completion of DNA synthesis. ATM also phosphorylates NBS (S343), BRCA1 (S1387), SMC1 (Structural maintenance of chromosome 1, at S957 and 966), and TopBP1 (S345). These complexes form a multi-protein complex BASC (BRCA1-associated genome surveillance complex). The loading of CDC45 is dependent on the MRN complex;

blockage of this complex renders cells radioresistant following gamma irradiation (Falck *et al.* 2002). ATR also phosphorylates Chk1 at S317 and S345 which phosphorylated Cdc25A and targets it for degradation (Zhou *et al.* 2002).

### 1.2.3. G2 Checkpoint

The G2 checkpoint stops the cell cycle prior to chromosome segregation. This checkpoint is primarily activated following the formation of a DSB, however high dose of UV do activate this checkpoint. This is perhaps due to the formation of lesions close to each other on the separate DNA strands. Chk1 and Chk2 are activated by ATM and ATR and phosphorylate WEE1 and the phosphatase Cdc25C on S216 (Peng *et al.* 1997) which creates a binding site for the 14-3-3 proteins. 14-3-3 then interacts with Cdc25C and sequesters it to the cytoplasm where it can no longer activate Cdc2 by removing the inhibitory phosphorylations (T14 and Y15) meaning that Cdc2/Cylin B1 complex remains in its inactive state and the entry to mitosis is blocked.

**Figure 2**

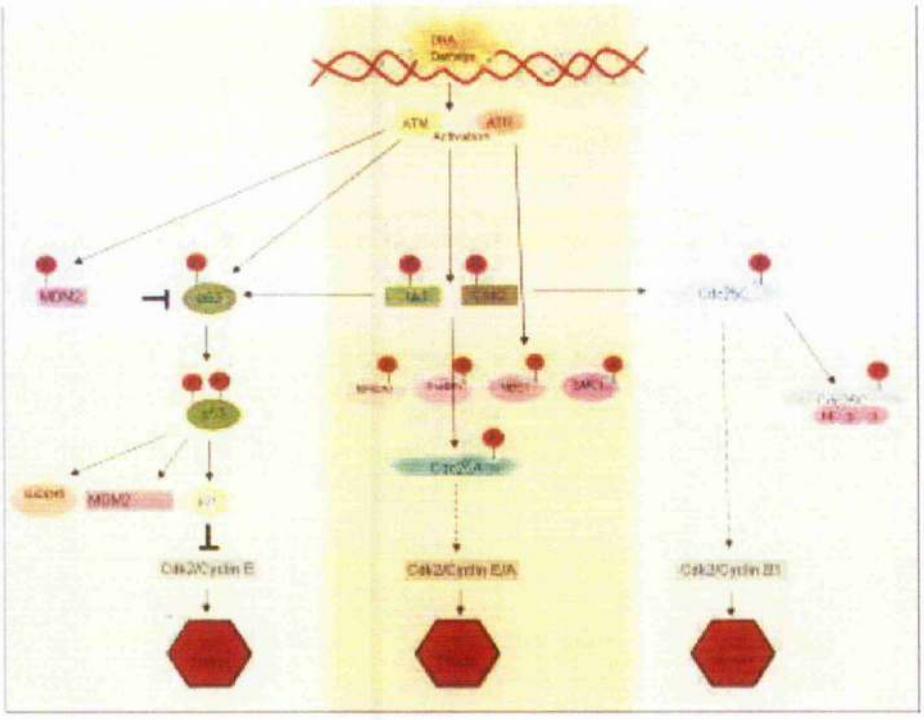



**Figure 2: Activation of the G1, S and G2 phase.** This diagram shows the activation of the cell cycle points see text for details.

## 1.3 Repair

Damage to DNA can be caused by a wide variety of endogenous and environmental sources. Radiation (gamma rays and x-rays), ultraviolet light, reactive oxygen species (ROS) by-products of cellular metabolism, and alkylating agents. The type of damage caused and the repair mechanism initiated depends on the damage agent (Figure 3). Base loss occurs spontaneously, indeed several thousand purines and hundreds of pyrimidines are lost a day. The primary amino group is unstable and can be converted to keto groups, changing cytosine to uracil. Chemical changes can occurs from reactive oxidative species ($H_2O_2$, •OH, -O) which are produced as by-products of normal cellular metabolism. X-Rays, and ionising radiation (IR), can cause double strand breaks and thymine oxidation producing thymine glycol. Photo damage occurs when UV light is absorbed by the bases and results in the formation of bonds between pyrimidines, causing cyclobutane pyrimidine dimers (SPD) TT being the most common, but T-C and CC dimers do occur. Errors during replication can result in mismatch, insertions or deletions. Bis-alkylating agents such as psoralens produce crosslinked strands. In addition to double strand breaks, IR can also cause normally transient topoisomerase-DNA interactions to be stabilised leading to protein-DNA crosslinks. The faithful repair of these DNA lesion is essential to prevent the loss/alteration of genetic material which might result in tumorigenesis. The different methods of DNA damage repair proceed as follows.

**Figure 3**

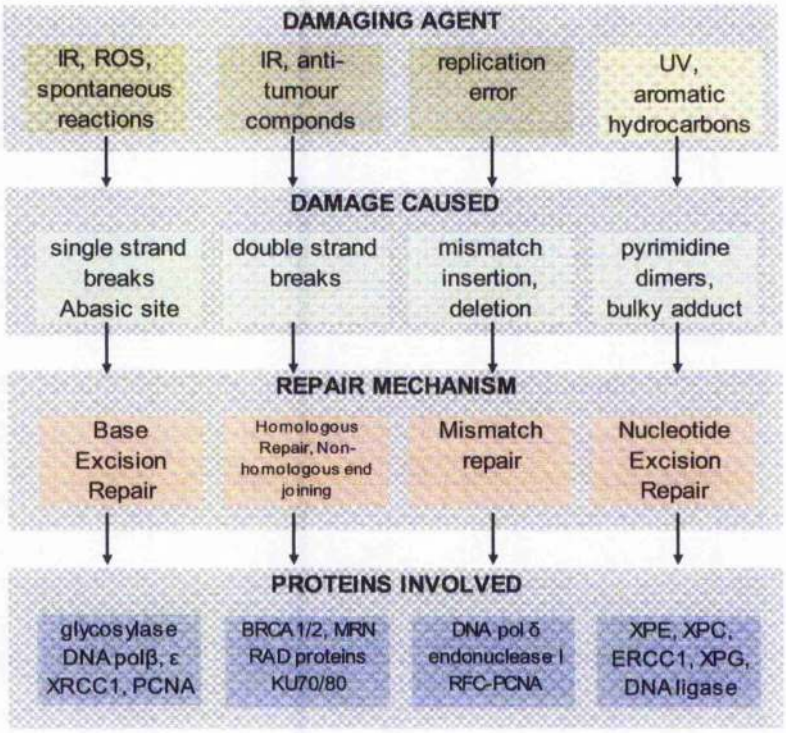



**Figure 3: Summary of the Types of DNA damage and the Repair Mechanisms.** This diagram shows the types of DNA damaging agents which cells are subjected to, the damage to DNA these damaging agents cause and the repair mechanisms which take place to repair the lesion.

### 1.3.1. Nucleotide Excision Repair

Nucleotide excision Repair (NER) removes bulky DNA lesions following ultra violet radiation or aromatic hydrocarbon exposure. This method of repair is activated by two mechanisms, either by surveying the whole genome for bulky lesions, or by responding to stalled RNA polymerases in transcription-coupled repair (TCR). In response to damage not on an actively transcribed DNA, the heterodimer DDB1-XPE bind to lesions induced by UV. XPE recruits XPC to the damage site, which then results in the dis-association of XPE from the DNA allowing XPC access to the site of the damage. The transcription factor TFIIH unwinds the DNA duplex in an ATP dependent manner. This unwinding of DNA then recruits other repair proteins; RPA which binds to the DNA ends; XPG which makes the 3' incision and ERCC1 which makes the 5' incision. The gap between the incisions is then filled by DNA polymerase δ and ε, via PCNA and RFC and ligated by DNA ligase I. TCR follows the same repair process although it does not require XPE but does require RNA polymerase.

### 1.3.2. Mismatch Repair

Mismatch repair corrects mistakes made during replication and accounts for 99% of all repair. MMR proteins follow behind the replication fork and remove mismatched nucleotides, insertions and deletions caused by template slippage or DNA

polymerase misincorporation. The RFC-PCNA complex identifies gaps in DNA that occur near to mismatched nucleotides and binds DNA at the 3' ends. MSH2 (MutS*alpha*) and MLH1 (MutL*alpha*) then cause another nick in the DNA, resulting in two cuts either side of the mismatched nucleotides. The DNA including the mismatch is degraded in a 5' to 3' direction by exonuclease I and the gap is then filled as in BER and NER by DNA polymerase δ and the ends are ligated by DNA ligase I.

### 1.3.3. Base Excision Repair

Damage which is caused by endogenous sources such as reactive oxygen species (ROS), the by-products of normal metabolism are repaired by BER. The type of DNA damage that occurs include; depurination (cleavage of the link between deoxyribose and the purine bases), deamination (removal of the amino group) of cytosine, the formation of covalent adducts and damage to the DNA backbone (Review see Wilson *et al.* 1998). BER can repair these lesions either by a short (1-2 nucleotides) patches or long (2-10 nucleotide) patches. The initiation of both is carried out by DNA glycosylase which removes the base resulting in an apurinic or aryprimidinic (AP) site. Endonucleases then cleave the phosphodiester bond. For short patches the gap is filled by DNA Polβ, and ligated by DNA ligase III via XRCC1. Long patches are replicated by Pol δ and Pol ε, via PCNA and then ligated by DNA ligase III.

### 1.3.4. DSB repair, Homologous Repair (HR) and Non-Homologous End Joining (NHEJ)

Double strand breaks are a direct result of ionising radiation or reactive oxygen species (ROS). ROS may cause DSB by several mechanisms; by creating long "nick" in DNA or by a free radical reaction on one strand which then starts a chain reaction resulting in a crosslink and eventually a DSB, in addition these "nicks" in DNA can form DSB during replication. DSB are repaired by one of two mechanisms; either by homologous recombination (HR) or by non-homologous end joining (NHEJ). Which repair takes place depends on what point during the cell cycle the break occurs; HR occurs primarily during S-G2 phase when the sister chromatid is available (Takata *et al.* 1998) and NHEJ occurs outside S-phase.

HR is error free using a homologous sister chromatid as a template for the damaged DNA. Mre11/Rad50/NBS1 (MRN) complex facilitates the loading of RAD52 to the ends of the breaks (Sonaoda *et al.* 2001). The importance of the MRN complex is shown by the failure of cells from NBS patients where NBS is mutated, to repair DSB resulting in an increase in cancer predisposition for sufferers. RAD52 senses the broken DNA ends and recruits RAD51, which binds the single stranded overhangs. With the aid of RPA, RAD53, Rad54, BRCA1 and BRCA2 RAD51 searches for the homologous template on the sister chromatid. RAD51 binds RAD53 and via BRCA1 and BRCA2 facilitates the strand invasion. MMS4 cleaves the 3'

non-homology and following branch migration results in the formation of a Holliday junction, which is then resolved by resolvase (Figure 4a).

The second method of DSB repair; NHEJ is more error prone as no homologous template is used and occurs when the sister chromatid is not available outside S-phase. Genetic alterations can result from the ligation of unrelated DNA sequences creating fusion proteins. One such fusion protein is the oncogenic BCR-Abl protein which is found in chromic myeloid leukaemia (CML) and acute lymphocyte leukaemia (Laurent *et al.* 2001), which results from a fusion of the serine/threonine kinase BCR (Breakpoint cluster region) and the oncogene ABL, BCR activates ABL giving rise to tumourigenesis. To repair DSBs by NHEJ, the heterodimeric protein complex Ku70-Ku80 binds the DNA ends and recruits DNA-PK, XRCC4 and DNA ligase IV (Jackson *et al.* 2000). DNA-PK phosphorylates XRCC4 and the Ku heterodimer aligning the broken DNA ends and then following alignment the ends are then ligated by DNA ligase IV via XRCC4 (Figure 4b).

**Figure 4**

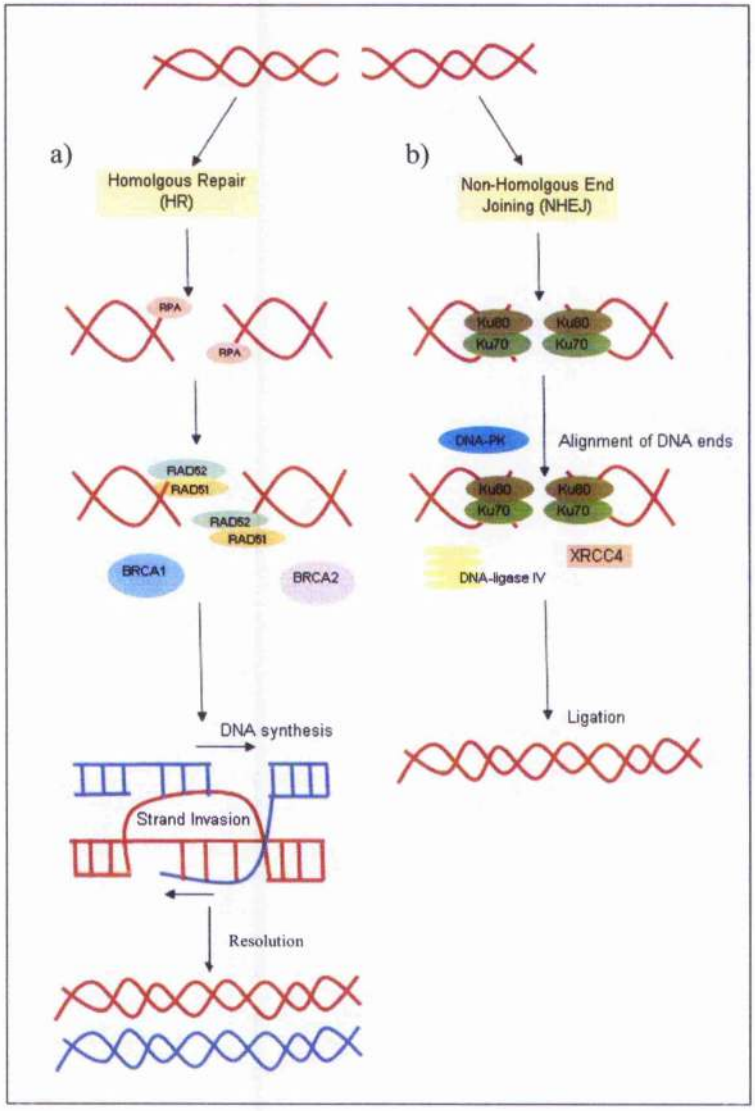



**Figure 4: Repair of DSB by Homologous Repair (HR) and Non-homologous End Joining (NHEJ).** This diagram shows the mechanism and proteins involved in HR (a) and NHEJ (b).

## 1.4. Apoptosis

If the damage a cell receives is too extensive to be repaired the cell undergoes apoptosis, and a failure to induce apoptosis in damaged cells plays a critical role in tumorigenesis. The apoptotic pathway is a signal cascade involving sensors, transducers and effector proteins. The key effectors of which are the cysteine aspartyl proteases (caspases), and the BCL2 proteins. Two methods by which cells may undergo programmed cell death particularly of relevance to the data presented in this thesis are via p53 and E2F1. As discussed, following DNA damage and the activation of ATM the transcription factor p53 is stabilised leading to the induction of several cell cycle regulators including GADD45 and p21, and target genes required for the apoptotic pathway including Bax, CD95, and Death receptor 5 (Wu *et al.* 1999, Oda *et al.* 2000) triggering apoptosis. Stabilisation of p53 by CHK2 following DNA damage promotes its translocation to the mitochondrial membrane, and this disruption of the mitochondrial membranes leads to activation of the caspases and the induction of apoptosis. Similarly to p53, E2F1 is also phosphorylated by ATM following DNA damage, leading to the stabilisation and activation of E2F1. This stabilisation is dependent several proteins including Mdm2 (Blattner *et al.* 1999) and TopBP1 (Discussed later 1.6). Liu and colleagues showed that E2F1 is regulated by TopBP1 via the recruitment of the chromatin remodelling complex; SWISNF (Discussed 1.5.2.) to inhibit E2F1 transcriptional activity and

that this regulation of E2F1 by TopBP1 is essential for E2F1-dependent apoptosis following DNA damage (Liu *et al.* 2004).

## 1.5. Role of Chromatin in DNA Damage Response

The activation of the DNA damage response depends upon the modification of chromatin (Review Costelloe *et al.* 2006). The nucleosome is the basic unit of chromatin and consists of a histone octamer core (Figure 5a) around which is wrapped 147bp of DNA (Kornberg *et al.* 1999). This tight packing of DNA can inhibit the accessibility of repair proteins to the lesion (Hara *et al.* 2000); therefore the chromatin surrounding DNA damage must be modified during the DNA damage response to ensure efficient repair. Chromatin is modified in two ways during the DNA damage response; by histone modification and by ATP-dependent remodelling enzymes.

### 1.5.1. The Modification of Histones

Histones contain serine, threonine, lysine and arginine polar residues, which are displayed on the surface of the nucleosome and are modified during the DNA damage response. In response to DNA damage a variant of histone H2, H2AX is phosphorylated at S139 (γH2AX) by the protein kinases (ATR, ATM and DNA-PK). ATM is the most important of the three kinases, as ATM-/- fibroblasts fail to

form γH2AX foci (Celeste *et al.* 2003) and the ATM inhibitor wortmannin inhibits H2AX phosphorylation (Paull *et al.* 2000). This phosphorylation of H2AX occurs in the region surrounding double strand breaks (a Mb on either side (Lowndes *et al.* 2005)) but it is not phosphorylated specifically at the break (Berkovich *et al.* 2007). This phosphorylation of H2AX aids the recruitment of the repair proteins including BRCA1, NBS1 and MDC1, and acts as a docking site; MDC1 via its BRCT domain binds phosphorylated H2AX directly (Stucki *et al.* 2005). The importance of H2AX phosphorylation in the recruitment of repair proteins is shown in mice lacking H2AX which show an increase in cancer incidence (Celeste *et al.* 2003) and fail to produce damage foci (Bassing *et al.* 2004). In addition the phosphorylation of H4 (S1) by casein kinase II is localised to the sites of DSB and promotes NHEJ in S. *cerevisiae* (Cheung *et al.* 2005).

The addition of methyl groups ($CH_3$) to lysine residues of the histones removes the positive charge and disrupts the DNA-histone interaction, and is essential for the recruitment of the BRCA1 homologues in yeast; methylation of H3 (79) is essential for the recruitment of the Rad9 in budding yeast (Huyen *et al.* 2004), and Crb2 is recruited via H4 K20 methylation in fission yeast (Sanders *et al.* 2004).

Similarly to methylation the addition of an acetyl group to the lysine residues within the histones disrupts the DNA-histone bonds (Narlikar *et al.* 2002), and increases access of the repair proteins (Downey *et al.* 2006). This acetylation is carried out by two groups of enzymes; histone acetyltransferases (HAT) which acetylates the lysine

residues and histone deacetyltransferase (HDAC) enzymes which remove the acetyl group. One HAT enzyme in humans Tip60 has two roles in the DNA damage response; firstly it acetylates ATM activating the kinase (Jiang *et al.* 2006) and secondly it acetylates H4 and H2AX facilitating the loading of the repair proteins BRCA1 and RAD51 to the sites of DNA damage (Loizou *et al.* 2006).

The ubiquitination of histones is important in the regulation of the DNA damage response, as the ubiquitin associated proteins localise to γ-H2AX foci and of H2AX, H3 and H4 acetylation is induced following both IR and UV damage (Wang *et al.* 2006). As discussed previously following DNA damage H2AX is acetylated by the HAT enzyme Tip60, this acetylation of H2AX has now been shown to be an essential event in the subsequent ubiquitination of H2AX by the ubiquitin conjugating enzyme (UBC13), and the modification of chromatin structure (Ikura *et al.* 2007).

### 1.5.2. Modification of Chromatin by ATP-dependent Chromatin Remodellers

The modification of the core nucleosomes by ATP-dependent chromatin remodellers use ATP to disrupt the bonds between DNA and the nucleosomes, sliding the nucleosome resulting in a looping out of the surrounding DNA. This results in a more "open" chromatin state increasing accessibility for the repair proteins. These enzymes belong to a large family of enzymes which contain a central ATPase

catalytic subunit. Studies in yeast have shown that these chromatin remodellers are recruited to the sites of DNA damage (Shim *et al.* 2005) and are essential for both the recruitment of RAD51 to the site of DNA damage and the loading of RAD51 onto ssDNA (Tsukuda *et al.* 2005). In addition these complexes are also required for strand invasion during HR (Chai *et al.* 2005). The mechanism of chromatin remodelling by these enzymes is linked to other histone modifications as the actions of Brg1 (Brahma related gene 1) the ATPase subunit of the chromatin remodeller SWISNF (Switch Mating Type, Sucrose Non-Fermenting) binds the acetylated lysine following DNA damage (Shen *et al.* 2007).

**Figure 5**

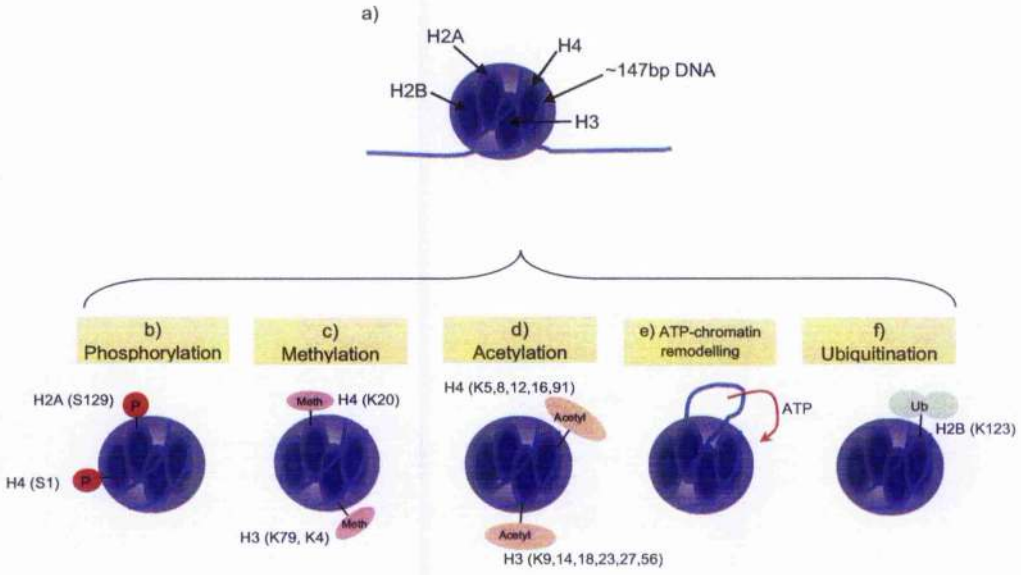



**Figure 5: Summarisation of chromatin modifications during the DNA damage Response.** a) Each nucleosome contains a histone octamer core containing histones H2A, H2B, H3 and H4, with 147bp of DNA wrapped around. b) Phosphorylations of histones H2AX (S129) and H4 (S1). b) Methylation of H4 (K20), and H3 (K79, K4). c) Acetylation of histones H4 (K5, K8, K12, K16, and K91), and H4 (K9, K14, K18, K27 and K56). e) Chromatin remodelling by the chromatin remodelling enzymes results in a looping out of the DNA surrounding the nucleosome. f) Ubiquitination of H2B (K123).

## 1.6. TopBP1

TopBP1 is an essential nuclear protein, as downregulation of TopBP1 results in decreased cell viability (Yamane *et al.* 2002), this protein, and its role in transcription, replication and repair provides the main focus of this thesis. TopBP1 was first identified as an interacting protein for Topoisomerase II β using a yeast two hybrid assay (Yamane *et al.* 1997). Following DNA damage TopBP1 is phosphorylated by ATM (Yamane *et al.* 2002) and localises to the sites of DNA damage (Yamane *et al.* 2002), co localising with other sensory proteins including NBS1 of the MRN complex (Morishima *et al.* 2007), BRCA1, PML (Xu *et al.* 2003) and the 9-1-1 subunit Rad9 (Greer *et al.* 2003). TopBP1 is also involved in the activation of the transducers following DNA damage; TopBP1 interacts directly with the phosphorylated S345 of Rad9 via BRCT 1 and 2 (Delacroix *et al.* 2007), facilitating the loading of the 9-1-1 complex and the activation of Chk1 via ATR-ATRIP and ultimately the activation of the cell cycle checkpoints. Further to this XTopBP1 has been shown to interact directly with XATR-XATRIP via amino acids 978-1192 and activate ATR kinase activity, this interaction is dependent on all three proteins and point mutant of TopBP1 W1138R still binds XATR-ATRIP but fails to activate XATR kinase activity (Kumagai *et al.* 2006). TopBP1 may also monitor recombination at DSB, as it co-localises with ATR, during meiosis (Perera *et al.* 2004).

### 1.6.1. Structure of TopBP1

TopBP1 contains eight BRCT (Breast Cancer Susceptibility Gene 1 [BRCA1] Carboxyl Terminus) domains (Yamane *et al.* 1999) which were originally identified within the carboxyl terminus of BRCA1 and as discussed (1.1.1.) BRCT domains are found in a variety of proteins involved in cell cycle control, DNA repair and transcriptional regulation [BRCA1 (Glover *et al.* 2006), NBS (Kobayashi *et al.* 2004) and RAD9 (Hammet *et al.* 2007)]. BRCT domains consist of hydrophobic tandem repeats, which are involved in interacting with other proteins, phosphorylated peptides (Glover *et al.* 2004), as well as single and double stranded DNA (Yu *et al.* 2003). Therefore following DNA damage, BRCT domains can alter their interacting partners and potentially interacting directly with damaged DNA, making their role in the DNA damage response essential for the correct regulation of the response. In addition no other protein has as many as eight BRCT domains indicating that TopBP1 plays an essential role in the processes described.

### 1.6.2. Homologues of TopBP1

DNA repair pathways are integral to all cellular organisms and are highly conserved in eukaryotes, the fission yeast *Schizosaccaromyces Pombe* (Rad4), budding yeast *Saccharomyces Cerevisiae* (Dbp11), *Drosophila melanogaster* (Mus101), *Xenopus laevi* (XTopBP1) and *Caernorhabditis elegans* (F37D6.1) all have TopBP1

homologues. Therefore further insight into roles of TopBP1 in the DNA damage response comes from the study of these proteins. Figure 6 shows the amino acid sequence similarity and BRCT domains which are conserved by the TopBP1 homologues discussed within this chapter.

### 1.6.2.1. Fission Yeast *Schizosaccaromyces Pombe* Homologue Rad4/ Cut5

The *Schizosaccaromyces Pombe* TopBP1 homolog was first identified as a radiation sensitive mutant ($Rad4^{TopBP1}$) and later as a "cell ultimately torn" mutant, Cut5 in a separate yeast screen (Saka *et al.* 1993). This fission yeast homologue of TopBP1 contains four BRCT domains and has roles in the S phase checkpoint and the DNA damage response. Saka *et al.* showed that $Rad4^{TopBP1}$ was essential for S-M checkpoint, and the initiation of replication in mutants of $Rad4^{TopBP1}$ show a cell cycle defect resulting in an increased the percentage of cells in S-phase (Spiga *et al.* 2004). Rad9 phosphorylation at Thr412 and Ser423 in response to DNA damage activates $Rad4^{TopBP1}$ binding via BRCT three and four (Furuya *et al.* 2004). $Rad4^{TopBP1}$ associates with $Rad26^{ATRIP}$ and $Rad3^{ATR}$ (Taricani *et al.* 2006) which then activates Chk1. The $Rad3^{ATR}$-$Rad26^{ATRIP}$ phosphorylation of Chk1 requires Crb2 (Saka *et al.* 1997). Crb2 contains one BRCT domain and forms foci in response to DNA damage (Du *et al.* 2003). $Rad4^{TopBP1}$ binds Crb2 via BRCT I and II, and using a yeast two hybrid assay Du *et al.* 2006 showed Crb2 binds $Rad4^{TopBP1}$ via its BRCT domain and that $Rad4^{TopBP1}$ is essential to recruit Crb2 to the sites of DNA damage.

### 1.6.2.2. Budding Yeast *Saccharomyces Cerevisiae* Homologue Dpb11

The budding yeast *Saccharomyces cerevisiae* TopBP1 homologue Dpb11 (DNA polymerase B possible subunit 11) contains four BRCT domains and was identified as an interacting partner of DNA polymerase II (Araki *et al.* 1995). Dpb11 like $Rad4^{TopBP1}$ has roles in both DNA replication and DNA damage. Dpb11 co-immunoprecipitates with Pol ε in S-phase extracts but not G1 extracts (Masumoto *et al.* 2000) and forms a complex with DNA polymerase II (Araki *et al.* 1995) recruiting it to the origins of replication (Masumoto *et al.* 2000). Chromatin immunoprecipitation showed that Dpb11 associates with DNA pol ε, at the origins of replication; and this association with these origins is dependent on MCM and RPA. Dpb11 interacts with Ded1 (Rad9 homologue) of the Ded1-Rad17-Mec3 (similar to human 9-1-1) complex via BRCT III and IV (Wang *et al.* 2002) mutant Dpb11-1 cells carry a truncated Dpb11 (lacking C-terminal) which has a defective DNA damage response as it cannot interact with Ded1.

### 1.6.2.3 *Drosophila Melanogaster* Homologue Mus101

The mutagen sensitive 101 (Mus101) gene was identified through a sensitivity to DNA-damaging agents screen in *Drosophila melanogaster* (Yamamoto *et al.* 2000). This sensitivity is specific to double strand breaks and not to UV radiation, and results in defective replication repair and a failure to recondense heterochromatin.

Mus101 encodes seven BRCT domains when aligned with TopBP1, and sequence alignment of these two proteins revealed that BRCT6 was the region absent in Mus101 (Yamamoto *et al.* 2000).

**Figure 6**

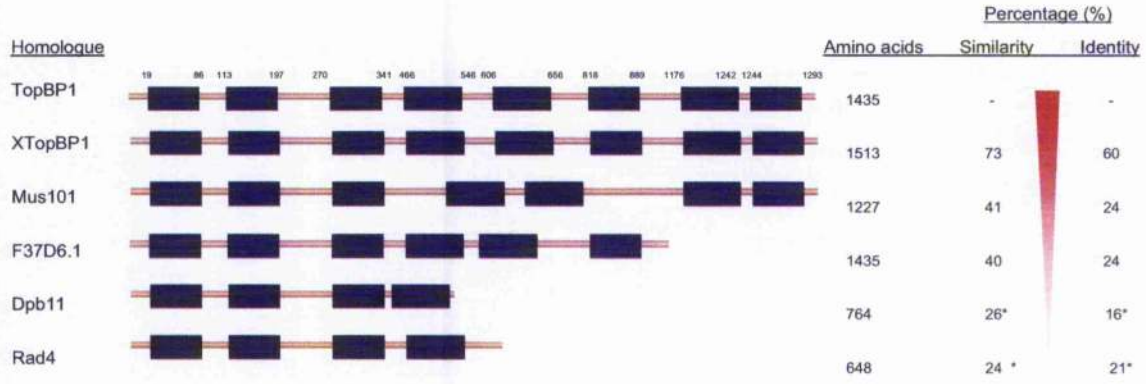



**Figure 6: Diagrammatic Representation TopBP1 homologues.** This diagram shows the conservation between the TopBP1 homologues. The number of BRCT domains in each protein is indicated (Blue box). The number of amino acids in each TopBP1 homologue and the percentage of both similar and identical residues (Compared against the human TopBP1) are indicated on the right of the diagram (Garcia *et al.* 2005). The sequence comparison of Dpb11* and Rad4* were performed using ClustalW™ .

### 1.6.2.4. *Xenopus Laevi* Homologue

The *Xenopus Laevi* homologue of TopBP1 (XTopBP1) provides more evidence of a role of TopBP1 in the DNA damage response and replication. XTopBP1 binds chromatin after the initiation of replication (Hashimoto *et al.* 2003). The N-terminal of XTopBP1 encompassing BRCT 1-4 is required for the initiation of replication; depletion of endogenous Xenopus XTopBP1 abolished replication (as shown by the incorporation of radio-labelled dCTP in xenopus sperm DNA), however replication was restored with the addition of XTopBP1 containing BRCT 1-4 but not with the addition of a C-terminal (BRCT5-8) XTopBP1. The C-terminal region (BRCT 5-8) was however shown to be the region essential for checkpoint activation via phosphorylation of XTopBP1 S1131 (Hashimoto *et al.* 2006), as phosphorylation of Chk1 was abolished in XTopBP1 depleted extracts until the addition of the C-terminal XTopBP1 (Hashimoto *et al.* 2006). XTopBP1 interacts directly with XRad9 via BRCT I and II and not only is this region essential for the binding of XRad9 this region is also essential for the checkpoint signalling as no phosphorylation of XChk1 is found in nuclear fractions lacking XTopBP1 (Lee *et al.* 2007). Immunodepletion of XTopBP1 from Xenopus egg extracts results in a loss of Rad1 phosphorylation by ATR (Lupardus *et al.* 2006) and a failure to phosphorylate Chk1 and induce cell cycle arrest, however Chk2 is not similarly effected.

### 1.6.2.5. *Caernorhabditis elegans* Homologue F37D6.1

The *Caernorhabditis elegans* (C.elegans) TopBP1 homologue F37D6.1 contains 8 BRCT domains. C.elegans provide a tool for studying genes involved in the DNA damage response as these genes can be specifically knocked down in these animals by feeding E.coli clones expressing double stranded RNA. Van Haaften and colleagues used this model system to investigate genes essential for the DNA damage response (Van Haaften *et al.* 2004). They used a strain of C.elegans (NL1832) which show an increase in DSB, and knocked out 74 genes involved in the DNA damage response to determine if the knock down of any of these genes resulted in a lethal phenotype. They identified three genes which resulted in lethality; one of which was the TopBP1 homolog F37D6.1. They also showed that knockdown of F37D6.1 resulted in an increase in chromosome aberrations following gamma irradiation, demonstrating that TopBP1 is essential for the repair of DSB following DNA damage.

### 1.6.3. TopBP1-A Possible Transcription Factor?

Research indicates that TopBP1 is possible a transcriptional regulator. TopBP1 interacts with and represses the transcription and apoptotic effects of E2F1 in a Brg1/Brm dependent manner as repression of E2F1 was not seen in the Brg1/Brm compromised C33a cell line (Liu *et al.* 2003). Lui *et al.* 2003 showed that TopBP1 directly interacts with E2F1 via BRCT6, and is recruited to E2F1 responsive promoter. TopBP1 interacts with the HPV16 viral transcription/replication protein E2 and co-activates transcription and replication (Boner *et al.* 2002). In addition TopBP1 directly interacts with Miz1 via BRCT 7 and 8, and the interactions of these two proteins is essential for the repression of Myc targeted genes (Herold *et al.* 2002, Myc review see Eisenman *et al.* 2001). The transcription factor c-myc belongs to a family of genes that are found in human cancers resulting from an increase in the expression of the proteins (He *et al.* 1998).

As discussed earlier (1.1.1) BRCA1 plays an essential role in the regulation of the DNA damage response (Zhang *et al.* 1998). BRCA1 contains two BRCT domains within the carboxyl terminal and a domain spanning this region of BRCA1 (amino acids 1560-1863 including the BRCT domains) can act as a transcriptional activation domain when fused to GAL4 DNA binding domains (Monteiro *et al.* 1996). In 1997 Monteiro and colleagues found that a common disease predisposing mutation (Ser1613Cys) within the transactivation domain could not activate transcription in

yeast compared to a common non-disease variant of the same residue (Monteiro *et al.* 1997). This work demonstrated that BRCA1 tumour suppressor activity was directly linked to its transcriptional activity. Further analysis of these BRCT domains has shown that they are frequently the region mutated in early onset breast cancer (Futreal *et al.* 1994) resulting in a loss of BRCT structure (Glover *et al.* 2006). This is supported by the studies in mice in which truncation of the BRCT domains resulting in a loss of BRCT structure causes tumours in mice (Ludwig *et al.* 2001). This precedence for the role of BRCT domains in transcriptional regulation and function set by BRCA1 suggested that the ability of TopBP1 to modulate transcription was perhaps via one of the eight BRCT domains.

### 1.6.4. TopBP1 and Breast Cancer

The importance of a faithful functioning DNA damage response is demonstrated by the disease states which are a direct result of defects within DNA damage response proteins. Table 2 summarises some of these diseases which arise from a failure to respond correctly to DNA damage and indicates the phase of the DNA damage response where these proteins function. In addition to similarities between the BRCT containing proteins TopBP1 and BRCA1 in sensing DNA damage and repair, there is increasing evidence that like BRCA1, TopBP1 plays a critical role in breast cancer implicating it as a possible breast cancer susceptibility gene.

10% of all breast cancer cases are familial cases; of which 50% are a result of either BRCA1 or BRCA2 mutations and 5% are a result of other gene alterations (p53 (Malkin *et al.* 1990), ATM (Swift *et al.* 1991)) however the susceptibility genes in the remaining 35% are still unaccounted for. Karppinen *et al.* provided the first evidence for TopBP1 as a breast cancer susceptibility gene (Karppinen *et al.* 2006). To investigate the possible involvement of TopBP1 in hereditary breast cancer they screened for germline polymorphisms within the TopBP1 gene in 125 familial breast cancers and compared the results to 187 non-hereditary breast cancers and 697 control samples. They identified a novel variant Arg309Cys, the incidence of which was increased compared to both healthy and non-familial breast cancer controls. In addition none of the familial samples carrying this polymorphism contained either a BRCA1 or BRCA2 mutation, thus implicating TopBP1 as a breast cancer susceptibility gene.

Karihtala *et al.* investigated the relationship between the levels of ROS and the expression of repair enzymes in benign, hyperplasia, in situ tumours and stage I breast tumours to identify if expression of these repairs enzymes correlated with the amount of damage within these cells (Karihtala *et al.* 2006). The measurement of 8-hydroxydeoxyguanosine (8-OHdG) was used as a marker of DNA damage by ROS because 8-OHdG is produced following an imbalance in $H_2O_2$ redox reactions when the enzymes involved are overwhelmed by the accumulated DNA damage. 8-OHdG expression increased from benign to invasive tumours, consistent with previous results which showed 8OHdG levels increased 15 fold in breast carcinomas

(Musarrat *et al.* 1996). TopBP1 associated with 8OHdG but not with other markers of damage and the expression of TopBP1 also increased during cancer progression from hyperplasia to invasive tumours (10% hyperplasia, 8% in situ (low grade), 45% in situ (high grade) and 35% of stage I tumours). This correlation between an increase in TopBP1 staining and cancer progression was also observed by Morris *et al.* 2007. They stained 123 feline mammary lesions for TopBP1 and found that the percentage of cells with TopBP1 staining increased as the histology of the lesion increased from benign to high grade carcinomas (10 samples in the high grade lesions showed strong TopBP1 staining in 80-100% of cells compared to only 1 sample in non-neoplastic samples). Furthermore, an increase in TopBP1 cytoplasmic staining was observed as the malignancy increased ($P< 0.001$). Aberrant cytoplasmic staining of TopBP1 in breast cancer was also observed in another study in which 9/59 human breast cancer samples (ranging from grade I to grade III invasive carcinomas) showed either nuclear and cytoplasmic or solely cytoplasmic staining (Going *et al.* 2007).

**Table 2**

| Disease | Protein | Function | Reference |
|---|---|---|---|
| | | **Sensor** | |
| Testicular tumors | Rad17 | RFC1-like | von Delmling *et al*. 1999 |
| Non-small cell lung carcinoma | Rad9 | PCNA-like | Maniwa *et al*. 2005 |
| A-T-like disorder (A-TLD) | Mre11 | DSB recognition/nuclease | Stewart *et al*. 1999 |
| Nijmegen breakage syndrome | NBS1 | DSB recognition/nuclease | Varon *et al*. 1998 |
| Breast Cancer | TopBP1 | BRCT containing | Going *et al*. 2007 |
| Breast and Ovarian Cancer | BRCA1 | BRCT containing | Hartikainen *et al*. 2007 |
| | | **Transducers** | |
| Ataxia-telengiestctasia (AT)<br>Familial Breast Cancer | ATM | PI3 Kinases | Broeks *et al*. 1998<br>Broeks *et al*. 2000 |
| Seckel Syndrome<br>Breast Cancer | ATR | PI3 Kinases | Alderton *et al*. 2004<br>Durocher *et al*. 2006 |
| Colorectal Cancer | Chk1 | Ser/Thr Kinase | Madoz-Gúrpide et al 2007 |
| Li-Fraumeni Syndrome<br>Familial Breast Cancer<br>Prostate Cancer | Chk2 | Ser/Thr Kinase | Allinen *et al*. 2001<br><br>Dong *et al*. 2003 |
| | | **Effector** | |
| Li-Fraumeni Syndrome<br>Breast/Pancreatic Cancer | p53 | Transcription Factor | Capponcelli *et al*. 2005<br>Malkin *et al*. 1990 |
| Thyroid lymphoma<br>Breast Cancer | Cdc25A | Phosphatase | Ito *et al*. 2005<br>Ito *et al*. 2004 |
| Thyroid lymphoma | Cdc25B | Phosphatase | Ito *et al*. 2005 |
| Prostate Cancer | Cdc25C | Phosphatase | Ozon *et al*. 2005 |
| Breast Cancer<br>Ovarian Cancer | BRCA2 | Repair | Fackenthal *et al*. 2002<br>Hartikainen *et al*. 2007 |
| Colorectal Cancer<br>Colorectal Cancer<br>Turcot's syndrome | MSH2<br>MLH1 | Repair<br>Repair | Froggatt *et al*. 1999<br>Lipkin *et al*. 2004<br>Hamilton *et al*. 1995 |
| Falconi Anaemia | FANCD2 | Repair | Futaki et al. 200[illegible] |
| Hodgkin lymphoma | Cdk1 | Kinase | Bai *et al*. 2005 |

**Table 2: Diseases associated with defects within the DNA damage response.** This table lists the DNA damage response proteins which are altered/lost in the disease indicated. The reference for each protein-disease link is also given.

Although TopBP1 is a possible transcription factor and a suspected breast cancer susceptibility gene the domains and protein partners involved are not clearly defined, therefore this thesis has three main aims:

I. To identify any transcriptional regulatory domains within TopBP1, such domains modify chromatin and therefore could be involved in all aspects of TopBP1 function; transcription, replication and repair.

II. To identify functional TopBP1 interacting partners.

III. To identify the genes regulated by TopBP1 under "normal" conditions and in response to double strand breaks.

# 2 - Materials and Methods

## 2.1. Materials

### 2.1.1. Antibodies

**Abcam Plc (Cambridge, United Kingdom)**

Anti-Sheep IgG horseradish peroxidase linked whole molecule (raised in Donkey)

Catalogue Number: Ab6900

**Amersham International plc (Buckinghamshire, United Kingdom)**

Anti-Mouse IgG horseradish peroxidase linked whole molecule (raised in sheep)

Catalogue Number: NXA931

**Applied Biosystems (California, USA)**

Anti-mouse IgG (whole molecule) FITC conjugate, developed in goat

Catalogue Number: T5393

**BD Transduction Laboratories (Oxford, United Kingdom)**

Anti-TopBP1 (amino acids 204-216) murine monoclonal antibody

Catalogue Number: 611875

**Santa Cruz Biotechnology Inc (Heidelberg, Germany)**

Anti-Brg1 (amino acids 209-296) polyclonal rabbit antibody

Catalogue Number: sc-10768

Anti-Brg1 N-terminus polyclonal goat antibody

Catalogue Number: sc-8749

**Sigma-Aldrich Company Ltd. (Dorset, United Kingdom)**

Anti-Rabbit IgG (whole molecule) peroxidase developed in goat

Catalogue Number: A6154

**Upstate (Dundee, United Kingdom)**

Anti-phospho-H2A.X (Ser139) developed in rabbit

Catalogue Number: 07-164

**Dr. Stefan Roberts (University of Manchester, United Kingdom)**

The Anti-GAL4 DNA binding domain polyclonal sheep antibody was a kind gift from Dr. Stefan Roberts (University of Manchester).

TopBP1 polyclonal antibody R1180 against amino acids 861-1287 was raised in a rabbit, method of preparation of the antibody and the preimmune have been described previously (Boner *et al.* 2002).

## 2.1.2. Bacteriology

**Becton Dickinson Labware (Oxford, United Kingdom)**

15ml polypropylene tubes - Catalogue Number: 348206

50ml polypropylene tubes - Catalogue Number: 358206

20ml sterile syringe - Catalogue Number: 301031

BD Bacto™ Agar - Catalogue Number: 214010

**Bibby Sterlin Ltd (Staffordshire, United Kingdom)**

90mm bacteriology petri dishes - Catalogue Number: 502014

**Institute of Comparative Medicine Central Services (University of Glasgow, United Kingdom)**

Luria Broth (L-Broth), 1 litre: 10g Tryptone, 10g NaCl, 5g yeast extract completed $ddH_2O$)

Luria Bertai agar, 1 litre: 10g Tryptone, 10g NaCl, 5g yeast extract, 15g bacteriological agar completed with $ddH_2O$).

**Invitrogen Ltd (Paisley, United Kingdom)**

Max efficiency® DH5α™ chemically competent cells - Catalogue Number: 18258

Subcloning efficiency™ DH5α™ chemically competent cells - Catalogue Number: 18265

**Sigma Chemical Co. Ltd (Dorset United Kingdom)**

Ampicillin - Catalogue Number: 10047

Isopropyl-β-D-1-thiogalactosidase (IPTG) - Catalogue Number: 15502

Kanamycin - Catalogue Number: K1876

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) - Catalogue Number: 16664

Typtone microbiology tested - Catalogue Number:T7293

### 2.1.3 Cell lines

**C33a** cells are derived from a HPV negative cervical carcinoma and are defective for both p53 and pRb function. C33a cells were obtained from Cancer Research UK (Cancer Research United Kingdom (CRUK), London).

**Human Embryonic Kidney 293T (HEK293T)** are derived from the 293 cell line in which the SV40 T-antigen was inserted. HEK293T cells were a kind gift from Dr. Brian Willet, University of Glasgow.

**MCF7** cells derived from a Human Caucasian breast adenocarcinoma were purchased from Cancer Research UK (CRUK, London United Kingdom).

**MRC5** cells are a primary human foetal derived cell line was purchased from CRUK (CRUK, London United Kingdom).

**U2OS** are an oesteosarcoma cell line, expressing wild type p53 and were purchased from CRUK (CRUK, London United Kingdom).

### 2.1.4 Chemicals and Reagents

**Amersham International PLC (Buckinghamshire, United Kingdom)**

Enhanced Chemiluminescence (ECLplus) detection substrate - Catalogue Number: PN2132

Hyperfilm X-ray film - Catalogue Number: 93184

**Applied Biosystems (Warrington, United Kingdom)**

Hi-Di™ Formamide - Catalogue Number: 4336697

**Invitrogen Ltd (Paisley, United Kingdom)**

Agarose (ultrapure electrophoresis grade) - Catalogue Number: 41025

**Roche Biosystems (Basel, Switzerland)**

Complete Protease Inhibitor Cocktail Tablets - Catalogue Number: 12481800

**Sigma Chemicals Co. Ltd (Dorset, United Kingdom)**

1-(4, 5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT) - Catalogue Number:M2003

3',5',5''-Tetrabromophenolsulfophethalein (Bromphenol Blue) - Catalogue Number: B0126

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) - Catalogue Number: H4034

3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo] phenylazo)-2, 7-naphthalenedisulfonic acid sodium salt (Ponceau S) -Catalogue Number: P7170

Acetic acid sodium salt (Sodium acetate) - Catalogue Number: S2889

Bicinchonoinic Acid (BCA) solution - Catalogue Number: B9643

Boric Acid ~99% - Catalogue Number:B6768

β-Mercaptoethanol - Catalogue Number: M6250

Caffeine 99% tissue culture grade - Catalogue Number: C8960

Copper (II) sulphate (pentahydrate 4% (w/v) solution) - Catalogue Number: C2284

Ethidium Bromide - Catalogue Number: E1510

Magnesium Chloride (MgCl2) anhydrous beads - Catalogue Number: 449172

N-lauroylsarcosine solution - Catalogue Number: L7414

Nonident P-40 (NP40) - Catalogue Number: I8896

Phenol: Chloroform: Isoamyl Alcohol (25:24:1) - Catalogue Number: P3803

Sodium Acetate - Catalogue Number: S2889

Sodium Chloride - Catalogue Number: S7653

Tween 20 (Polyoxethylene sorbitan nonolaurate) - Catalogue Number: P1379

Tris Base (2-Amino-2-(hydroxymethyl)-1, 3-propanediol) - Catalogue Number: T6066

Thiazolyl Blue Tetrazolium Bromide (MTT) - Catalogue Number: M5655

**University of Glasgow Stores (Glasgow, United Kingdom)**

Crude ethanol

**VWR International (Leicestershire, United Kingdom)**

Absolute 99.7-100% AnalaR® ethanol - Catalogue Number: 10107EP

AnalaR® Sodium Hydroxide pellets - Catalogue Number: 102524X

AnalaR® Glycine molecular biology grade - Catalogue Number: 101194M

AnalaR® D-(+)-Glucose - Catalogue Number: 101176K

AnalaR® Glycerol - Catalogue Number: 10118EU

Crystal Violet - Catalogue Number: 340245L

Dimethyl sulfoxide (DMSO) - Catalogue Number: 8029122500

Ethylene diamine tetra acetate (EDTA) disodium salt - Catalogue Number: 30031.294

Hydrochloric acid - Catalogue Number: 101252F

Methanol AnalaR® - Catalogue Number: 10158FK

Propan-2-ol (Isopropanol) Molecular Biology Grade - Catalogue Number: 437423R

Sodium dodecyl sulphate (SDS) AnalaR® - Catalogue Number: 108073J

### 2.1.5 Enzymes

**Cambrex Bioscience (Wokingham, United Kingdom)**

Takara DNA ligation Kit (version V) - Catalogue Number 6022

**Invitrogen Ltd (Paisley, United Kingdom)**

BamH1 and reaction buffer (REact® 3) - Catalogue Number: 15201

EcoR1 and reaction buffer (REact ® 3) - Catalogue Number: 15202

Kpn1 and reaction buffer (REact ® 4) - Catalogue Number: 15232

Platinum ® Taq DNA polymerase - Catalogue Number: 10966

**New England Biolabs (Hitchin, United Kingdom)**

Antarctic phosphatase and reaction buffer - Catalogue Number: MD289L

**Promega Ltd (Southampton, United Kingdom)**

*Pfu* DNA Polymerase 10X Reaction Buffer with $MgSO_4$ - Catalogue Number: M7741

dATP 100mM (40µmol) - Catalogue Number: U1205

dCTP100mM (40µmol) - Catalogue Number: U1225

dGTP100mM (40µmol) - Catalogue Number: U1202

dTTP 100mM (40µmol) - Catalogue Number: U1235

### 2.1.6 Kits

**Applied Biosystems (Warrington, United Kingdom)**

BigDye® Terminator v3.1 Cycle Sequencing Kit - Catalogue Number: 4337455

**Invitrogen Ltd (Paisley, United Kingdom)**

ChargeSwitch® Plasmid ER Mini Kit - Catalogue Number: CS10100

ChargeSwitch® PCR Clean-Up Kit - Catalogue Number: CS12000

Purelink® plasmid maxiprep kit - Catalogue Number: K210006

NuPAGE® 4-12% Bis-Tris 12 well 1.0 mm gels - Catalogue Number: NP0302BOX

NuPAGE® 4-12% Bis-Tris 10 well 1.0 mm gels - Catalogue Number: NP0301BOX

NuPAGE® Sample Reducing Agent (10X) - Catalogue Number: NP0009

NuPAGE® Antioxidant - Catalogue Number: NP0005

NuPAGE® MES SDS Running Buffer (20X) - Catalogue Number: NP0002

NuPAGE® Transfer Buffer (20X) - Catalogue Number: NP0006

**Promega Ltd (Southampton, United Kingdom)**

Luciferase Assay System - Catalogue Number: E1500

Reporter Lysis Buffer (5x solution) - Catalogue Number: E3971

**Qiagen Ltd (Crawley, United Kingdom)**

QIAquick Gel Extraction Kit - Catalogue Number: 28704

QIAquick PCR Purification Kit - Catalogue Number: 28104

QIAGEN Plasmid Midi Kit - Catalogue Number: 12162

RNAeasy RNA extraction Kit - Catalogue Number: 74104

QIAprep Spin Miniprep Kit - Catalogue Number: 27106

## 2.1.7 Miscellaneous

**Corning® Life Sciences (Leicestershire, United Kingdom)**

96 Well Clear Flat Bottom Microplate - Catalogue Number: 3635

**Decon Laboratories Ltd (Hove, United Kingdom)**

Decon 75® Liquid

**Elkay International (Basingstoke, United Kingdom)**

1.5ml microcentrifuge tubes with attached cap - Catalogue Number: MICR050

0.5ml microcentrifuge tubes with attached cap - Catalogue Number: MICR051

Plastic Pasteur pipettes - Catalogue Number: P511

**EquiBio (Kent, United Kingdom)**

2mm electroporation cuvettes - Catalogue Number: ECU-102

**Kimberley Clark (Brighton, United Kingdom)**

Safeskin Disposable Purple Nitrile Exam Gloves - Catalogue Number: 014602

**Morrisons Supermarket (Anniesland, United Kingdom)**

Marvel dried skimmed milk

**Sigma Chemicals Co. Ltd (Dorset, United Kingdom)**

Protein G Sepharose® Fast Flow in 20% ethanol - Catalogue Number: P3296

**Thermo Labsytems Corporation (Warwickshire, United Kingdom)**

Luminoskan Acsent Luminometer and Ascent software (version 2.4.2)

**Ultra Violet Productions (UVP) (Cambridge, United Kingdom)**

UVP Multi-doc gel documentation system

High performance ultra-violet transilluminator (UVP VB-26)

**Vector Laboratories, Ltd., (Peterborough, United Kingdom)**

VECTASHIELD Mounting Medium with DAPI - Catalogue Number: H-1200

## 2.1.8. Molecular Weight Markers

**Invitrogen Ltd (Paisley, U.K.)**

100 bp DNA Ladder - Catalogue Number: 15628

1Kb DNA Ladder - Catalogue Number: 15615

See Blue® Pre-stained protein standard - Catalogue Number: LC5925

## 2.1.9. Plasmids

**pACT** vector expresses the herpes simplex virus VP16 activation domain amino acids 411-456 under control of the cytomegalovirus (CMV) immediate early promoter (Promega Ltd Southampton, United Kingdom).

**pBIND** vector contains the yeast GAL4-DNA binding domain upstream of a multiple cloning region. (Promega Ltd Southampton, United Kingdom).

**pCGBrg1 (4638)** vector expresses wild type Brg1 under the control of the cytomegalovirus (CMV) promoter, and was a kind gift from Dr. Christian Muchardt, referred to as Brg (wt) in the text.

**pCGBrg1 ATP mutant (4639)** vector expresses ATP mutant Brg1 under the control of the cytomegalovirus (CMV) promoter, and was a kind gift from Dr. Christian Muchardt, referred to as Brg (-/-) in the text.

**pCGBrm (4102)** vector expresses wild type Brm under the control of the cytomegalovirus (CMV) promoter, and was a kind gift from Dr. Christian Muchardt, referred to as Brm (wt) in the text.

**pCGBrm ATP mutant (4137)** vector expresses ATP mutant Brm under the control of the cytomegalovirus (CMV) promoter, and was a kind gift from Dr. Christian Muchardt (Pasteur Institute, Paris) and is referred to as Brm (-/-) in the text.

**pCMV** mammalian expression vector was a kind gift from Peter Howley (Harvard University, Boston, USA).

**pE2F1** vector expresses the full length E2F1 under the control of the cytomegalovirus (CMV) promoter, and was a kind gift from Dr. Kevin Ryan (Beatson Institute for Cancer Research, Glasgow).

**pGBKT7** expresses proteins fused to amino acids 1-147 of the Gal4 DNA binding domain, in yeast proteins are expressed from the ADH1 promoter.

**pG5luc** contains five GAL4 binding sites upstream of a minimal TATA box, upstream of the firefly luciferase gene (Promega Ltd Southampton, United Kingdom).

**pGL3 control** contains SV40 promoter and enhancer sequences driving the expression of firefly luciferase (Promega Ltd Southampton, United Kingdom).

**pGL3 basic** lacks promoter and enhancer sequences (Promega Ltd Southampton, United Kingdom).

**ptk6E2luc** contains the thymidine kinase promoter from HSV-1 cloned into the pGL3 luciferase vector upstream of 6 HPV-16 E2 binding sites (Vance *et al.* 1999).

**pTopBP1** expresses the full length TopBP1 under control of the cytomegalovirus promoter and was a kind gift from Dr. Kazuhiko Yamane.

**p73luc** luciferase vector contains E2F1 binding sites has been described previously (Irwin et al. 2000), and was a kind gift from Dr. Kevin Ryan (Beatson Institute for Cancer Research, Glasgow).

## 2.1.10. Tissue Culture

**Amaxa Biosystems (Cologne, Germany)**

Nucleofector™ II device

Cell line Nucleofector Kit V- Catalogue Number VCA - 1003

**Corning incorporated (Leicestershire, United Kingdom)**

100mm x 20mm non-pyrogenic cell culture dishes - Catalogue Number: 3296

60mm x 15mm non-pyrogenic cell culture dishes - Catalogue Number: 4301

25ml disposable serological polystyrene pipettes - Catalogue Number: 4251

10ml disposable serological polystyrene pipettes - Catalogue Number: 4101

5ml disposable serological polystyrene pipettes - Catalogue Number: 4135

6 well flat bottom non-pyrogenic cell culture cluster - Catalogue Number: 3506

12 well flat bottom non-pyrogenic cell culture cluster - Catalogue Number: 3512

24 well flat bottom non-pyrogenic cell culture cluster - Catalogue Number: 3524

96 well flat bottom non-pyrogenic cell culture cluster - Catalogue Number: 3596

External thread cryogenic vials - Catalogue Number: 430287

Cell scraper - Catalogue Number: 3010

**Greiner Bio-one Ltd (Stonehouse, United Kingdom)**

50ml polypropylene centrifuge tubes - Catalogue Number: 210201

15ml polypropylene centrifuge tubes - Catalogue Number: 188261

**Invitrogen Ltd (Paisley, United Kingdom)**

DMEM 1 x w/GlutaMAX™/Glucose Na Pyr - Catalogue Number: 11995

Distilled water - Catalogue Number: 15230

Foetal bovine serum - Catalogue Number: 16141

Penicillin-Streptomycin Solution (5000:5000) liquid - Catalogue Number: 15070

Trypsin-EDTA (1x) 0.05% trypsin 0.53mM EDTA·Na - Catalogue Number: 25300

**Nunc™ Part of Thermo Fisher Scientific (Hereford, UK)**

25cm tissue culture flasks with filtered caps - Catalogue Number: 136196

80cm tissue culture flasks with filtered caps - Catalogue Number: 178905

175cm tissue culture flasks with filtered caps - Catalogue Number: 178883

**Promega Ltd Southampton, United Kingdom**

Phosphate buffered saline (PBS) tablets - Catalogue Number: P4417

## 2.1.11. Yeast Materials

**Clontech Ltd (Wokingham, United Kingdom)**

AH109 Yeast strain - Catalogue Number: 630444

**Invitrogen Ltd (Paisley, United Kingdom)**

BL21 E. *coli* competent cells - Catalogue Number: C6070

**Sigma Aldrich Ltd (Dorset, United Kingdom)**

Sodium carbonate ($Na_2CO_3$) - Catalogue Number: S7795

Magnesium sulfate ($MgSO_4$) - Catalogue Number: M2643

Poly (ethylene glycol) (PEG) - Catalogue Number: 81150

Sodium trichloroacetate (TCA) - Catalogue Number: 45670

6-Aminopurine hemisulfate salt Adenine sulfate salt - Catalogue Number: A9126

L-histidine monohydrate - Catalogue Number: H8000

L-Leucine - Catalogue Number: L8912

L-Tryptophan - Catalogue Number: T0254

Potassium Chloride (KCl) - Catalogue Number: P9333

Ammonium sulfate molecular biology grade ≥99.0% - Catalogue Number: A4418

Lithium Acetate - Catalogue Number: 517992

Yeast Nitrogen Base - Catalogue Number: 51483

# 2.2 Methods

The protocols used in this thesis are described in the following section. The manufacturers and distributors details for the materials used are given in the previous section.

## 2.2.1 Molecular Biology

### 2.2.1.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis was typically carried out using 1% agarose gels. The appropriate amount of electrophoresis grade agarose (Invitrogen) was weighed and dissolved in 0.5 x TBE Buffer (10 x TBE: 900mM Tris base, 900mM boric acid, 25mM EDTA, pH8.0) by heating. For a 200ml solution 5µl (10mg/ml) ethidium bromide was added, and the solution cooled to room temperature, before pouring into the assembled gel cast containing an appropriate sized comb. Once cooled and set the gel was placed into the electrophoresis tank, and submerged in 0.5 x TBE Buffer (as above). Sample volumes typically ranged between 10-30µl and contained 1 x loading buffer (10 x loading buffer: 65% (w/v) sucrose, 10mM Tris-HCl pH 7.5, 10mM EDTA, 0.3%(w/v) bromophenol blue). Samples were loaded into individual wells, along side a 1Kb or 100bp ladder (depending upon predicted sample size) as a molecular weight marker. The sample DNA was separated by electrophoresis using 50V until the separate bands and sizes were easily distinguishable. The DNA was

visualised by UV light and photographed using a UVP Gel Documentation System (UV Productions).

### 2.2.1.2. DNA /RNA concentration determination

The concentration of plasmid DNA was measured using an absorbance measurement at 260nm (Biotech spectrophotometer -model UV1101). An optical density reading (OD) of 1 being equivalent to 50μg/ml of double stranded DNA. RNA concentration was determined similarly and OD of 1 is equivalent to 40μg/ml RNA. A 260nm/280nm absorbance ratio was measured for all plasmid preparations a reading of between 1.8 and 2.1 indicative of a pure sample.

### 2.2.1.3. Restriction enzyme digests

Restriction digests were performed using the required restriction enzyme and the appropriate buffer following the manufacturer's instructions. In general, the restriction digests were completed in a total volume of 30μl. This typically contained 5-10μl sample DNA, 1μl restriction enzyme, 3μl restriction enzyme buffer, completed to 30μl with distilled water. Digests were incubated at 37°C for 2-3 hours.

### 2.2.1.4. DNA purification using phenol chloroform

Restriction digests, sequencing and ligation reactions were purified using phenol: chloroform to remove all residual contaminants. Sample volumes were typically completed to 100/200µl prior to extraction. An equal volume of phenol: chloroform: isoamyl alcohol (24:25:1 v/v/v) was added to the sample, vortexed well and centrifuged for 10 minutes at 15000g 4°C. This resulted in the separation of the aqueous and organic phases; the top aqueous phase was removed and pipetted into clean microcentrifuge tubes and the bottom organic phase was discarded.

### 2.2.1.5. Ethanol Precipitation of DNA

Following purification using phenol: chloroform DNA was precipitated using ethanol precipitation. 1/10th the sample volume of 3M sodium acetate, pH 5.2 and 2.5x the sample volume 100% ethanol, was added to the sample. The samples were vortexed and left to incubate at –20°C for 30-60 minutes. Following this precipitation the samples were centrifuged at 15000g, at 4°C for 20 minutes, and the ethanol discarded. The resulting pellet was then washed by adding 1x sample volume 70% ethanol and centrifuging for a further 20 minutes under the same conditions. The ethanol was again discarded and the pellet dried either by the use of a vacuum, or naturally until no ethanol remained. The precipitated DNA was then resuspended in 20µl distilled $H_20$.

### 2.2.1.6. Phosphatase Treatment

Following restriction digests cut vectors were de-phosphorylated using antarctic phosphatase™ (New England Biolabs) to prevent vector re-ligation. 1µl phosphatase was added to the restriction digest and incubated at 37°C for 15 minutes. The phosphatase and restriction enzymes were inactivated by heating to 70°C.

### 2.2.1.7. DNA ligation

Following restriction enzyme digests and dephosphorylation of the vector, the vector and insert were ligated in a typical volume of 10µl. Typically this contained; 4µl insert, 1µl de-phosphorylated vector, and 5µl ligase (Takara). Ligations were incubated at 16°C for 1 hour or overnight.

### 2.2.1.8. Transformation of Chemically competent Bacterial Cells

DH5α chemically competent E. *coli* were transformed during cloning or and prior to plasmid maxiprep extraction. 1µl of a ligation reaction or 10ng plasmid DNA was added to 20µl DH5α cells and incubated on ice for 30 minutes. Following the incubation the cells were heat shocked at 42°C for 1 minute and placed back on ice for 5 minutes. 100µl SOC medium (25ml bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was added to each tube and they were incubated at 37°C whilst shaking (225rpm) for 1 hour.

Following the incubation the samples were plated out on antibiotic resistant agar plates, inverted, and incubated overnight at 37°C. Colonies were picked the following day and grown in 5ml L-broth (2.1.2.), 50mg/ml antibiotic, overnight at 37°C with shaking (225rpm) prior to both small and large scale preparations of plasmid DNA.

### 2.2.1.9. Electroporation of E. *coli*

40µl electrocompetent BL21 cells (Invitrogen) were incubated with 1µg plasmid DNA and transformed by electroporation using 2.5µF, 200Ω 1.8kV. This was then transferred to 500µl SOC medium and incubated at 37°C for 1 hour with agitation (225rpm) before plating out on to kanamycin resistant plates.

### 2.2.1.10. Large Scale preparation of Plasmid DNA (Maxiprep)

Large scale preparation of plasmid DNA was carried out according to the manufacturer's instructions. Briefly, 1ml of a 5ml overnight bacterial culture in L-broth ampicillin/kanamycin was used to inoculate 100ml of L-broth with selective antibiotic in a 2-litre glass conical flask. This bacterial culture was incubated at 37°C 225rpm overnight. Following incubation the bacterial cells were pelleted by centrifugation at 5000g for 30 minutes at 4°C. The resulting supernatant was discarded, and the bottles inverted for 5 minutes at room temperature to remove any excess. The bacterial pellet was resuspended in 10ml resuspension buffer (50mM

Tris-Cl pH 8.0, 10mM EDTA, 100µg/ml RNase A) by vortexing and pipetting, before adding 10ml lysis buffer (200mM NaOH, 1% SDS) and mixing gently by inversion 6 times. The lysis reaction was allowed to continue for 5 minutes at room temperature. 10ml chilled precipitation buffer (3.0M potassium acetate pH 5.0) was then added and mixed gently be inversion before incubation on ice for 30 minutes. The resulting solution containing the genomic DNA, proteins and cell debris in the form of a white fluffy precipitate was centrifuged at 5000g for 30 minutes. Following centrifugation the supernatant was filtered to remove the precipitate and the cleared lysate was added to the extraction column. The column had been equilibrated by adding 15ml equilibration buffer (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol (v/v) and 0.15% Triton r X-100 (v/v)) before the addition of the lysate. The lysate was left to drain by gravity flow and a total of 60ml wash buffer (1.0M NaCl, 50mM MOPS pH 7.0, 15% isopropanol) per column was added and left to drain by gravity flow. The wash buffer is essential to remove contaminants from bacterial strains which have a high amount of carbohydrate. The DNA now captured on the resin was eluted by adding 15ml elution buffer (1.25M NaCl, 50mM Tris-Cl (pH 8.5) and 15% isopropanol), and collected in 50ml centrifuge tubes. 12ml isopropanol was then added and to the eluted DNA and the sample centrifuged at 5000g 4°C for 1 hour. The resulting supernatant was decanted and the cell pellet washed in 10ml 70% ethanol before centrifugation at 5000g 4°C for 30 minutes. The pellets were allowed to air dry before being resuspended in 300µl distilled water, and stored at –20°C until required.

### 2.2.1.11. Small scale Plasmid Purification (Miniprep)

Bacterial colonies were picked and grown overnight in 5ml L-broth at 37°C with shaking (225rpm). 1ml of culture was then transferred to a microcentrifuge tube. The samples were pelleted at 15000g at 4°C for 1 minute, and the supernatant discarded. The bacterial pellets were resuspended in 300µl resuspension buffer (50mM Tris-Cl (pH 8.0), 10mM EDTA and 100µg/ml RNase A) by pipetting. 300µl lysis buffer (200mM NaOH, 1% SDS) was then added and mixed gently by inversion to allow the lysis reaction to proceed for 5 minutes at room temperature. Following lysis 300µl chilled precipitation buffer (3.0M potassium acetate pH 5.0) was then added and mixed by inversion resulting in a cloudy precipitate. This solution was then centrifuged for 10 minutes at 15000g, 4°C with the formation of a compact white pellet. The supernatant was then pipetted into an eppendorf containing 40µl magnetic beads, and incubated for 1 minute at room temperature. This was placed into a magnetic rack for 1 minute with the formation of a tight pellet. The supernatant was removed and 1ml wash buffer added to resuspend the magnetic beads. This was then placed on the magnetic rack for 1 minute to form a tight pellet and the supernatant discarded. This wash process was repeated once before the addition of 50µl elution buffer. Samples were then placed on the magnetic rack for 2 minutes and the purified DNA was removed and transferred to an eppendorf.

### 2.2.1.12. Polymerase chain reaction (PCR)

PCR reactions were routinely carried out in a volume of 50µl. Using 1-10ng of template DNA, 10pmol of each primer (forward and reverse), 200µM each dNTP (dATP, dTTP, dCTP, and dGTP), 1µl polymerase and 5µl 10x Pfu polymerase reaction buffer, completed to 50µl with distilled water. Amplification was carried out using a MJ Research PTC200 gradient cycler. The cycle conditions were routinely as follows: 95°C for 10 minutes (denaturation step), 94°C for 1 minute, 55°C for 1 minute (annealing step), and 72°C for 3 minutes (elongation step). This cycle was repeated 25 times after which samples underwent 94°C for 1 minute 50°C for 1 minute, 72°C for 10 minutes and held till required at 4°C. The elongation step was changed depending on the desired size of PCR product.

**Table 3: 5' Primers for cloning TopBP1 into pBIND**

| Primer | Sequence 5'-3' | Restriction Site |
|---|---|---|
| TopBP1 2 | CGCGGATCCGTCACCACCAGCGATGTGTC | BamH1 |
| TopBP1 113 | CGCGGATCCGTATTTTTCTTGGTTGCATA | BamH1 |
| TopBP1 181 | CGCGGATCCGTGACAGTATTGAGAAAGGT | BamH1 |
| TopBP1 253 | CGCGGATCCGTACACTTGAAAATCTAGAA | BamH1 |
| TopBP1 341 | CGCGGATCCGTG AGTGTTTCAGTAAAGGT | BamH1 |
| TopBP1 460 | CGCGGATCCGTACAATAACTGAAGAAGCG | BamH1 |
| TopBP1 470 | CGCGGATCCCGCGTCTGAAGCCAGTACGG | BamH1 |
| TopBP1 500 | CGCGGATCCGTCTGAGCAGAACTGTTGCG | BamH1 |
| TopBP1 586 | CGCGGATCCAGTGCAAGTGTTCAAGAATAC | BamH1 |
| TopBP1 635 | CGCGGATCCGTGAGACTGCTAGAACGGGA | BamH1 |
| TopBP1 710 | CGCGGATCCGTCAACATGCCAGACAGGTC | BamH1 |
| TopBP1 798 | CGCGGATCCGTCTTTCTGCCAGCCCTCAA | BamH1 |
| TopBP1 1169 | CGCGGATCCGTACGAATAGAGGAGACTCAT | BamH1 |
| TopBP1 540 | GCGGGATCCGTTTTGATCCAAAGTCGAAT | BamH1 |

**Table 4: 3'Primers for cloning TopBP1 into pBIND**

| Primer | Sequence 5'-3' | Restriction Site |
|---|---|---|
| TopBP1 258 | CGGGGTACCTTCTAGATTTTCAAGTGT | Kpn1 |
| TopBP1 591 | CGGGGTACCGTATTCTTGAACACTTGC | Kpn1 |
| TopBP1 803 | CGGGGTACCTTGACCCATGGCAGAAAG | Kpn1 |
| TopBP1 914 | CGGGGTACCGATATCCAAGCTCATTTT | Kpn1 |
| TopBP1 1435 | CGGGGTACCGTGTACTCTAGGTCGTTT | Kpn1 |
| Top 675 | CGGGGTACCAGTATCTGAATTTAGATTGAT | Kpn1 |

**Table 5: Primers for cloning TopBP1 into pGBKT7**

| Primer | Sequence 5'-3' | Restriction Site |
|---|---|---|
| 3' Top 258 (pGBkT7) | GCCGGATCCGTTAGATTTTCAAGTGTAGG | EcoR1 |
| 3' Top 591 (pGBkT7) | GCCGGATCCGTGTATTCTTGAACACTTGC | EcoR1 |
| 5' Top 253 (pGBkT7) | CGGGAATTCACACTTGAAAATCTAGAA | BamH1 |
| 5'Top 2 (pGBkT7) | CGCGGATCCGTCACCACCAGCGATGTGTC | BamH1 |
| 3' Top 258 (pGBkT7) | CGCGGATCCGTTAGATTTTCAAGGTAGG | EcoR1 |

**Table 6: Primers for cloning VP16-GAL4/VP16-TopBP1-GAL4**

| Primer | Sequence 5'-3' | Restriction Site |
|---|---|---|
| 5' Top 258/VP16 | GTTCGGGGGGGCCGTCGATAGATTTTCAAGTGTAGG | - |
| 5' VP16/Top 258 | CCTACACTTGAAAATCTATCGACGGCCCCCCGACC | - |
| 5' VP16 | CGCGGATTCGTTCGACGGCCCCCCCGAGC | BamH1 |
| 3' Vp16 Act | CGGGGTACCTCCCGGACCCGGGGAATC | Kpn1 |

### 2.2.1.13. PCR Purification

PCR products were purified either using QIAquick PCR clean up (Qiagen) or ChargeSwitch® PCR Clean-Up Kit (Invitrogen). Both protocols are briefly described below.

### 2.2.1.13.1. QIAquick PCR clean up Protocol

Briefly, 500µl Buffer PB added to 100µl PCR sample and vortexed. This mixture was pipetted into a column and centrifuged for 1 minute at 15000g, 4°C and the flow through discarded. 750µl Buffer PE was then added to the centre of the gel column and centrifuged at 15000g, 4°C for 1 minute. The flow through was discarded, and the column placed into a clean microcentrifuge tube. Depending on the

concentration of the PCR product required for further protocols the DNA was eluted by adding 30/50µl EB Buffer, before centrifuging for 1 minute under the previous conditions. The purified DNA then stored at -20˚C until required.

### 2.2.1.13.2. ChargeSwitch® PCR Clean-Up

Briefly 50µl PCR purification buffer was added to the 50µl PCR product and incubated with 10µl of magnetic beads for 1 minute. The solution was placed on the magnetic rack forming a tight pellet. The supernatant was then removed and the pellet was washed twice with 150µl wash buffer. The excess wash buffer was then removed and the pellet resuspended with 20µl elution buffer and incubated at room temperature for 1 minute. The solution was then placed onto the magnetic rack until a tight pellet formed. The supernatant containing the purified DNA was then removed and stored at -20˚C until required.

### 2.2.1.14. Gel Extraction

Restriction digest products were occasionally following agarose gel electrophoresis using Purelink™ Gel Extraction protocol (Invitrogen). The area of the gel containing the DNA to be purified was cut out and weighed. To this slice 30µl gel solubilisation buffer per 10mg of gel was added. The gel was then heated to 50°C for 15 minutes with occasional agitation to ensure it was completely dissolved. The solution was then placed into a gel extraction column and centrifuged for 1 minute at 12000g. The

flow through was discarded and the column was washed twice with 700µl wash buffer at 12000g for 1 minute. 50µl warm (70°C) elution buffer was then added to the centre of the column before a final spin (12000g). The purified DNA stored at -20°C until required.

### 2.2.1.15. DNA sequencing

To check plasmids for mutation and that inserts were cloned in frame, plasmids were routinely sequenced using an applied Biosystems 3100 automated sequencer. The region of interest was sequenced using a primer complementary to the appropriate region. Reactions were carried out in 0.5ml PCR tubes and contained 0.5µg plasmid DNA, 3.2pmol primer, 2µl Big Dye Terminator Reaction mix and 4µl 5 x Big Dye Buffer (Applied Biosystems) completed to 14µl with distilled water. Samples were heated to 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. This cycle was repeated 25 times and the sequencing reaction held at 4°C till required. PCR reactions were purified using phenol: chloroform ethanol precipitation (as described 2.2.1.4.). Following purification the reactions were resuspended in 25µl formamide and analysed using an ABI 300 automatic sequencer.

### 2.2.1.16. BCA / CuSO4 Assay

Bovine serum albumin (BSA) protein standard solutions were made up at the

following concentrations from a stock (2mg/ml) solution; 80, 100, 200, 400, 1000 and 2000µg/ml. 10µl of each standard and protein lysate to be measured were loaded into a 96 well plate. 200µl developing solution was then added (5ml BCA (Bicinchoninic Acid Sigma) 100µl Copper II Sulphate pentahydrate 4% w/v solution (Sigma)). The plate was then incubated at 37°C for 30-60 minutes, before the absorbance was measured at 462nm. The absorbance readings for the BSA standards were used to derive a standard curve, from which the sample concentrations were deduced.

### 2.2.1.17. Western Blotting

Protein concentrations were determined using a BCA/$CuSO_4$ assay as described (2.2.1.16.). Equal amounts of protein were added to 2µl NuPage™ sample reducing agent (10X) and 5µl NuPage™ sample buffer (4x) (Invitrogen) and heated to 70°C for 10 minutes. The pre-cast gels either 10/12 well were placed into the gel tank filled with 1x MES running buffer. 400µl 10X antioxidant was added to the running buffer before loading the sample. 10µl See-Blue® kDa marker (Invitrogen) was also loaded as marker using fine pipette tips. SDS-Page gels were run for 1 hour at 200V. The buffer was discarded and the gel cast was carefully cracked open and the top plate peeled of to leave the gel in place. The wells were removed and the gel was transferred to a nitrocellulose membrane using one of three methods depending on the size of the protein of interest (Indicated in results). 1) Wet transfer; 7 blotting pads were soaked in 1 x transfer buffer (50ml transfer buffer (TB), 100ml methanol

completed to 1 litre with distilled water). 3 pads were placed in the blotting module followed by a filter paper also soaked in transfer buffer. Following the filter paper the gel was placed on top before placing the nitrocellulose membrane and another filter paper on top. Any air bubbles from this stack were removed by rolling before the addition of 4 more transfer buffer soaked blotting pads. The blot module was run at 30V for 1 hour. 2) Semi-dry transfer; the gel and two blotting pads were soaked in transfer buffer for 10 minutes. Two blotting pads were placed onto the semi-dry blotter and rolled to remove any air bubbles; a wet filter paper, the gel and then another filter paper were added to the blotting pads and again rolled before the addition of two blotting pads. This gel stack was then run at 10V for 2 hours. The semi-dry transfer was a better method of transfer for large proteins (>150kD). 3) Dry transfer; using Invitrogen i-blot transfer following the manufacturer's instructions. Following transfer the nitrocellulose membrane was incubated with 5% milk PBS-T (1 x PBS 0.1% tween) to remove any non-specific bands. Following blocking the primary antibody appropriately diluted in 5% milk PBS-T was incubated for 1 hour with gentle agitation at room temperature. The antibody was then removed and the membrane washed in PBS-T for 15 minutes, and 3 x 5 minutes at room temperature with gentle agitation. The secondary antibody was then added diluted in 5% milk PBS-T and incubated at room temperature for 1 hour with gentle agitation. The antibody was then removed and the wash steps repeated. The proteins were detected using ECLplus (GE Healthcare) according to the manufacturer's instructions. Briefly 1ml detection reagent A and 25µl detection reagent B was mixed and added to the membrane and incubated at room temperature for 5 minutes. The excess was

removed and the membrane placed into a cassette and developed with X-ray hyperfilm (Amersham).

### 2.2.1.18. Stripping membranes

Membranes were stripped in order to remove any traces of a previous antibody before reprobing. The membrane was submerged in stripping buffer containing 100mM 2-Mercaptoethanol, 2% SDS, and 62.5mM Tris-HCl pH 6.7, and incubated at 50°C for 30 minutes with occasional agitation. Following this incubation the membrane was washed twice for 10 minutes with PBS-T at room temperature, and blocked for 1hour in 5% milk PBS-T, before adding the desired antibody.

### 2.2.1.19. Coomasssie staining

Following separation by SDS-PAGE (2.2.1.17), the gel was removed from the case placed in 100ml $ddH_2O$ and heated at 900W for 1 minute in microwave. The gel was then incubated at with gentle agitation for 1 minute at room temperature. This wash step was repeated 3 times and the water discarded. 30ml simply Blue Safe Stain (Invitrogen) was added to the gel and heated at 900W for 1 minute. The gel was incubated in the stain for 10 minutes at room temperature, and then washed with 100ml $ddH_2O$ for 10 minutes. The wash was discarded and 20ml 20% NaCl added to destain for 1 hour at room temperature with occasional agitation. The resulting stained gel was dried between papers and stored at room temperature.

### 2.2.1.20. Immunoprecipitation

Cell lysates were harvested as described (2.2.2.7.). The antibody for the immunoprecipitation was added at a 1:250 dilution and incubated at 4°C overnight with rotation. Following the incubation 30µl Protein G-beads were added to the reaction and incubated for 1 hour with rotation at 4°C. The lysates were then centrifuged at 80g for 1 minute. 15µl supernatant was removed into an eppendorf as a control for antibody binding, and the remaining supernatant discarded. The beads were washed 4 times in 500µl lysis buffer, centrifuged at 80g for 1 minute and the supernatant discarded following each wash. The beads were then resuspended in 15µl PBS, 5µl sample buffer (Invitrogen NuPAGE™) and 2µl sample reducing agent (Invitrogen NuPAGE™). Samples were then heated to 70°C for 10 minutes and separated on a SDS-PAGE gel 200V for 1 hour and the proteins detected by western blotting (2.2.1.17.).

### 2.2.1.21. Flow Cytometry

Cells were plated out and treated/transfected as required. Cells were washed with 5ml PBS, 1ml trypsin was added and the plated incubated at 37°C for 15 minutes. The trypsin was inactivated with the addition if 4ml ice cold IFA buffer and the cells transferred to a 15ml falcon tube and centrifuged at 80g for 10 minutes. The supernatant was aspirated leaving 500µl to resuspend the pellet. On a vortex at low speed 4.5ml ice-cold 70% ethanol was added dropwise, keeping the samples on ice.

The samples were then incubated on ice for 30 minutes, before centrifugation at 80g 4°C. The cell pellet was then washed in 5ml PBS and spun again. The pellet was then resuspended in 500µl propidium iodide, stored at 4°C in the dark until required. To analyse the cell cycle profile samples were run using EXPO32ADCXL4.color program. To induce an early S-phase arrest and synchronise cell populations, 5µg/ml aphidicolin was added to the plates for 16 hours prior to release and harvest.

### 2.2.1.22. Chromatin Immuno-Precipitation (ChIP) Assay

Cells were plated out as described and transfected/treated as described. 36hrs post transfection cells were fixed using 190µl 37% formaldehyde added directly to the plates at a final concentration of 1%. The plates were incubated at 37°C for 10 minutes and a final concentration of 0.125M glycine added to stop the crosslinking and the plates incubated on ice. Cells were harvested by scraping into a 15ml falcon the cells were pelleted at 80g for 5 minutes. The resultant pellet was washed twice with 5ml PBS/0.5%NP40 at 100g, 4°C. The samples were then incubated on ice with 40ml high salt buffer (HSB; 0.5%NP40, PBS 1M NaCl) for 30 minutes. Following this incubation the cells were washed twice with PBS/0.5% NP40 with centrifugation at 200g 4°C. The pellet was then resuspended in 40ml low salt buffer (LSB; 0.1% NP40, 10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.1M NaCl) and incubated on ice for 30 minutes before being washed twice with PBS/0.5% NP40 with centrifugation at 200g 4°C. The pellet was resuspended in 1ml LSB and passes through a 26G needle. 300µl sarcosyl was added to the sample and the volume

competed to 3ml using LSB. The sample was then pipetted into a 50ml falcon tube containing 40ml LSB, 100mM sucrose and centrifuged at 700g 4°C for 10 minutes. The supernatant was discarded and the process repeated. The supernatant was discarded and the pellet resuspended in 2ml TE (10mM Tris pH 8.0, 1mM EDTA). Samples were then sonicated on ice using Biosonicator 12x on high 15 seconds on 15 seconds off. Following sonication 200µl 11X NET buffer (1.65M NaCl, 5.5mM EDTA, 5.5% NP40, 550mM Tris-HCl pH 7.4) was added to the sample and the sample split into two eppendorfs and centrifuged at 13000g for 5 minutes at 4°C. The resultant supernatants' were transferred to eppendorfs and 10% sample volume removed and stored at 4°C for the input DNA sample. One eppendorf was incubated with the desired antibody and the second incubated with a non-specific antibody as a control over night at 4°C with rotation. Following the antibody incubation 50µl dry bead volume protein G beads were added and the samples incubated with rotation for 1 hour at 4°C. The samples were then transferred to a chromatography column and washed twice with 10ml RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1mM EDTA pH 8.0), the washes drain by gravity flow. Following the RIPA wash the beads were washed twice with 10ml LiCl buffer, then twice with 10ml TE. The beads were removed from the column using 0.5ml TE and transferred to an eppendorf. The beads were centrifuged at 2000rpm for 30seconds and resuspended in 200µl TE, 1%SDS to elute the DNA from the beads. The beads were incubated with TE,1%SDS at room temperature for 10 minutes and centrifuged at 5000g for 1 minute. The resultant supernatant was transferred to an eppendorf and the beads eluted again using a further 200µl TE, 1%SDS as

described. This second elution was added to the first and the samples centrifuged at 5000g for 1 minute and the supernatant transferred to an eppendorf. 5µl 10mg/ml proteinase K was added to each sample and incubated at 42ºC overnight or 60 ºC for 4 hours to reverse the crosslinks. Samples were then phenol: chloroform purified twice as described (2.2.1.4.) and ethanol precipitated (2.2.1.5). The pure pellet was resuspended in 40µl filtered TE, and stored at 4°C. Immunoprecipitated DNA was quantified by PCR performed on the input and bound fractions, using primers 5'-GAGGACAACGGGGACAGTAA-3' and 5'-TCCACCAGAAAAACTCCAGC-3' for p73 promoter and 5'-GACAGGTACGGCTGTCATCA-3' and 5'-AACGGCAGACTTCTCCTCAG-3' for the β-actin control,); cycling parameters were 95 °C for 3 min, 25 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, and 72 °C for 5 min as described (2.2.1.12.).

### 2.2.1.23. RNA Extraction

RNA was extracted using Qiagen RNAeasy protocol according to the manufactures instructions. Briefly, cells were plated out as described and treated/transfected. To harvest the RNA the culture medium was removed and 350µl lysis buffer (RLT Qiagen) was added and the cells harvested by scraping. This sample was transferred to a QIAshredder column to homogenise the sample and centrifuged at 15000g for 2 minutes. 350µl 70% ethanol was then added and the sample including any precipitate that might have formed were transferred to an RNAeasy column and centrifuged for 15 seconds at 12000g. The flow through was discarded and the

column washed with 700µl wash buffer (RW1 Qiagen) with centrifugation at 8000g for 30 seconds. The flow through was discarded and the column washed with 500µl RPE (Qiagen) at 10,000rpm for 30 seconds. The column was then transferred to a new collection tube and 50µl RNAase free water was added directed onto the column membrane, and centrifuged at 15000g for 1 minute. The concentration of RNA was determined (2.2.1.2.) and the quality checked on a 1% agarose gel (2.2.1.1.).

### 2.2.1.24. HAT Assay Protocol

2 x$10^5$ 293T were plated out and transfected using a calcium phosphate precipitation protocol as described (2.2.2.2.). 36 hours following transfection the medium was removed and the cells harvested by incubating the cells with 1ml trypsin, at 37 °C for 10 minutes. 5ml culture medium was then added to inactivate the trypsin and the cells collected into a 15 ml centrifuge tube and centrifuged at 80g for 5 minutes. The cell pellets were washed in 1ml cold PBS and centrifuged for an additional 5 minutes at 80g. The pellets were then lysed in 100µl NP40 lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0 containing 1/10 volume protease inhibitor cocktail tablet) and incubated on ice for 30 minutes. Cell debris was pelleted by centrifugation at 15000g for 10 minutes. The supernatant was transferred to a new eppendorf and the concentration of protein determined using a BCA/$CuS0_4$ assay (as described 2.2.1.16.). 50µg of each sample was completed to 400µl with NP40 lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0 containing 1/10 volume

protease inhibitor cocktail tablet), and 1µl of the appropriate antibody was added. The samples were then incubated at 4°C with rotation for 1 hour. Following the incubation 10µl pre-swollen (5ml PBS overnight) protein sepharose-G beads were added and incubated for 1 hour at 4°C with rotation. The samples were then centrifuged at 80g for 1 minute and the pellet washed 5X with 500µl NP40 lysis (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0). After the final wash the pellet was resuspended in 40µl HAT buffer (Promega) and transferred to a 96 well plate. 50µl of HAT assay buffer was then added to each including a HAT assay buffer only sample as a negative control. 5µl substrate I and 5µl substrate II were added before the addition of 5µl enzyme mix to start the reaction. The plate was incubated at 37 °C and the absorbance at 450nm taken every 15 minutes until the enzyme reaction is complete.

## 2.2.2. Tissue Culture

### 2.2.2.1. General Growth

All cell culture experiments were carried out in a room specifically for the purpose and under strict sterile conditions. Cell culture work was performed inside a flow hood (Class II Microbiology Safety Cabinets, Gelaire BSB4). 293T, C33a, MCF7, MRC5 and U2OS cell lines were grown in DMEM 10% FCS, 1% Penicillin Streptomycin in an atmosphere at 37°C containing 5% CO2 (v/v) (Napco model 5410, Napco Scientific). Cell lines were split 1/10 twice a week in 35ml DMEM 10% FCS, 1% Penicillin Streptomycin.

### 2.2.2.2 Calcium Phosphate Transient Transfection

Cells were transiently transfected using a calcium phosphate precipitation technique. Approximately 3 x $10^5$ C33a/MCF7/MRC5 or 2 x $10^5$ 293T/U2OS cells per 60mm culture dish were plated out in 5ml DMEM (containing 10% foetal calf serum and penicillin). The following day, the 2µg of the DNA of interest was completed to 450µl with distilled water. 50µl of 2.5M $CaCl_2$ was then added forming an opaque precipitate in between 15 and 30 minutes. This 500µl solution was then added drop wise to 2 x HEPES Buffered saline (50mM HEPES, 280mM NaCl, and 1.5 mM $Na_2HPO_4.2H_2O$ to pH 7.02 with a few drops of 0.1M NaOH). The solution was left until the formation of the precipitate. As samples were carried in duplicate, 500µl of the precipitate was added to each duplicate monolayer, and left to incubate for 16-18 hours. The cell monolayer was then washed twice with 3ml PBS, and 5ml fresh medium was added before an additional 24-hour incubation.

### 2.2.2.3. Lipofectamine 2000™ Transfection

For siRNA knockdown experiments, siRNA oligos against TopBP1 and luciferase were designed (Dharmacon). Two transfections solutions were prepared; firstly 2.5µl of the desired oligo was added to 497.5µl optimem (Invitrogen), and secondly 15µl lipofectamine 2000™ was added to 485µl optimem. These two separate cocktails were incubated at room temperature for 5 minutes before being mixed and

incubated for a further 20 minutes at room temperature. Following the incubation 0.5 x $10^6$ MCF7 cells were added to the lipofectamine/oligo solution and plated out onto a 60mm tissue culture plate incubated at 37°C 5% $CO_2$ (v/v).

### 2.2.2.4. Luciferase assay

Cells were transfected using the calcium phosphate precipitation protocol as described (2.2.2.2.). The cells were removed from the incubation and the medium removed. The tissue culture dishes were washed twice with 3ml PBS removing all excess PBS from the second wash by pipetting. 300µl of 1 x Reporter Lysis Buffer (Promega) was added to each plate and left to incubate at room temperature for 10 minutes. The resultant cell lysate was scraped using a sterile plate scraper into a clean microcentrifuge tube. These were centrifuged at 13000g for 10 minutes at 4°C. The supernatant was then pipetted into fresh microcentrifuge tube, and stored at –70°C until required. To assay luciferase activity 80µl of each sample was loaded into a 96 well plate and the luminescence measured using the Luciferase Assay System (Promega) and a Luminoskan Ascent plate reader (Thermo Labsystems).

### 2.2.2.5. Colony Survival Assay

3 x $10^5$ MCF7 cells per 100mm culture dish were plated out in 7ml DMEM (containing 10% foetal calf serum and penicillin), and siRNA treated as described (2.2.2.3). 48 hours later the cells were trypsinised, counted and 2000 cells per

100mm culture dish were plated out in 7ml DMEM (containing 10% foetal calf serum and penicillin). 12 days later the medium was removed and the cells washed twice with 5ml PBS. The surviving colonies were visualised by staining with 2mls 0.5% crystal violet solution (25% methanol, $ddH_2O$) for 20 minutes at room temperature with gentle shaking. The plates were washed with tap water until the solution ran clear, dried overnight at room temperature before the colonies were counted.

### 2.2.2.6. MTT Cell Viability Assay

The MTT assay provides an additional method of measuring cell viability. Cell lines to be assayed were plated out and transfected using Lipofectamine 2000™ as described (2.2.2.3.). 36 hours post transfection, the cells were harvested by trypsinisation and 500 cells plated out into each well of a 96 well plate (DMEM 10% FCS, 1% Penicillin Streptomycin). At the time points indicated, the medium was removed and 100µl 10% MTT solution (1-(4, 5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan) was added to the cells and the plate incubated at 37°C for 2 hours until the formation of a precipitate. The media was then removed and the precipitate resuspended in 100µl DMSO. The absorbance (560nm) of each well; samples and negative control (MTT only) were measured.

### 2.2.2.7. Preparation of protein extracts

For the detection of proteins using western blotting 2 x $10^5$ 293T/U2OS or 3 x $10^5$ C33a/MCF7/MRC5 cells were plated out on 10cm tissue culture plates in 7ml DMEM 10% FCS, 1% Penicillin Streptomycin. Cells were transfected using the calcium phosphate precipitate as described (2.2.2.2.). 36 hours post transfection; 1ml trypsin was added to each plate and incubated at 37°C for 10 minutes. The cells were then transferred to a 15ml centrifuge tube and spun at 80g for 5 minutes. The resultant pellet was washed in 5ml PBS and the cell pellet was lysed in 300µl NP40 lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0 containing 1/10 volume protease inhibitor cocktail tablet), and incubated on ice for 30 minutes. The lysate was then centrifuged at 15000g and the resultant supernatant containing the cell proteins was transferred to a clean microcentrifuge tube and stored at -80°C until required, the pellet was discarded.

### 2.2.2.8. Inhibitors and Damaging Agents

Cells were treated with varying doses (Gy = 1Gy corresponds to the absorption of one joule of energy by one kilogram of matter) of gamma irradiation from a $Co^{60}$ source at the Beatson Institute for Cancer Research (Switchback Road, Glasgow) 48 hours after plating out. Cells were transported and damaged 4 hours prior to harvesting for luciferase transcription assays. Control plates which were not treated were also transported as a control. The PIKK inhibitors, caffeine (2mM)

wortmannin (100nM) and LY294002 (100μM) were added to cell culture medium one hour prior to gamma/mock irradiation at the concentrations described.

## 2.2.3. Yeast Protocols

### 2.2.3.1. Synthetic Dropout media (SD) Preparation

0.85g Yeast Nitrogen Base, 2.5g Ammonium sulphate and 10g Agar were dissolved in 350ml $ddH_2O$ and autoclaved. Following sterilisation 25ml 40% glucose and 5ml of each 100x stock amino acid required for the media was added (Table 7). The volume was competed to 500ml with $ddH_2O$ and the plates were poured and stored at 4°C until required. For SC-trp plates adenine, histidine and leucine were added. For SC-trp-his/ade adenine and leucine, or adenine and histidine were added respectively. For SC-trp-his-ade media only leucine was added.

**Table 7: Amino acid stocks**

| Amino Acid | 100x stock | Manufacturer |
|---|---|---|
| L-adenine hemisulphate salt | 2g in 1000ml | Sigma |
| L-histidine HCL monohydrate | 2g in 1000ml | Sigma |
| L-leucine | 10g in 1000ml | Calbiochem |
| L-tryptophan | 2g in 1000ml | Calbiochem |

### 2.2.3.2. TCA preparation of cell lysates

Yeast cultures were grown until an $OD_{600nm}$ was reached, and the cells were then pelleted and resuspended in 1ml 0.3M NaOH and incubated at room temperature for 10 minutes with occasional agitation. The cell lysate was then incubated on ice for 10 minutes before the addition of 150µl TCA (w/w) and a further incubation of 10 minutes on ice. The samples were then centrifuged at 15000g for 10 minutes at 4°C, the supernatant removed and the pellet centrifuged again for a further 10 minutes under the same conditions. 15µl sample reducing buffer (Invitrogen) was added to resuspend the pellet and the sample heated to 100°C before a brief spin to pellet any debris. The proteins were then separated by SDS-PAGE separation and transferred (as described 2.2.1.17.) and probed for the presence of GAL4 fusion proteins.

### 2.2.3.3. Liquid β-GAL Assay

Overnight yeast cultures were centrifuged for 3 minutes at 15000g and the pellet washed in 1.5ml Z Buffer (60mM $Na_2HPO_4$, 40mM $Na_2H_2PO_4$, 10mM KCl, 1mM $MgSO_4$ pH7.0) and centrifuged again under the same conditions. The resulting pellet was resuspended in 300µl Z Buffer (as above) and freeze thawed in liquid nitrogen for 15 seconds. 700µl Z buffer containing 50mM β-mercaptoethanol, and 160µl O-Nitro-D-Galactoside (10mg/ml) was added and the timing started. Absorbance readings at 420nm and 550nm were taken at 5 minute intervals and the reaction terminated by the addition of 400µl $Na_2CO_3$. The units used in the assay are as follows:

$$\text{Units} = \frac{1000 \times [(OD_{420}) - 1.75 \times (OD_{550})]}{\text{Time (Hours)} \times \text{Volume Culture} \times (OD_{600})}$$

### 2.2.3.4. Transformation of Yeast by Heat Shock

40µl AH109 were mixed with 1µg plasmid DNA before the addition of 300µl LiPEG (100mM Lithium acetate, 10mM Tris pH 8.0, 1mM EDTA and 40% PEG 3350). The cells were vortexed and incubated at room temperature for 20 minutes. Following the incubation 35µl DMSO was added and the sample incubated at 42°C for 15 minutes. The cells were then centrifuged for 3 minutes at 80g and the

resulting supernatant discarded. The pellet was resuspended in 75µl $H_20$ and the cells plated out onto kanamycin resistant agar plates and incubated at 37°C overnight. AH109 contained the following;

**AH109** MATa, trp1-190, leu2-3,112, ura3-52, his3-200, galΔ, gal80Δ, LYS2; GAL1UAS_GAL1TATA_HIS3, GAL2UAS_GAL2TATA_ADE2, URA3:MEL1UAS_MEL1TATA_LacZ

### 2.2.3.5. Making Electrocompetent Yeast

20ml L broth (2.1.2.) was inoculated with a single BL21 colony and grown overnight at 37°C 225rpm. From this culture 8mls was used to inoculate 400ml L broth (2.1.2.) which was grown until and $OD_{600}$ of 0.6 was reached. The bacteria were then pelleted with centrifugation at 2000g for 15minutes, washed twice with 500ml ice cold water and centrifuged again under the same conditions. The pellet was then resuspended in 20ml filter sterilised ice cold glycerol and centrifuged at 2000g for 10 minutes 4°C. The pellet was then resuspended in 2ml filter sterilised glycerol and stored at -80°C until required.

### 2.2.3.6. Testing for Transactivation by Replica plating

A single colony from each construct was picked and inoculated into 500µl $ddH_2O$. This solution was used to spot the colony onto SC-trp plates with 100 grid squares,

one colony per square, and incubated at 30°C for 3 days. This plate was used as a master plate to replica plate onto plates lacking tryptophan and adenine (SC-trp-ade), tryptophan and histidine (SC-trp-his) and plates lacking tryptophan, adenine and histidine (SC-trp-his-ade). Colonies which grew on this triple dropout media indicated that the TopBP1 constructs were capable of transactivating the transcription of the reporter genes.

## 2.2.4 Microscopy

### 2.2.4.1. Plating cells and Fixation

For immunofluorescence MCF7 cells were plated out at 2 x $10^5$ cells per 60mm plate onto glass 15mm diameter coverslips. 72 hours later the medium was removed and the cells fixed in 4% formaldehyde at 37°C for 10 minutes. The cells were then washed three times with 5ml PBS and the excess removed. The coverslips were then permeablised using 1ml ice cold methanol per 60mm plate and incubated for 10 minutes on ice. The coverslips were then washed with 5ml of PBS three times and stored in 1ml PBS at 4°C until staining (2.2.4.2.).

### 2.2.4.2. Staining coverslips

Coverslips were fixed (2.2.4.1.) and stained for the presence of γH2AX foci. The fixed coverslips were incubated with 20µl, 1/250 dilution of monoclonal γH2AX antibody in PBS for 1 hour at room temperature. The coverslips were then washed five times with 1ml PBS before a further incubation with 20µl secondary anti-mouse FITC conjugate (1/400 dilution in PBS) for 1 hour at room temperature in the dark. Following this incubation the coverslips were washed five times with 1ml PBS and the excess removed. The coverslips were placed cell surface down onto a glass microscope slide and immunofluorescence detected using Leica confocal software.

## 2.2.5 Statistics

### 2.2.5.1 Mean and Standard Error

The mean ± the standard error of the mean (SEM) are shown in several experiments as a control for experimental reproducibility. The standard error of the mean (SEM) gives a measure of the precision of the mean giving a representation of the spread of the data, indicating how far the mean of sample is from the true mean.

$$SEM = \frac{\text{Standard Deviation } (\sigma)}{\sqrt{[\text{Sample number } (n)]}}$$

Calculated using Microsoft Excel using =STDEV (X-Y)/SQRT (COUNT(X,-Y))

### 2.2.5.1 Statistical Significance

Statistical significance for the data presented in this thesis was typically calculated using a Students-T-test to give a significance value (p). This was carried out using Microsoft Excel (T-TEST function). The cut off for all experiments was taken as 0.05, therefore if two sets of data return a p value of <0.05 there is a less than 5% chance that the result observed is due to chance, and the two groups are therefore statistically significantly different.

# 3- Results

## 3.1. TopBP1 Contains Multiple Transcriptional Modification Domains

TopBP1 plays an essential role in signalling the DNA damage response and repair and has been proposed as a transcriptional regulator, both activating and repressing transcription. TopBP1 co-activates transcription with the HPV16 E2 protein when E2 is bound to target promoters (Boner *et al.* 2002) and represses the transcriptional activity of E2F1 (Liu *et al.* 2003). Following UV damage cell cycle arrest is induced by the inhibition of Cdk via the up-regulation of p21 by Miz1, TopBP1 has been shown to bind Miz1 and regulate p21 transactivation (Herold *et al.* 2002). These regions which modify chromatin in order to facilitate these functions of TopBP1 have

not been identified therefore this chapter investigated if TopBP1 contains any transcriptional regulatory domains.

### 3.1.1. Identification of a Transcriptional Activation Domain

In order to investigate if TopBP1 contains transcriptional regulatory domains, TopBP1 sequences were cloned into the GAL4 DBD (DNA binding domain) expression vector pBIND (Figure 7a). The ability of these GAL4-TopBP1 fusion proteins to regulate transcription from the luciferase reporter pG5luc, which contains five GAL4 binding sites, was determined (2.2.2.4.) and the results are shown in Figure 7b. This experiment was carried out in 293T cells, and the luciferase activity was normalised against total protein present in each sample using a BCA/$CuSO_4$ assay (2.2.1.16.). All luciferase assays described were carried out at least three times in duplicate, and the results show the mean ± S.E.M. (Standard Error of Mean, 2.2.5.1.). The plasmids encoding all the GAL4-TopBP1 plasmids were sequenced and the correct protein expression was determined by transfecting the plasmids into 293T cells and preparing total protein extracts (2.2.2.7.). Figure 7a lists the predicted mass of each protein in kDa (Right hand side). Equal amounts of each lysate were separated by SDS/PAGE (2.2.1.17.) and the proteins detected using an anit-GAL4 DBD antibody (2.1.1.).

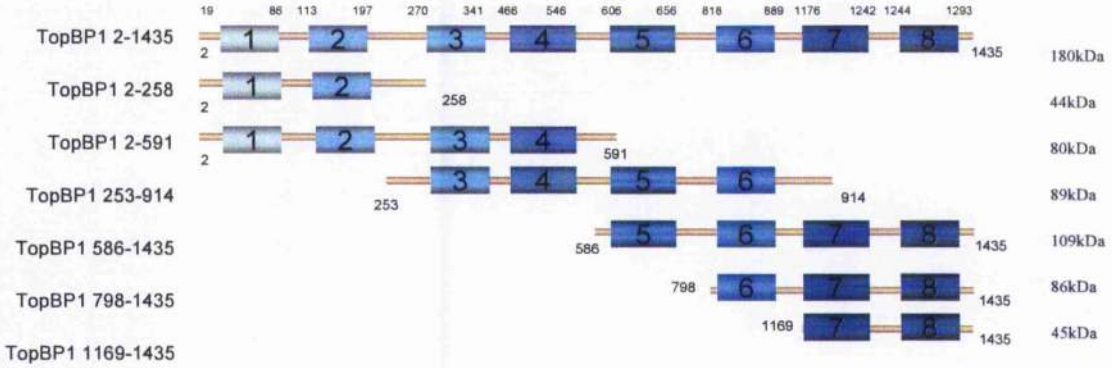
As shown in Figure 8 all TopBP1 fusion proteins are expressed, although some more strongly than others. This was repeated at least twice for each construct and similar

results were always obtained. The GAL4 fusion protein, TopBP1 amino acids 586-1435 shows a significantly reduced level of expression and a reduction in transcriptional activity compared to the other constructs although this will be discussed later (3.1.3.). It is clear from Figure 7b there are transcriptional repression domains encoded by TopBP1, when normalised against the empty vector pBIND (Equivalent to 1). TopBP1 2-591 shows significantly higher activity compared to TopBP1 2-258 suggesting there may be a domain between amino acids 258-591 containing transcriptional activation properties.

To investigate whether TopBP1 amino acids 258-591 contains activation domain additional GAL4-TopBP1 fusion expression plasmids were constructed (Figure 9a). TopBP1 253-591 shows a 50 fold increase in transcription compared to the empty vector pBIND (Figure 9b), suggesting that a transcriptional activation domain is contained between amino acids 253-591. Similar to the results which identified the BRCA1 transactivation domain (Monteiro *et al.* 1996), the full length fusion shows no increase in transcription, the activation domain is only revealed using protein truncations. This region of TopBP1 contains both the third and fourth BRCT domains, therefore these domains were deleted from the activator construct to create, TopBP1 253-591 ΔBRCT3 and TopBP1 253-591ΔBRCT4. The removal of BRCT3 has no significant effect on the transcriptional activation ($p = 0.96$), although the removal of BRCT4 from TopBP1 253-591 significantly alleviates 24% of the activity ($P=0.03$), indicating that the activation domain lies partially within BRCT4. The expression of these GAL4-TopBP1 fusion proteins is shown in Figure 14b.

**Figure 7**

a)


19 86 113 197 270 341 466 546 606 656 816 889 1176 1242 1244 1293
TopBP1 2-1435
2
1435
180kDa
TopBP1 2-258
258
44kDa
TopBP1 2-591
591
80kDa
TopBP1 253-914
253
914
89kDa
TopBP1 586-1435
586
109kDa
TopBP1 798-1435
798
86kDa
TopBP1 1169-1435
1169
45kDa

b)

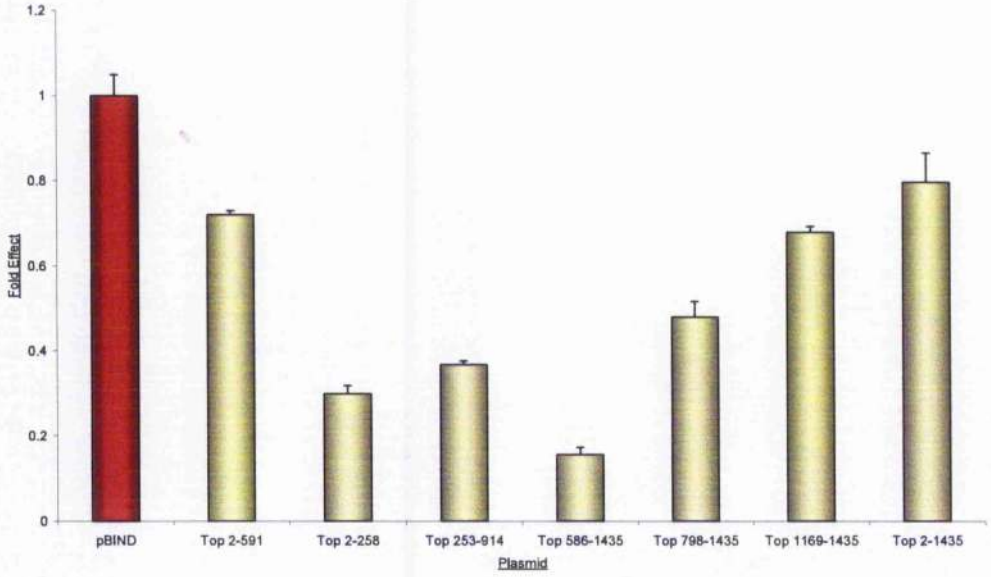

Fold Effect
1.2
1
0.8
0.6
0.4
0.2
0
pBIND
Top 2-591
Top 2-258
Top 253-914
Top 586-1435
Top 798-1435
Top 1169-1435
Top 2-1435
Plasmid

**Figure 7: Transcriptional Analysis of GAL4-TopBP1 fusion proteins.** a) Schematic representation of the GAL4-TopBP1 fusion proteins, designed for the initial study of transcriptional activation and repression. Shaded boxes correspond to the location of BRCT domains. Numbers correspond to amino acid number of TopBP1. Amino acid numbers in fusion name indicates the first and last amino acid of TopBP1 contained in each construct. The predicted size of these proteins in kDa is shown on the right of each construct. b) Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the constructs indicated in a). All luciferase assays were carried out in duplicate at least three times; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 8**

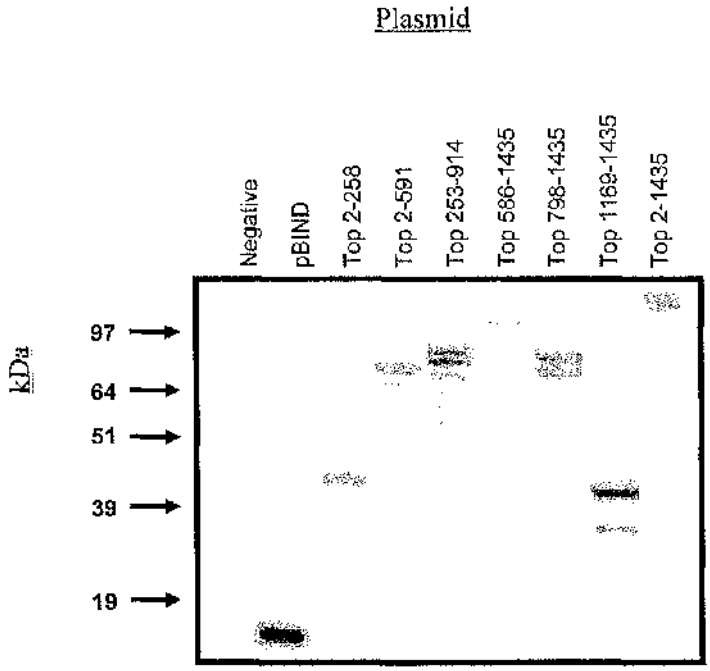
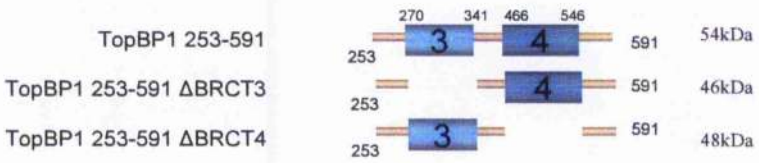



**Figure 8: Protein Expression of GAL4-TopBP1 Fusions.** Western blot showing the expression of the GAL4-TopBP1 fusion proteins indicated in Figure 5a) in 293T cells using anti-GAL4 DBD antibody. Cell lysates were harvested as described (2.2.2.7.). The location of the molecular weight marker (kDa) is shown on the left of the blot.

**Figure 9**

a)


TopBP1 253-591
TopBP1 253-591 ΔBRCT3
TopBP1 253-591 ΔBRCT4
270
341
466
546
3
4
253
591
54kDa
46kDa
48kDa

b)

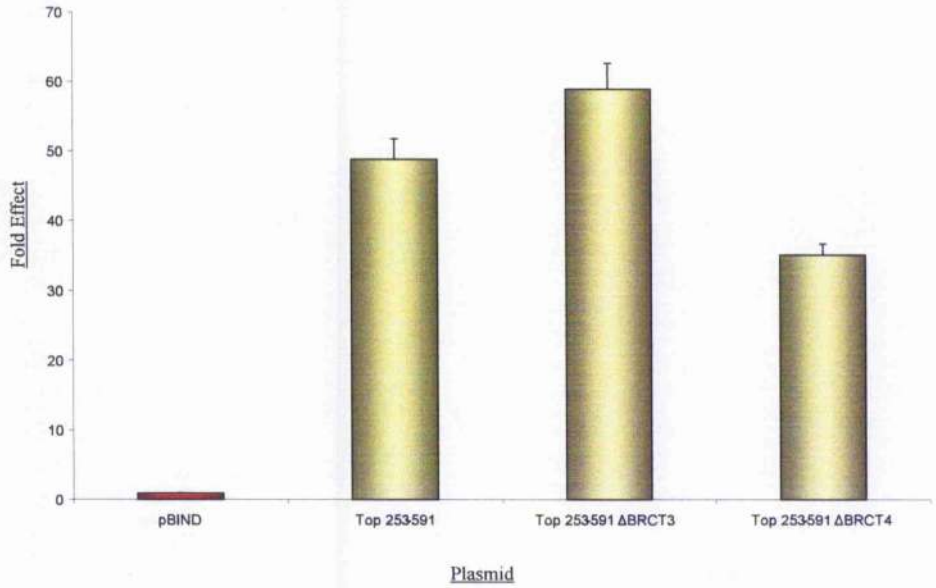
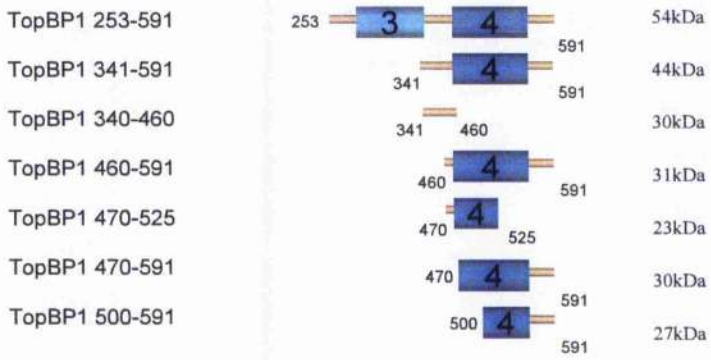

Fold Effect
70
60
50
40
30
20
10
0
pBIND
Top 253-591
Top 253-591 ΔBRCT3
Top 253-591 ΔBRCT4
Plasmid

**Figure 9: Identification of a Transcriptional Activation Domain.** a) Schematic representation of the GAL4-TopBP1 fusion proteins. Numbers correspond to amino acid number of TopBP1. The predicted size of these proteins (kDa) is shown on the right of each construct. b) Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 amino acid 253-591, ΔBRCT3 and ΔBRCT4 fusion proteins ±S.E.M.

In order to further characterise the activation domain within TopBP1 amino acids 253-591, a further series of truncations were cloned into pBIND (Figure 10a). The BRCT domains present in each of these constructs is indicated, and the predicted size of these proteins is listed on the right of the diagram. The ability of these fusion proteins to activate transcription from the luciferase reporter pG5luc was assayed as previously described and the mean ± S.E.M. of these fusion proteins is shown in Figure 10b. TopBP1 253-591, 341-591 and 460-591 show a strong increase in transcription, compared to the empty vector pBIND. In contrast TopBP1 470-525, TopBP1 340-460, and TopBP1 500-591 show no increase in transcriptional activation compared to the empty vector indicating that an essential region of the activation domain lies within TopBP1 amino acids 460-500. Figure 11 shows a multiple sequence alignment using the ClustalW™ program (European Bioinformatics Institute (EBI) an outstation freely available from European Molecular Biology Laboratory (EMBL)) of the human TopBP1 sequence from amino acids 460-500, comparing the sequences from a variety of species as indicated. * indicates identical residues conserved across species, : indicates residues in which the charge of the amino acid is conserved, the hydrophobic residues which are conserved are indicated by •. Similar to other transcriptional activation domains, this activation domain in TopBP1 contains hydrophobic pockets interspersed with acidic residues, (Roberts *et al.* 1993).

**Figure 10**

a)


TopBP1 253-591
TopBP1 341-591
TopBP1 340-460
TopBP1 460-591
TopBP1 470-525
TopBP1 470-591
TopBP1 500-591
253
3
4
591
341
4
591
341
460
460
4
591
470
4
525
470
4
591
500
4
591
54kDa
44kDa
30kDa
31kDa
23kDa
30kDa
27kDa

b)

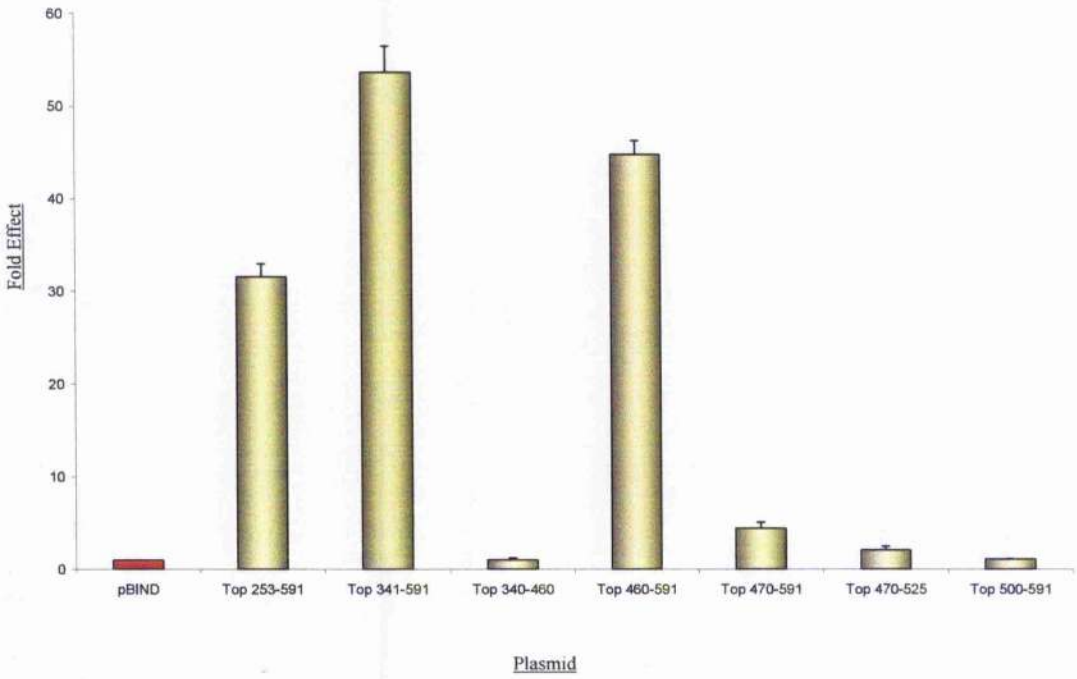
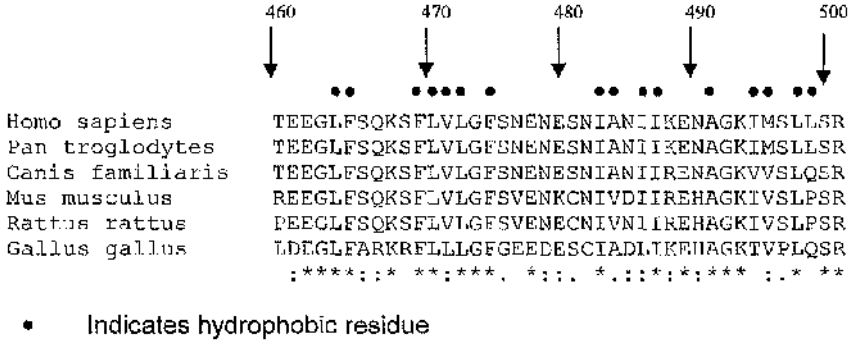

Fold Effect
60
50
40
30
20
10
0
pBIND
Top 253-591
Top 341-591
Top 340-460
Top 460-591
Top 470-591
Top 470-525
Top 500-591
Plasmid

**Figure 10: The Transcriptional Activation Domain resides partially within BRCT4.** a) Schematic representation of the GAL4-TopBP1 fusion proteins. Numbers correspond to amino acid number of TopBP1; the shaded boxes indicate the regions containing BRCT domains. The predicted size of these proteins in kDa is shown on the right of each construct. b) Luciferase assay showing the mean fold effect against the empty vector pBIND (being equivalent to 1) of the constructs shown in a) ±S.E.M.

**Figure 11**




• Indicates hydrophobic residue

**Figure 11: Sequence similarity across the Activation Domain is conserved across species.** ClustalW™ sequence alignment of TopBP1 amino acid residues spanning the activation domain, • indicates a hydrophobic residue which is conserved.* indicates a residue which is conserved across species,: indicates a residue in which the charge of that residue is conserved across species.

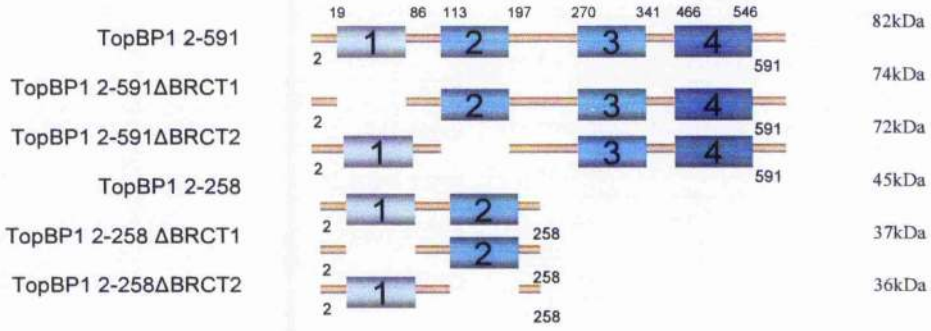
### 3.1.2 A Repression Domain within BRCT2 Represses the Activation Domain

The GAL4-TopBP1 fusion TopBP1 2-591 contains the activation domain identified within amino acids 460-500 (3.1.1) but it shows no increase in transcriptional activity compared to the empty vector pBIND (Figure 7b). This indicated that there was possibly a repressor element within amino acids 2-258 which was acting upon the activation domain to repress its activity within this construct. To investigate this hypothesis a further a series of TopBP1 constructs containing BRCT domain deletions were cloned into pBIND (Figure 12a), and the ability of these fusion proteins to activate transcription from the pG5luc luciferase reporter was assayed as previously described, the results are shown in Figure 12b. The removal of first and second BRCT domains from TopBP1 2-591 does not restore the transcriptional activity seen by TopBP1 253-591 (Figure 9b). However this repressor domain was revealed using further truncations; Figure 13a shows the regions covered by these fusion proteins. TopBP1 181-591 shows a significant increase in fold activation compared to pBIND a level which is similar to that seen by TopBP1 253-591; however TopBP1 113-591 shows no increase in transcription indicating that the repressor domain lies within amino acids 113-181, this region between amino acids 113 and 181 maps directly to BRCT2 of TopBP1, indicating that BRCT2 is the region which represses the activation domain.

However; if this was the only repressor element acting upon the activation domain within amino acids 460-470, TopBP1 2-591 Δ BRCT2 should show an increase in transcriptional activity as it contains the activation domain but not the BRCT2 repressor domain, however this is not the result observed. TopBP1 2-591 Δ BRCT2 does not show an increase in transcription (Figure 12b). One possibility is that there is an additional repressor element within amino acids 2-113 incorporating the first BRCT domain, which is also repressing the activity of the activation domain within the TopBP1 2-591 Δ BRCT2 fusion, but not the ability of TopBP1 181-591 to activate transcription. Figure 14 shows the expression of the GAL4-TopBP1 fusion proteins described in Figures 10, 12 and 13 using the anti-GAL4 DBD antibody. The expression of each fusion protein was confirmed in repeat experiments.

**Figure 12**

a)


TopBP1 2-591
TopBP1 2-591ΔBRCT1
TopBP1 2-591ΔBRCT2
TopBP1 2-258
TopBP1 2-258 ΔBRCT1
TopBP1 2-258ΔBRCT2
19
86
113
197
270
341
466
546
1
2
3
4
2
591
258
82kDa
74kDa
72kDa
45kDa
37kDa
36kDa

b)

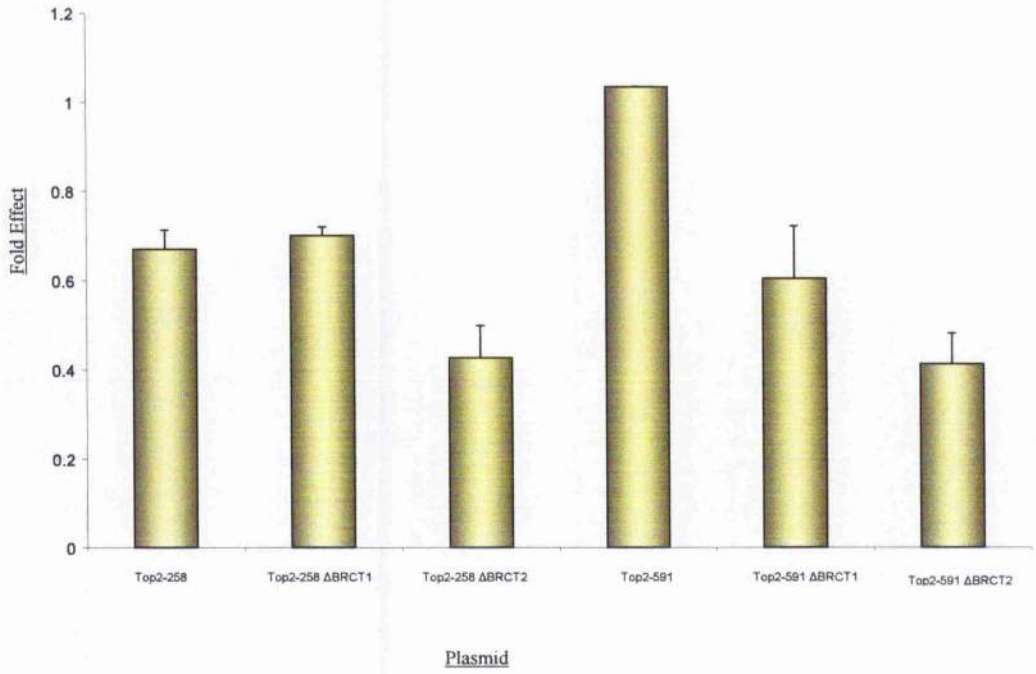

Fold Effect
1.2
1
0.8
0.6
0.4
0.2
0
Top2-258
Top2-258 ΔBRCT1
Top2-258 ΔBRCT2
Top2-591
Top2-591 ΔBRCT1
Top2-591 ΔBRCT2
Plasmid

**Figure 12: The Activation Domain is Repressed by an adjacent Repressor Domain.** a) Schematic representation of the GAL4-TopBP1 fusion proteins, numbers correspond to amino acid number, shaded boxes indicate the regions covered by BRCT domains. The predicted size of these fusion proteins is indicated on the right of the diagram. b) Luciferase assay showing the mean fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 BRCT deletion fusion proteins indicated in a) ± S.E.M.

**Figure 13**

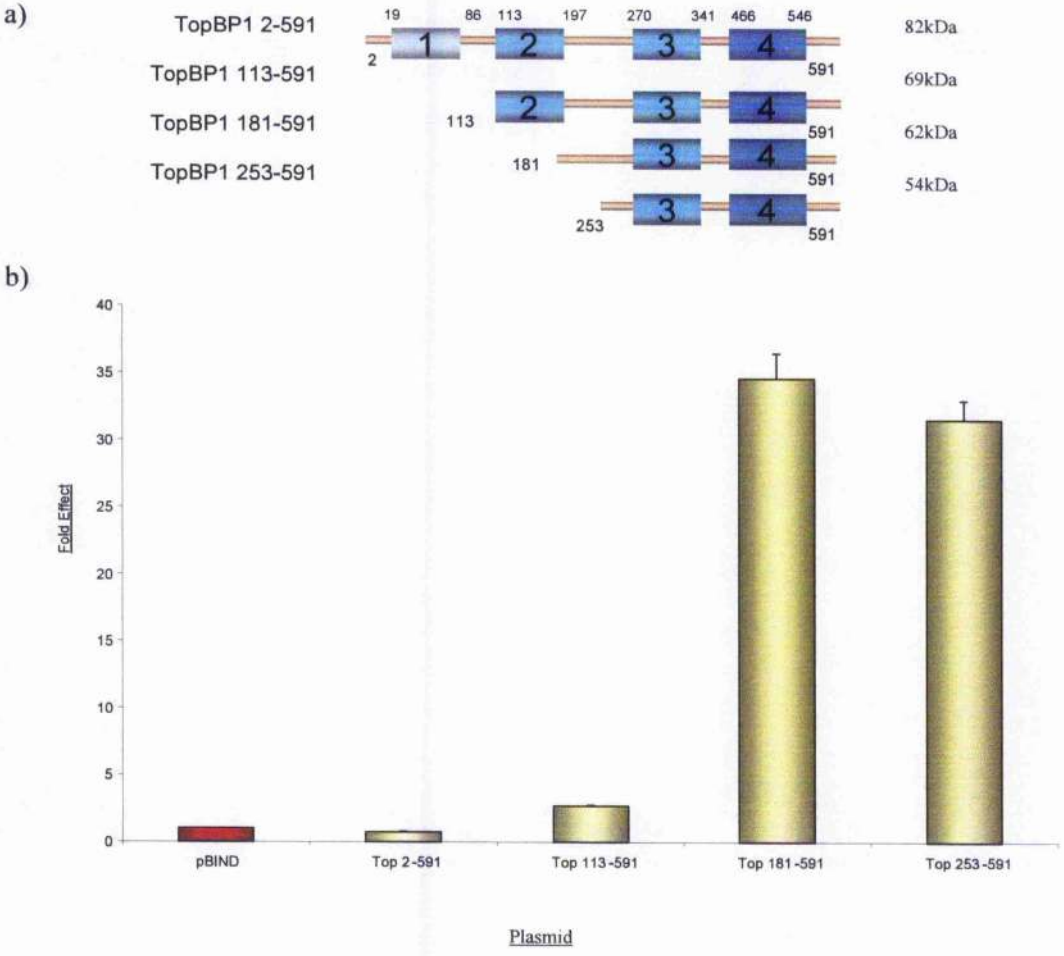

a)
TopBP1 2-591
TopBP1 113-591
TopBP1 181-591
TopBP1 253-591
19
86
113
197
270
341
466
546
2
591
113
591
181
591
253
591
82kDa
69kDa
62kDa
54kDa
b)
Fold Effect
40
35
30
25
20
15
10
5
0
pBIND
Top 2-591
Top 113-591
Top 181-591
Top 253-591
Plasmid

**Figure 13: The Repressor Domain maps directly to BRCT2.** a) Schematic representation of the GAL4-TopBP1 fusion proteins, shaded boxes indicate the regions containing BRCT domains, numbers correspond to amino acid number. The predicted size of these fusion proteins in kDa is indicated on the right of the diagram. b) Luciferase assay showing the mean fold effect change in transcription of the GAL4-TopBP1 fusion proteins shown in a) against the empty vector pBIND (being equivalent to 1) ± S.E.M.

**Figure 14**

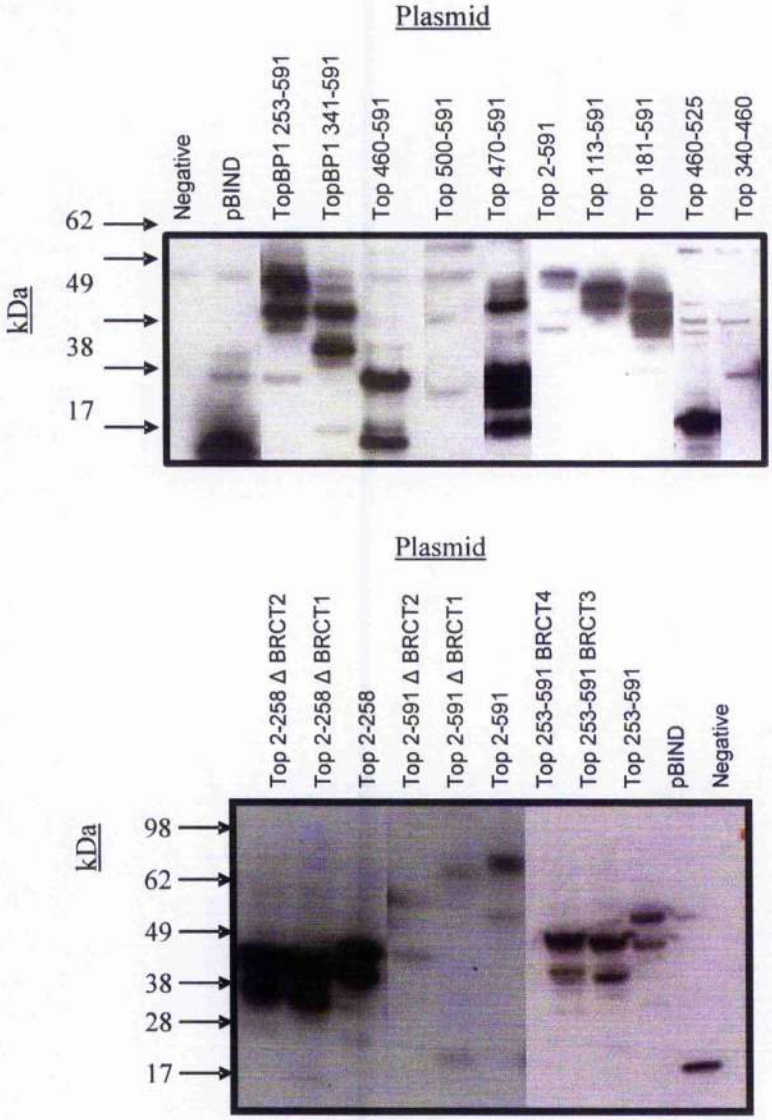

Plasmid
kDa
62
49
38
17
Negative
pBIND
TopBP1 253-591
TopBP1 341-591
Top 460-591
Top 500-591
Top 470-591
Top 2-591
Top 113-591
Top 181-591
Top 460-525
Top 340-460
Plasmid
kDa
98
62
49
38
28
17
Top 2-258 Δ BRCT2
Top 2-258 Δ BRCT1
Top 2-258
Top 2-591 Δ BRCT2
Top 2-591 Δ BRCT1
Top 2-591
Top 253-591 BRCT4
Top 253-591 BRCT3
Top 253-591
pBIND
Negative

**Figure 14: Expression of GAL4-TopBP1 fusion proteins**. Western blot showing the expression of the GAL4-TopBP1 fusion proteins indicated in Figures 10, 12, and 13). The plasmids were transfected into 293T cells and the protein lysates harvested as described (2.2.2.7.). Equal amounts of each lysates were then separated by SDS-PAGE and the fusion proteins detected using anti-GAL4 DBD antibody (2.2.1.17.). The location of the molecular weight marker (kDa) is shown on the left of each blot.

The repression of the activation domain by BRCT2 could be due to either an intramolecular interaction, in which BRCT2 physically masks the activation domain supressing its activity, or the repression could be due to the recruitment of a repressor complex to the site of the activator. To study which of these two possibilities is more likely, TopBP1 2-258 which contains the supressor was cloned in frame with the transcriptional activator VP16 as a fusion protein with the GAL4 DBD (VP16Rep), as a control the VP16 activation domain was cloned into GAL4 DBD (VP16 Activation Domain [VP16AD]). A diagrammatic representation of these fusion proteins is shown in Figure 15a. The ability of these GAL4-TopBP1 fusions to acitvate transcription from the pG5luc luciferase reporter was tested, and the results are shown in Figure 15b. As expected VP16 AD showed a strong 150 fold increase in transcription compared to GAL4 (pBIND) being equivalent to 1. VP16Rep showed a 6.5 fold increase in transcription compared to pBIND and was able to supress over 90% of the transcriptional activation of the VP16 AD ($p = 0.0045$) indicating that the repressor domain can suppress the ability of the adacent VP16 to activate transcription. This result indicates that the mechanism in which the BRCT2 repressor domain represses the activation domain of TopBP1 (amino acids 460-500) is likey due to the recruitment of a repressor complex and not by an intramocelular interaction as this repressor domain within TopBP1 can also repress the transcriptional activity of the VP16 transcriptional activation domain. The expression of the fusion proteins (Figure 15a) is shown in Figure 16.

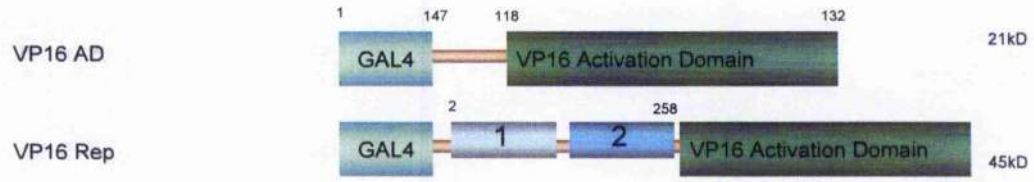
To more fully understand the mechanism of repression a luciferase transcription assay was carried out in which the cells were treated with trichostatin A (TSA) three hours prior to harvesting. TSA is a histone deactyltransferase (HDAC) inhibitor; these inhibtors cause a build up of acetylated histones, and result in an open chromatin state which is associated with transcriptional activation (Figure 17a) (Review Ekwall *et al.* 2005). Figure 17b shows the fold change in transcription from the pG5luc reporter of the GAL4-TopBP1 fusion proteins compared to the empty vector pBIND. In this experiment TopBP1 2-591 contains both the activation domain (amino acids 460-470) and the repression domain (amino acids 113-181), TopBP1 2-258 contains only the repression domain and TopBP1 253-591 only the activation domain. When treated with TSA pBIND shows a significant ($p=0.023$) increase in transcription compared to untreated cells, indicating that the GAL4 DNA binding domain has an activation domain kept silent by adacent repressor elements under the control of HDACs as they are revealed with TSA treatment. TSA did not alleviate the repression of the activation domain indicating that repression is HDAC independent.

It is possible that the activity of the activation domain may function in a histone acetyltransferase dependent (HAT) manner. Although chromatin activation and silencing will be discussed in more detail in chapter 3.2, the results presented here aim to ascertain if the TopBP1 activation/repression domains identified contain any HDAC or HAT activity. HAT enzymes acetylate the core histones and are characterised according to the histone acetylated (Kimura *et al.* 2005). This acetylation of the core histones results in the formation of a more "open" chromatin

structure providing access for other chromatin remodelling proteins, DNA damage proteins and transcriptional machinary. Recent evidence has shown that HAT enzymes also acetylate a larger number of substrates not just core histones (Yang *et al.* 2007). Therefore the GAL4-TopBP1 repressor and activator fusions were tested for HAT activity (2.2.1.24.) in 293T cells. In this assay the 293T cells were transfected with the GAL4-TopBP1 expression plasmids indicated in Figure 18, total cell lysates were prepared and immunoprecipitated (as described 2.2.2.7 and 2.2.1.20) using the anti-GAL4 antibody and the immunoprecipitate was used in this HAT assay. This assay indirectly measures the de-acetylation of acetyl-Coenzyme A to produce free CoA. The results are shown in Figure 18, active recombinant HAT serves as a positive control, and following a 60 minute incubation with the acetyl-CoA substrate shows a clear increase in HAT activity. However GAL4 alone (pBIND), TopBP1 253-591 and TopBP1 2-591 show no increase in HAT activity with the addition of the substrate although these constructs do have a higher basal activity than the negative control, indicating that the transcriptional activation and repression is HAT independent.

**Figure 15**

a)


VP16 AD
1
147
118
132
GAL4
VP16 Activation Domain
21kD
VP16 Rep
2
258
GAL4
1
2
VP16 Activation Domain
45kD

b)

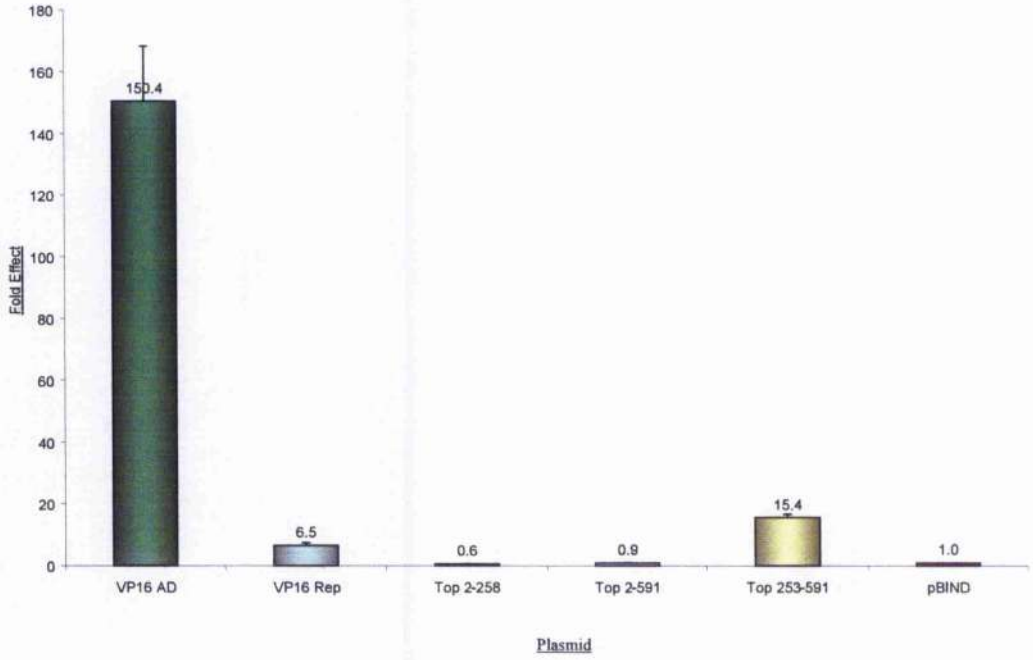

180
160
140
120
100
80
60
40
20
0
Fold Effect
150.4
6.5
0.6
0.9
15.4
1.0
VP16 AD
VP16 Rep
Top 2-258
Top 2-591
Top 253-591
pBIND
Plasmid

**Figure 15: Transcriptional Repression by BRCT2 is likely due to the recruitment of a Repressor complex.** a) Schematic representation of the GAL4-VP16 fusion (VP16 AD), and GAL4-VP16-TopBP1 2-258 (VP16Rep) fusion proteins. Numbers correspond to the start and end amino acid of each segment of the fusion, the predicted size of these proteins in kDa is shown on the right of the diagram. Shaded boxes in VP16Rep indicate the location of BRCT domains within the TopBP1 sequence. b) Luciferase assay showing the mean fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the constructs indicated in a), +/- S.E.M. This experiment was repeated three times in duplicate.

**Figure 16**

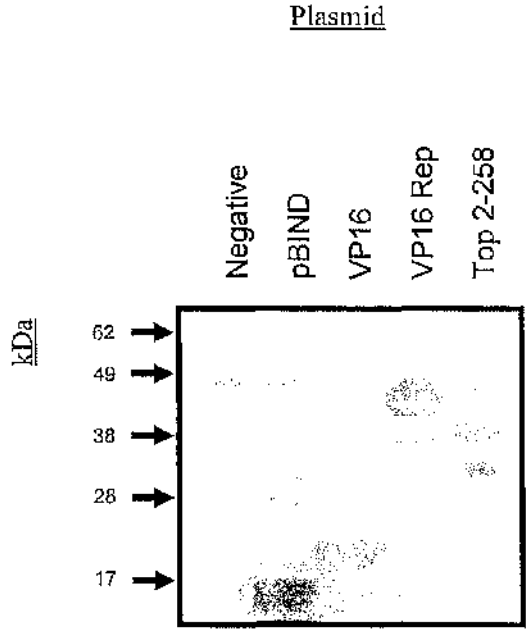
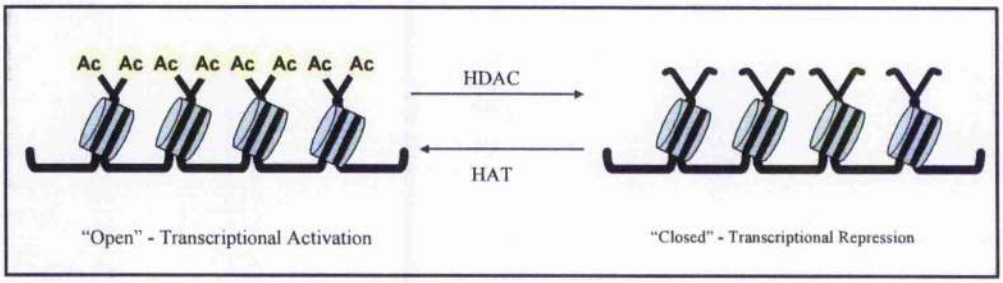



**Figure 16: Protein Expression of VP16-GAL4 Fusion Proteins.** Western blot showing the expression of the VP16-GAL4 fusion proteins indicated in 15a) using anti-GAL4 DBD antibody. The location of the molecular weight marker (kDa) is shown on the left of the diagram as a control.

**Figure 17**

a)


Ac Ac Ac Ac Ac Ac Ac Ac
HDAC
HAT
"Open" - Transcriptional Activation
"Closed" - Transcriptional Repression

b)

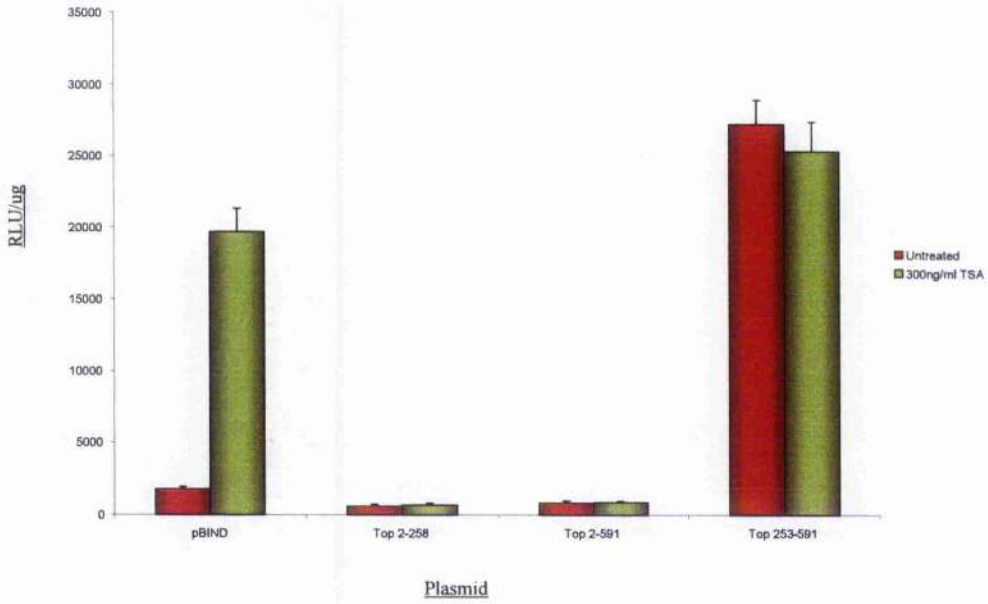

35000
30000
25000
20000
15000
10000
5000
0
RLU/ug
pBIND
Top 2-258
Top 2-591
Top 253-591
Plasmid
Untreated
300ng/ml TSA

**Figure 17: BRCT4 and BRCT2 Domains do not contain any intrinsic HDAC activity**. a) Diagram showing the mechanism of HDAC and HAT activity. HAT enzymes facilitate the acetylation of core histones resulting in an "open" chromatin structure allowing access for transcription factors and DNA repair proteins. HDAC remove the acetyl groups resulting in a "closed" chromatin structure. b) Luciferase assay of GAL4-TopBP1 fusion proteins TopBP1 amino acids 2-258, 2-591 and 253-591, either untreated or treated with 300ng/ml Trichostatin A for 3 hours prior to harvesting. Results are shown as relative light units (RLU) per μg of total protein in lysates, the activation and repression domains show no HDAC activity.

**Figure 18**

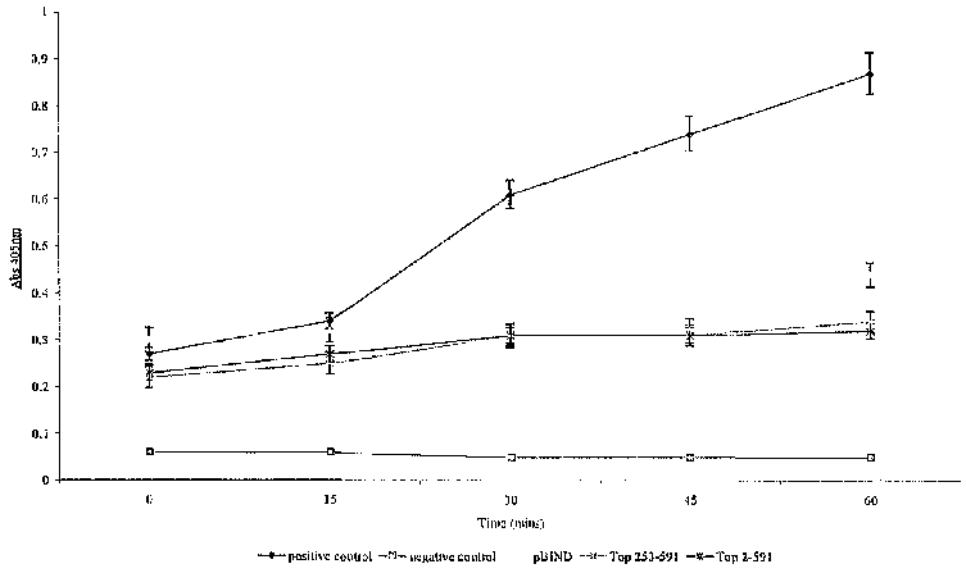



**Figure 18: BRCT4 and BRCT2 Transcriptional domains do not contain any HAT activity.** Histone acetyltransferase assay (2.2.1.24.), showing the mean absorbance at 450nm following the addition of the substrate (Time zero). This experiment was carried out in duplicate three times and this graph shows the mean ±S.E.M.

### 3.1.3 Identification of a Transcriptional Repression Domain within BRCT5

As shown in Figure 7b TopBP1 586-1435 repressed transcription from the pG5luc luciferase reporter. pG5luc was designed for the study of transcriptional activation and has a low background promoter activity; therefore a second luciferase reporter was constructed for the study of transcriptional repression with a more active promoter. The reporter ptkGAL46E2 contains the GAL4 binding domain from pG5luc upstream of a thymidine kinase promoter (2.1.9.), a diagrammatic representation of this luciferase reporter is shown in Figure 19. The incorporation of the E2 binding sites provides a more versatile reporter for future experiments investigating the role of TopBP1 in HPV16 E2 transactivation. Figure 20 shows the ability of the initial GAL4-TopBP1 fusion proteins (Figure 7a) to regulate transcription from the ptkGAL46E2 luciferase reporter in 293T cells. Compared to the empty vector (pBIND), TopBP1 586-1435 shows a 10 fold repression ($p = 0.01$), a repression stronger than the 5 fold observed using pG5luc (Figure 7b) therefore ptkGAL46E2 was used as the luciferase reporter for all assays in this chapter. However, as shown in Figure 8 TopBP1 586-1435 is poorly expressed compared to the other GAL4-TopBP1 fusion proteins. To determine if this repressive effect was merely due to lower levels of protein, a titration of TopBP1 586-1435 was carried out in 293T cells and the luciferase activity quantified as a fold effect against the reporter, ptkGAL46E2 being equivalent to 1 (Figure 21). Over a 20 fold range in concentration

the transcriptional repression shown by TopBP1 586-1435 increased as the concentration increased, increasing from a 5 fold repression at 0.1μg to a 15 fold repression at 2μg compared to the empty vector pBIND, indicating that the repressive activity contained within this construct is not due to the poor expression of the protein but a function of this region. Unlike TopBP1 586-1435, the GAL4-TopBP1 fusion protein 798-1435 shows a reduced repressive activity compared to pBIND, indicating a repression domain is situated within TopBP1 amino acids 586 and 798. To characterise the location of this repression domain further a series of GAL4-TopBP1 truncations focusing on the region within TopBP1 amino acids 586-798 were constructed (Figure 22a), and the activity of these constructs to regulate transcription is shown in Figure 22b. TopBP1 586-1435 shows a 10 fold repression; however TopBP1 635 and 710-1435 do not show a strong repressive activity indicating that the repression domain lies within amino acids 586-635. This region partly encompasses the fifth BRCT domain; amino acids 606-656. There is further evidence to support BRCT 5 as the region containing this repressor domain; as shown in Figure 22b, the removal of BRCT 5 from TopBP1 586-1435 (TopBP1 ΔBRCT5) alleviates the repressive activity of this construct. The results for GAL4-TopBP1 (amino acids) 586-803 in the luciferase transcription assays is also shown in Figure 22b, although transcriptional repression by this construct is less than that observed for TopBP1 586-1435, the deletion of BRCT5 from TopBP1 586-803 further reinforces the result seen for TopBP1 586-1435, as the deletion of BRCT5 completely removes any transcriptional repression. The expression of the fusion proteins indicated in Figure 22a is shown in Figure 23.

**Figure 19**

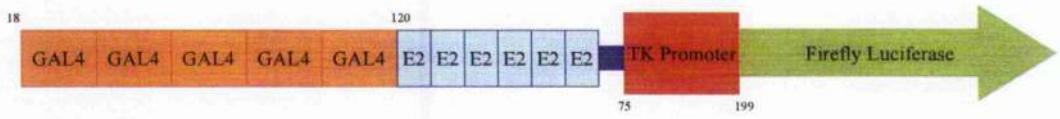



**Figure 19: ptkGAL46E2 Luciferase Reporter.** Diagrammatic representation of the luciferase reporter construct designed and cloned for the study of transcriptional repression. Five GAL4 binding sites (Orange Box) from pG5luc were cloned in frame into the reporter construct ptk6E2 (Vance *et al.* 1999). ptk6E2 luciferase activity (Green Arrow) is driven by the HSV thymidine kinase promoter from nucleotide 75-199 (Red Box) containing the TATA box downstream of six consecutive E2 binding sites (Blue Box) (Appendix page 339-349).

**Figure 20**

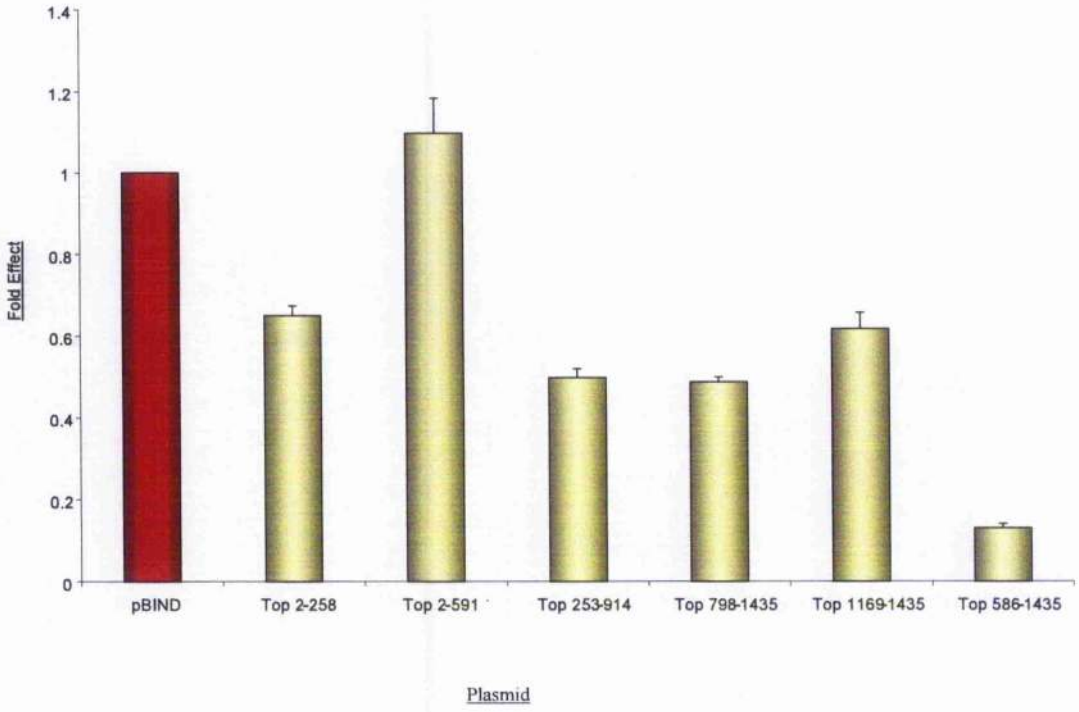



**Figure 20: Identification of a Second Transcriptional Repression Domain.** Luciferase assay showing the mean activity normalised against pBIND (Equivalent to 1) of the initial constructs shown in Figure 7a, using the reporter construct ptkGAL46E2 (Figure 19) ± S.E.M. Top 586-1435 shows a stronger repression using the ptkGAL46E2 luciferase reporter than was observed using pG5luc (Figure 7b).

**Figure 21**

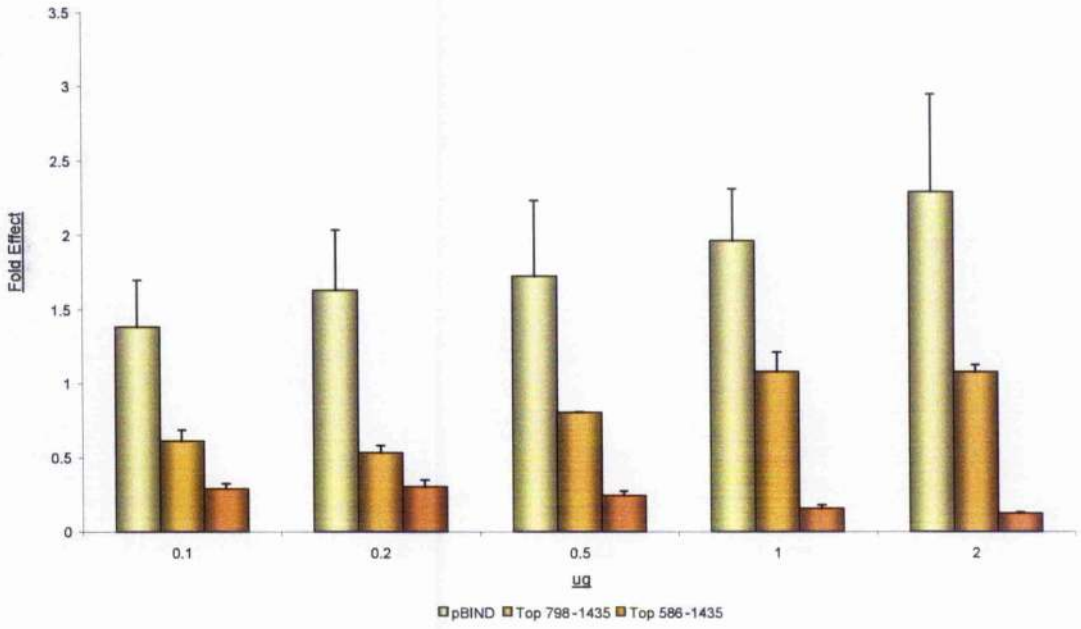
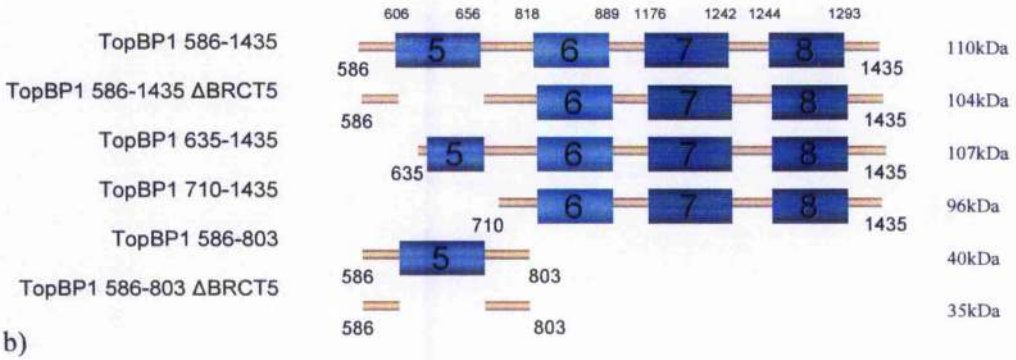



**Figure 21: Repression by TopBP1 586-1435 is not due to a lower level of Protein.** Titration of pBIND, TopBP1 586-1435 and TopBP1 798-1435 over a 20 fold range showing the repression of TopBP1 586-1435 throughout this range. Results are shown as a mean fold effect against ptkGAL46E2 (Equivalent to 1) ±S.E.M. This experiment was carried out three times in duplicate.

**Figure 22**

a)


606
656
818
889
1176
1242
1244
1293
TopBP1 586-1435
TopBP1 586-1435 ΔBRCT5
TopBP1 635-1435
TopBP1 710-1435
TopBP1 586-803
TopBP1 586-803 ΔBRCT5
5
6
7
8
586
1435
635
710
803
110kDa
104kDa
107kDa
96kDa
40kDa
35kDa

b)

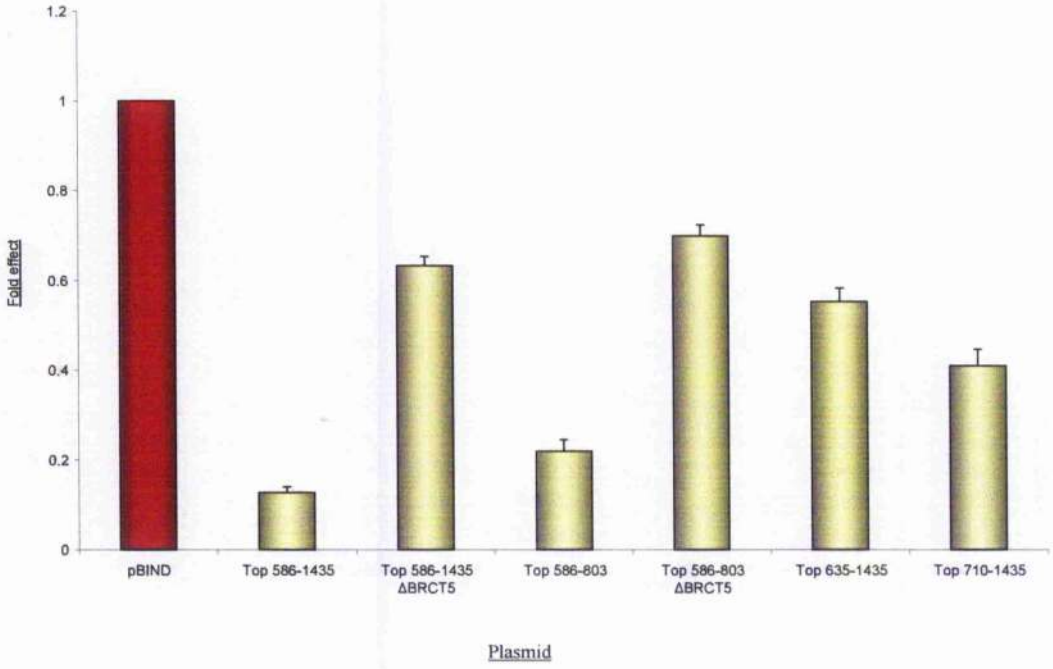

Fold effect
1.2
1
0.8
0.6
0.4
0.2
0
pBIND
Top 586-1435
Top 586-1435 ΔBRCT5
Top 586-803
Top 586-803 ΔBRCT5
Top 635-1435
Top 710-1435
Plasmid

**Figure 22: BRCT5 is responsible for Transcriptional Repression.** a) Schematic representation of the GAL4-TopBP1 fusion proteins. Shaded boxes indicate the regions covered by BRCT domains, and numbers correspond to amino acid number. b) Luciferase assay showing the mean fold effect change in transcription against the empty vector pBIND (being equivalent to 1), ± S.E.M of the constructs indicated in a).

**Figure 23**

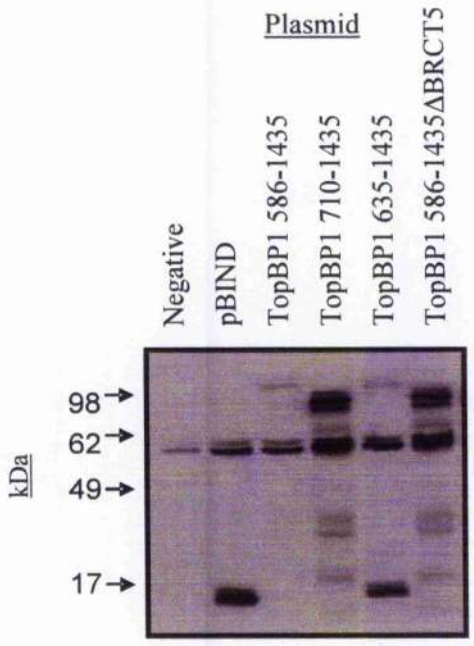



**Figure 23: Protein Expression of GAL4-TopBP1 Fusion Proteins.** a) Western blot showing the expression of the GAL4-TopBP1 fusion proteins indicated in Figure 22a using anti-GAL4 DBD antibody. The location of the molecular weight marker (kDa) is shown on the left of the blot.

### 3.1.4 The Transcriptional Repression Domain within BRCT5 can Repress the Activation Domain

The work described in chapter 3.1.2 suggested that the transcriptional activation domain within amino acids 460-500 is suppressed by the recruitment of a repressor complex (Figure 15). Chapter 3.1.3 identified a second repressor domain within BRCT5; therefore we tested whether BRCT5 could repress the adjacent activation domain. To investigate this a TopBP1 sequence was cloned into pBIND to create a GAL4-TopBP1 fusion protein containing the activation domain (amino acids 460-591) and the repression domain within BRCT5 (Figure 24a). The expression of this protein is shown in Figure 24b, using the anti-GAL4 DBD antibody. The ability of this fusion protein to activate transcription for the ptkGAL46E2 luciferase reporter was tested in 293T cells (Figure 24c). TopBP1 460-591 shows a significant 30 fold increase in transcriptional activation as previously shown in Figure 10b; however TopBP1 460-675 shows no increase in transcription indicating that the repressor domain contained within BRCT5 can also repress the activation domain within BRCT4 when placed in cis.

**Figure 24**

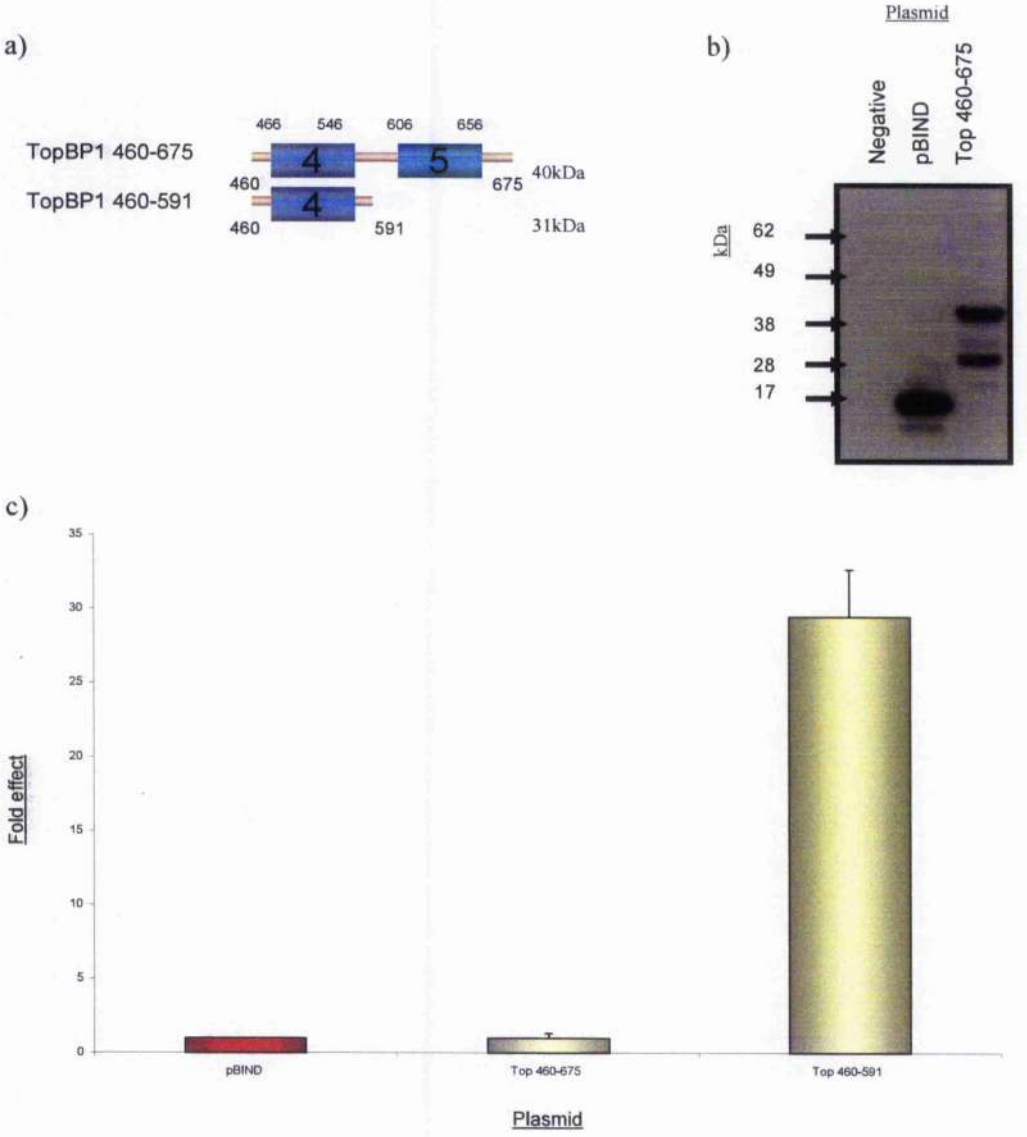

a)
TopBP1 460-675
TopBP1 460-591
466
546
606
656
4
5
460
675
40kDa
4
460
591
31kDa
b)
Plasmid
Negative
pBIND
Top 460-675
kDa
62
49
38
28
17
c)
Fold effect
35
30
25
20
15
10
5
0
pBIND
Top 460-675
Top 460-591
Plasmid

**Figure 24: The BRCT5 Repressor is Dominant over the BRCT4 Transcriptional Activation.** a) Schematic representation of the GAL4-TopBP1 fusion proteins. The predicted size of these proteins in kDa is shown on the right of each construct. Shaded boxes indicate the areas containing BRCT domains, numbers correspond to amino acid number. b) Western blot showing the expression of the GAL4-TopBP1 fusion proteins indicated in a) using anti-GAL4 DBD antibody. The location of the molecular weight marker is shown on the left. The expression of TopBP1 460-591 was shown previously (Figure 14). c) Luciferase transcription assay showing the mean fold change in transcription of the constructs indicates in a) normalised against the empty vector pBIND (being equivalent to 1) ± S.E.M.

### 3.1.5 The Domains identified within BRCT2 and BRCT4 are functionally conserved in Yeast

In order to determine the functional conservation of the repressor domain within BRCT2 and the activation domain within amino acids 460-500 of TopBP1, the ability of these domains to function in yeast was tested. TopBP1 sequences encoding amino acids 2-591, 2-258 and 253-591 were cloned into the yeast expression vector pGBKT7 to create yeast expression vectors encoding GAL4-TopBP1 fusion proteins (Figure 25a). These plasmids were transformed into the yeast strain AH109 (2.2.3.4), and plated out onto SC-Trp plates. Cell lysates were then prepared using a TCA preparation as described (2.2.3.2) and the proteins were separated by SDS-PAGE and probed for the presence of the GAL4 DBD (Figure 25b) to check the expression of these proteins in yeast. The predicated size of the GAL4-TopBP1 fusions is indicated by an *, although it is of note that in the case of TopBP1 2-258 although there is a band at the correct size, a larger protein is more evident.

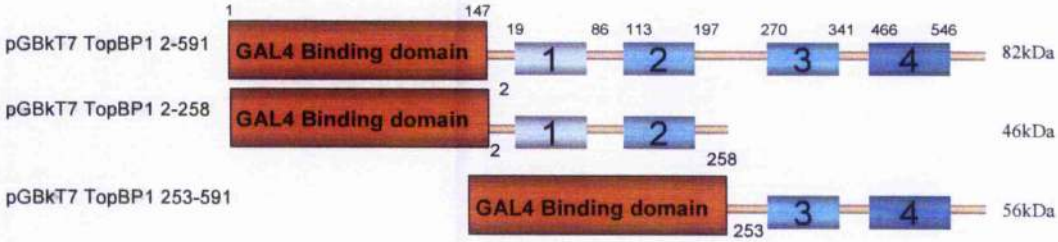
The ability of these fusion proteins to act as activators/repressors of transcription was tested in yeast using replica plating (2.2.3.6). The HIS3 and ADE2 genes are under the control of the GAL4 DNA binding sequences therefore only TopBP1 sequences which activate transcription would permit growth on the adenine and histidine amino acid depleted plates. Several clones expressing these GAL4-TopBP1 proteins were tested for their ability to grow on SC-Trp-His, Sc-Trp-Ade and SC-Trp-His-Ade

amino acid dropout plates. An example of the initial screen is shown in Figure 26, plate A shows the growth of all clones on SC-Trp media, B; SC-Trp-Ade, C; SC-Trp-His and D; SC-Trp-His-Ade amino acid dropout media. 2 clones for TopBP1 253-591 were tested but however only one of showed growth on all dropout media plates, however this clone did not express the fusion protein. A master plate is shown in Figure 26b, showing the location of each clone. GAL4-TopBP1 fusions 2-258, 2-591 and TopBP1 253-591 clone I were spotted onto SC-Trp and SC-Trp-His-Ade plates with pGBKT7 to confirm the results shown in Figure 26, the results are shown in Figure 27, only the TopBP1 sequence 253-591 allowed growth on the triple dropout medium, demonstrating that the activator within this region which was initially identified in mammalian cells (3.1.1) also functions in yeast.

The activity of the repressor domain identified within BRCT2 is also conserved in yeast. TopBP1 2-591 showed no growth on the triple dropout medium, even though this construct contains the activation domain within TopBP1 253-591, mimicking the result seen in the luciferase transcription assay in mammalian cells (Figure 7b, and Figure 9b). This result was confirmed using a liquid β-GAL assay (2.2.3.3) as the data shows (Figure 28) TopBP1 253-591 shows a 4 fold increase in activity. The β-GAL assay also suggests that the repressor domain can actively repress the yeast promoter as the activity of the TopBP1 2-258 construct was lower than the activity of TopBP1 2-591 similar to the initial observation in 293T cells (Figure 7b).

**Figure 25**

a)


pGBkT7 TopBP1 2-591
GAL4 Binding domain
1
147
19
86
113
197
270
341
466
546
1
2
3
4
82kDa
pGBkT7 TopBP1 2-258
GAL4 Binding domain
2
2
258
46kDa
pGBkT7 TopBP1 253-591
GAL4 Binding domain
253
3
4
56kDa

b)

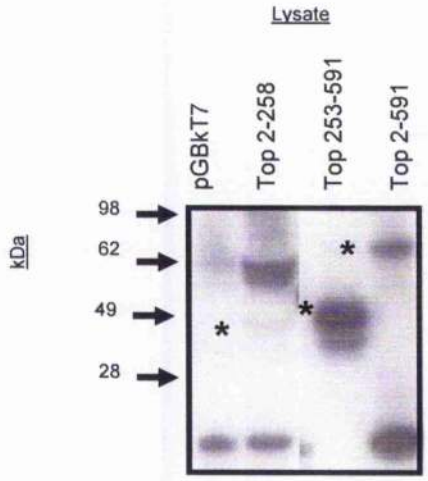

Lysate
pGBkT7
Top 2-258
Top 253-591
Top 2-591
kDa
98
62
49
28
*
*
*

**Figure 25: TopBP1 Activation and Repression Domains are Conserved in Yeast.** a) Diagrammatic representation of the TopBP1 constructs cloned into the yeast expression vector pGBKT7. Blue boxes correspond to BRCT domains and numbers correspond to amino acid number. The predicted size of the proteins is indicated on the right of the diagram. b) Western blot extracts from pGBKT7 lysates were harvested (2.2.3.3) and separated by SDS-PAGE and probed using anti-GAL4 DBD antibody. * indicates the predicted size of the proteins.

**Figure 26**

a)


A
C
B
D

b)


Top
TopBP1 2-258
TopBP1 2-258
TopBP1 2-591
TopBP1 2-591
TopBP1 253-591
TopBP1 253-591
TopBP1 253-591
TopBP1 253-591
TopBP1 2-591
TopBP1 2-591
pGBKT7
pGBKT7
Bottom

**Figure 26: Yeast Reporter Activation Screen**. a) Example of dropout media reporter screen, numbers of correspond to the amino acid start and end of TopBP1 present in the GAL4 fusion a diagrammatic representation of the TopBP1-GAL4 fusions is show in Figure 19. A) Sc-Trp plate B) Sc-Trp-Ade, C) Sc-Trp-His D) Sc-Trp-His-Ade. A master template is shown in b) indicating the location of the clones spotted onto the media dropout plates. Note only TopBP1 amino acids 253-591 shows any growth on the dropout media plates indicating that this construct can activate transcription in yeast.

**Figure 27**

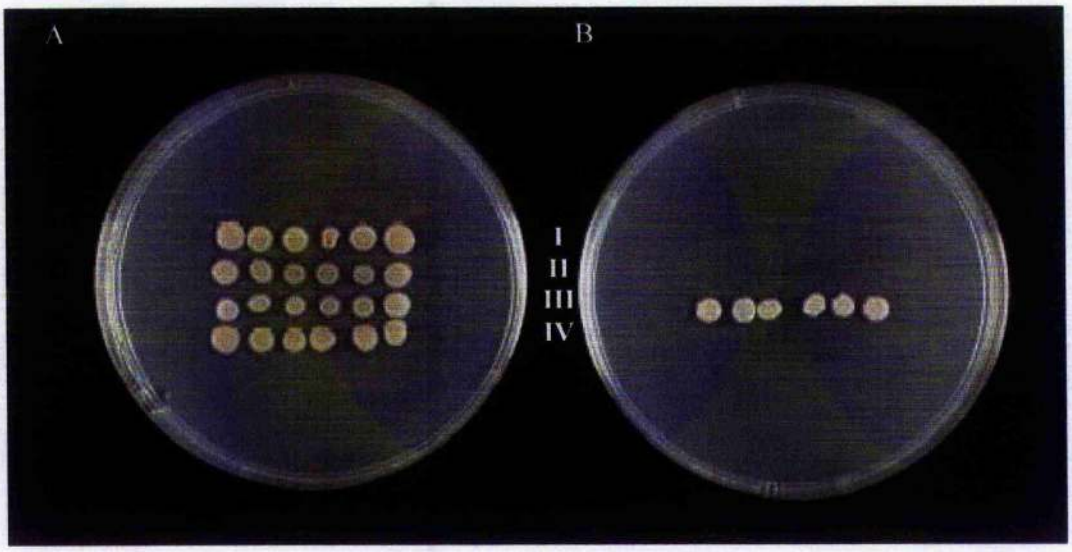



**Figure 27: TopBP1 Activation Domain functions in Yeast.** Plate A) Sc-Trp media (Full complement). Plate B) Sc-Trp-His-Ade dropout media (Deficient in tryptophan, adenine and histidine). Numerals correspond to the clones spotted, each clone has six repeat spots per plate; I represents pGBKT7, II; TopBP1 2-591, III; TopBP1 253-591, IV; TopBP1 2-258. All constructs including the empty vector grew on the full complement plate; however, growth is only present on the TopBP1 253-591 clone on the triple dropout plate indicating transcriptional activation.

**Figure 28**

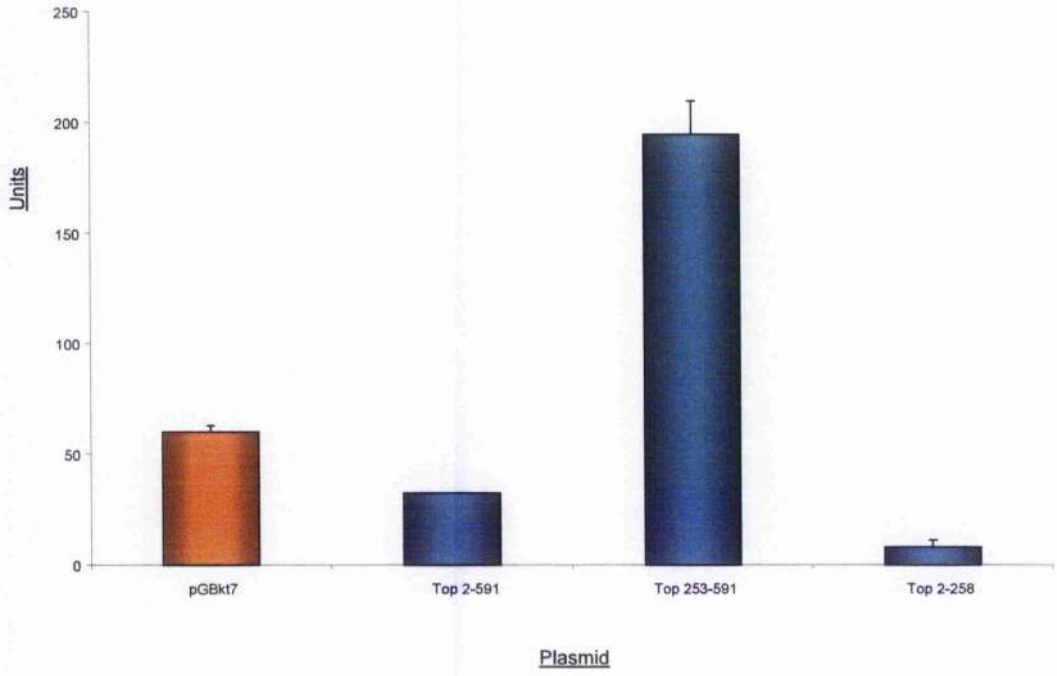



**Figure 28: BRCT2 Repression also functions to repress the Activation Domain in Yeast.** The ability of the fusion proteins to activate transcription in yeast was tested directly by monitoring levels of β-galactosidase activity. The promoter controlling the expression of this protein is controlled by GAL4 DNA binding sites. The results are expressed as optical density units following the β-galactosidase assay.

### 3.1.6 Summary Chapter 3.1

TopBP1 has roles in DNA damage signalling and repair and can act as both a transcriptional co-activator (Boner *et al.* 2002) and a transcriptional co-repressor (Liu *et al.* 2003). Although the role of TopBP1 in the DNA damage response and chromatin modification will be discussed in more detail in chapter 3.2, the results presented here have identified three domains within TopBP1 which regulate transcription.

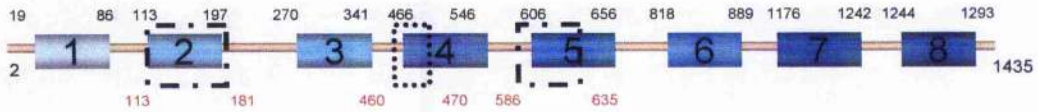
Figure 29a summarises the location of these transcriptional regulatory domains. A transcriptional activation domain lies between amino acids 460 and 500, partly encompassing the BRCT4 domain. This region of TopBP1 is rich in hydrophobic and acidic amino acids (Figure 11), characteristic of other transcriptional activation domains (Roberts *et al.* 1993). This is also similar to the amino acid content of the BRCA1 transactivation domain. Figure 29b shows a ClustalW sequence alignment of the TopBP1 and BRCA1 transactivation domains. Hydrophobic residues which are conserved between the two are indicated by •, and conserved residues are indicated by *. BRCT4 of TopBP1 lies within amino acids 466 and 546, which partly includes the activation domain similar to the BRCA1 activation domain which contains a BRCT domain (amino acids 1642-1736).

The transcriptional activation domain is suppressed by an adjacent repressor domain which maps directly to BRCT2. This repressor domain represses the transcriptional activation potential of BRCT4 most likely via the recruitment of a repressor complex and not by an intramolecular interaction. This is demonstrated in Figure 15 where the TopBP1 repressor was able to repress over 90% of VP16 transcriptional activity. The activation domain is also repressed by a second repressor domain, which exists on the C-terminal side of the BRCT4 activation domain (Figure 24). This repressor domain is a significantly stronger repressor than the BRCT2 repressor domain, repressing transcription over 10 fold compared to GAL4 (pBIND) alone (Figure 22).

Both the activation domain and the repressor domain contained within BRCT2 function in yeast (Figure 27), another feature similar to BRCA1 and other activation domains (Miyake *et al.* 2000). TopBP1 has a potential role in the generation of breast carcinomas, having aberrant expression in a significant number of tumours (Going *et al.* 2007). BRCA1 mutations in cancers are often found in the transactivation domain of BRCA1, which was identified using a similar GAL4 fusion protein assay (Monterio *et al.* 1996, 1997). It is unlikely given the significance of the BRCA1 mutations within the activation domain and their role in tumour suppression and transcriptional regulation that the TopBP1 domains identified here are an artefact of this established assay.

**Figure 29**

a)




b)

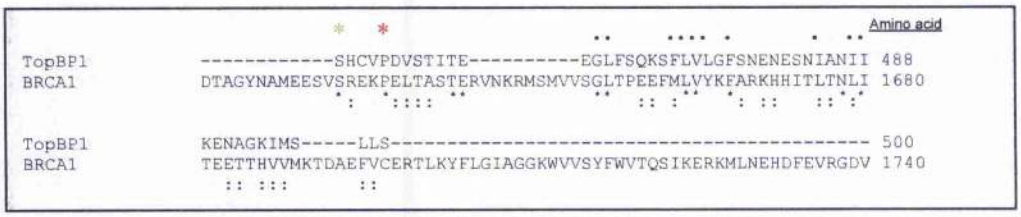



**Figure 29: Schematic Representation of the Activator and Repressor domains identified.** a) Blue boxes correspond to the location of BRCT domain, black numbers indicate the first and last amino acid contained within that BRCT domain. Red numbers and dash boxes indicate the first and last residue of TopBP1 contained in each of the transcriptional-activation/repression domain. b) Sequence alignment TopBP1 (amino acids 450-500) and BRCA1 (amino acids 1560-1863) activation domains. • indicates a conserved hydrophobic residue, * indicates residues which are conserved, : indicate residues in which the charge of the residue is conserved. A conserved serine residue is highlighted by a green *, and a conserved proline is highlighted by a red *.

## 3.2 TopBP1: DNA Damage and Chromatin Modification

### 3.2.1. Repression by BRCT5 is Dependent on Brg1 but Independent of Brm.

Chapter 3.1 discussed the identification of three transcriptional modification domains within TopBP1; an activation domain within amino acids 460-500, partly encompassing BRCT4, which is repressed by two adjacent repressor domains contained within BRCT2 and BRCT5. However the transcriptional activity of these domains is not universal to all cell lines; the activity of the GAL4-TopBP1 fusion proteins initially investigated (Figure 7a), was tested in C33a cells and the results are shown in Figure 30. The GAL4-TopBP1 fusion protein TopBP1 amino acids 586-1435, which contains the repressor domain within BRCT5, shows no repression in C33a cells; indeed it shows a two fold increase in transcription unlike the result previously observed in 293T cells (Figure 7b).

The modification of chromatin may be required for the functions of TopBP1 in transcriptional regulation and DNA damage repair (As discussed chapter 1.6.). The chromatin remodeller SWI/SNF was originally identified in yeast as a set of positive regulators of the HO gene (SWI; mating switch type) and the SUC2 gene (SNF; sucrose non-fermenting). The SWI/SNF mutliprotein complex consists of 10-12

subunits and modifies chromatin in an ATP dependent manner, by disrupting histone-DNA interactions. This complex either contains Brm (Brahma) or Brg1 (Brahma related gene 1) as the central ATPase catalytic subunit; C33a cells lack significant Brg1 and Brm activity (Muchardt *et al.* 1993).

Previous work on TopBP1 has shown an interaction with Brg1/Brm repressing the E2F1 activity (Liu *et al.* 2004). Therefore the ability of the repressor domain within BRCT5 to regulate transcription in the Brg1/Brm compromised C33a cell line was tested. Figure 31 shows a titration of GAL4-TopBP1 586-1435, GAL4-TopBP1 798-1435 and pBIND in C33a cells. Over a two hundred fold range of plasmid concentrations, keeping the reporter plasmid (ptkGAL46E2) constant at 1μg, TopBP1 586-1435 failed to repress transcription in C33a cells. These results suggested that transcriptional repression by BRCT5 could be mediated by the actions of either Brg1 or Brm; or both. In order to investigate this hypothesis further, Brg1 was restored to C33a by co-transfecting a Brg wild type expression plasmid (Muchardt *et al.* 1993), the GAL4-TopBP1 fusions and pTKGAL46E2. The results of this luciferase assay are shown in Figure 32; the data shows the mean of at least three experiments carried out in duplicate ± S.E.M. In this experiment the addition of Brg1 (wt) to C33a significantly restored the transcriptional repression ($p = 0.035$) observed previously in 293T suggesting that repression is dependent on Brg1. Figure 33 shows the same experiment in C33a cells with the addition of an ATPase mutant Brg1 plasmid (Brg1 (-/-) Muchardt *et al* 1993), this plasmid did not restore repression by BRCT5, indicating that the ATPase enzymatic activity of Brg1 is

essential for transcriptional repression by TopBP1 BRCT5. As the repression domain lies within BRCT5, Figures 32 and 33 also show the results for the TopBP1 BRCT5 deletion construct (GAL4-TopBP1 586-1435 Δ BRCT5), this construct shows no repressive activity and is not effected by either the addition of Brg1 (wt) or Brg1 (-/-).

To confirm the results seen in C33a cells similar experiments were carried out in 293T cells (a cell line which is not Brg1 or Brm compromised), with the addition of the Brg1 dominant negative (-/-) (Figure 34) and wild type plasmids (wt) (Figure 35). The addition of the Brg1 (-/-) ATPase mutant plasmid to 293T disrupts transcriptional repression by TopBP1 586-1435, but has no effect on either TopBP1 Δ BRCT5 or pBIND. The addition of Brg1 (wt) to 293T further confirms these results, as seen in Figure 35, when Brg1 (wt) is co-transfected with the GAL4-TopBP1 repressor construct Top 586-1435, even in Brg1 positive 293T cells the repressive activity of TopBP1 586-1435 is increased. The addition of Brg1 (wt) has no statistically significant effect on the activity of GAL4-TopBP1 586-1435 Δ BRCT5, in HEK293T or C33a cells indicating that transcriptional repression is dependent on Brg1 and BRCT5 of TopBP1.

**Figure 30**

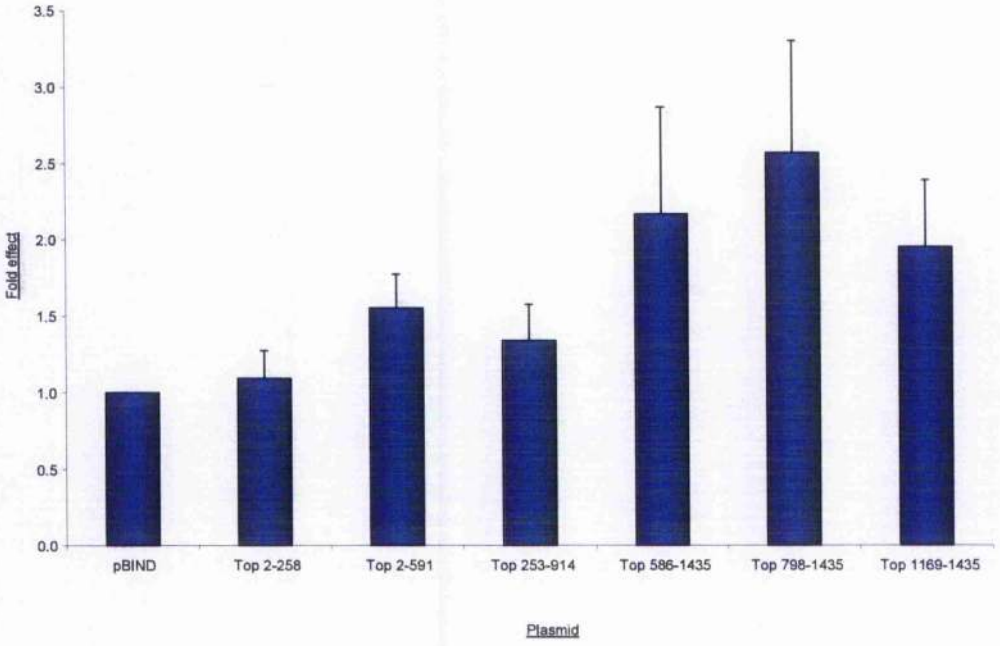



**Figure 30: Transcriptional Repression is not present in C33a.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs indicated in Figure 5b in C33a cells. All luciferase assays were carried out in duplicate and repeated at least three times; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 31**

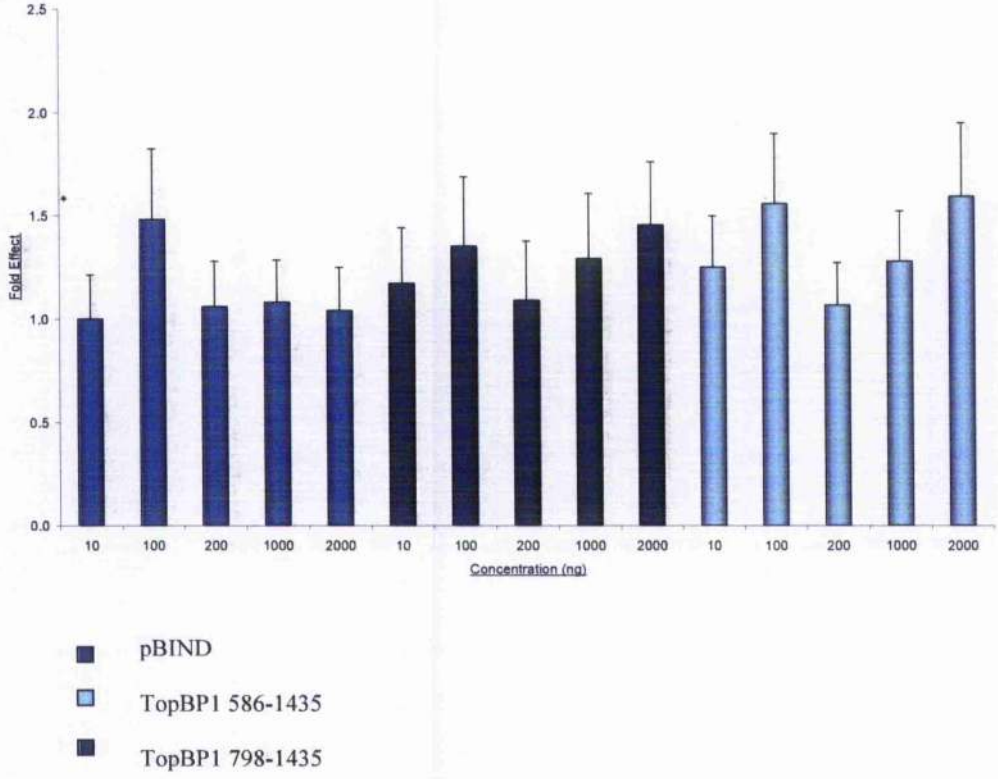



**Figure 31: Titration of GAL4-TopBP1 586-1435, GAL4-TopBP1 798-1435 and pBIND in C33a.** Luciferase assay showing the fold effect against the luciferase reporter pTKGAL46E2 (being equivalent to 1), in transcriptional activity of the empty vector pBIND and the GAL4-TopBP1 constructs 586-1435, and 798-1435 across varing concentrations. This graph shows the mean of triplicate experiments carried out in duplicate ± S.E.M.

**Figure 32**

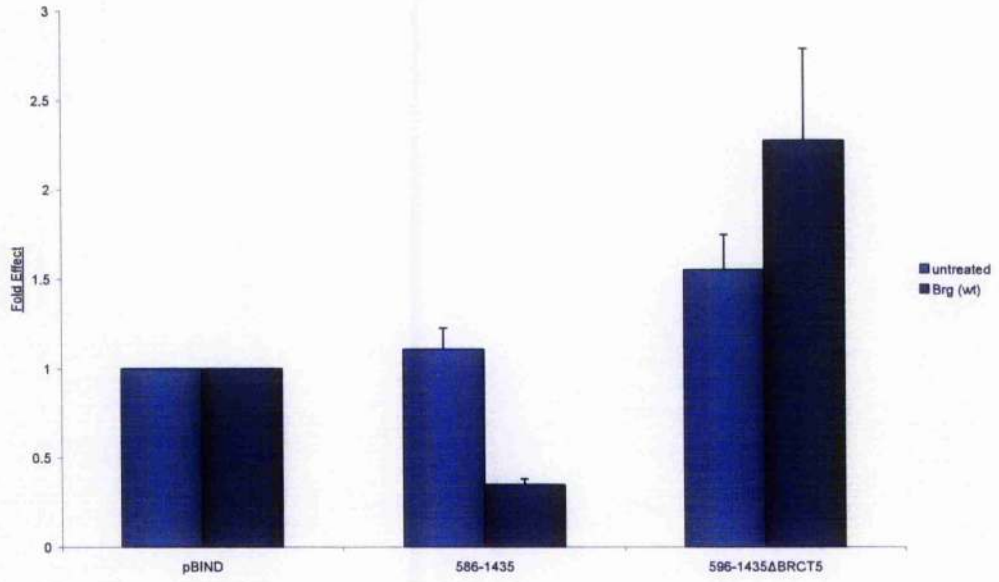



**Figure 32: The Addition of Brg1 (wt) restores Transcriptional Repression in C33a.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of Brg1 (wt) in C33a cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 33**

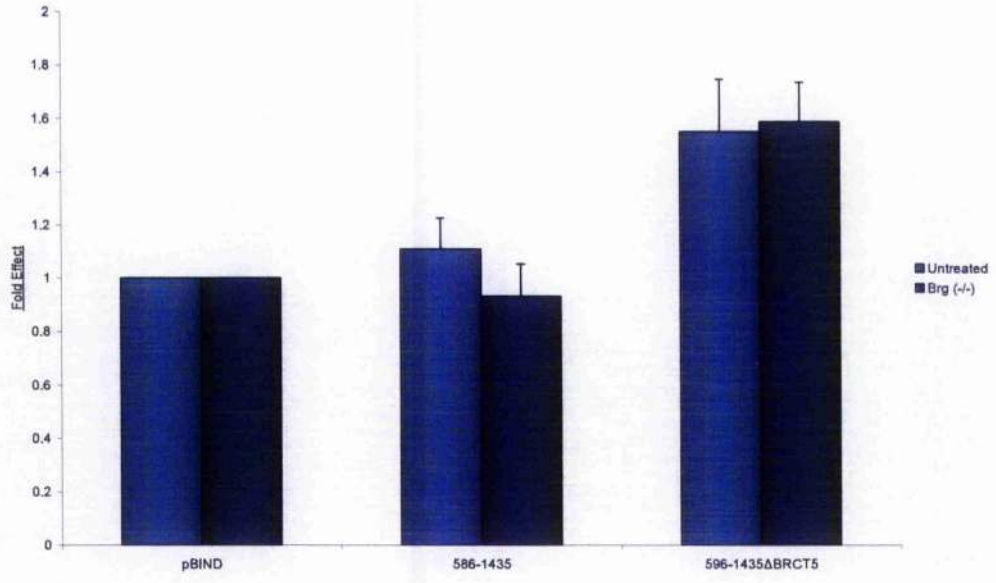



**Figure 33: The Addition of Brg1 (-/-) has no Effect on Transcriptional Repression in C33a.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of the Brg1 dominant negative plasmid (-/-) in C33a cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 34**

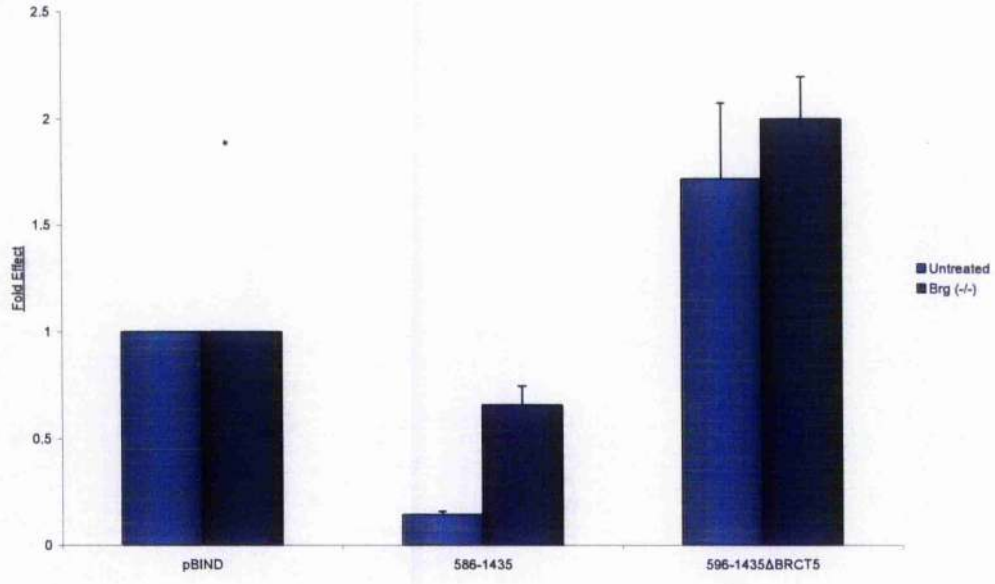



**Figure 34: The Addition of Brg1 (-/-) removes Transcriptional Repression in Brg1 positive 293T cells.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of the Brg1 dominant negative plasmid (-/-), in 293T cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 35**

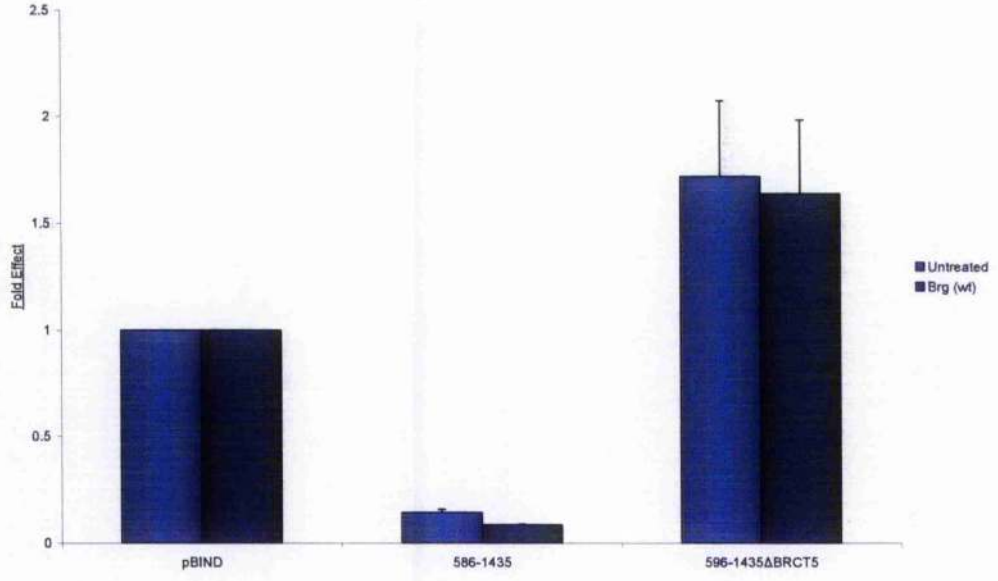



**Figure 35: The Addition of Brg1 (wt) increases Transcriptional Repression in Brg1 positive 293T cells.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of Brg1 (wt) in 293T cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

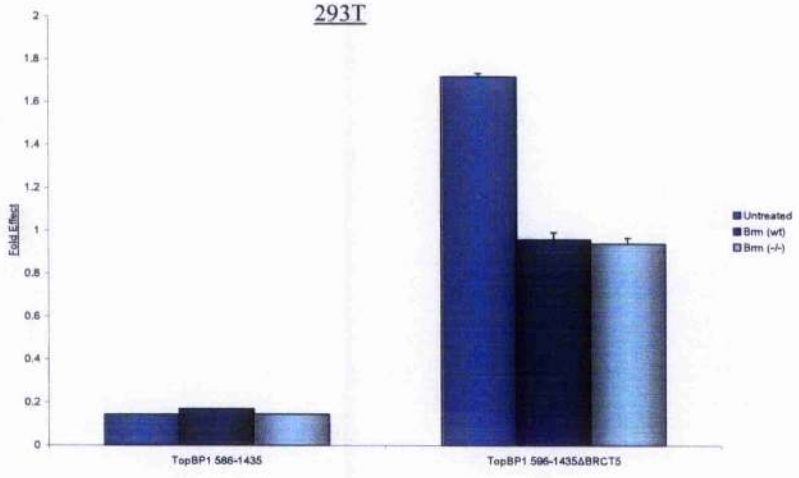
As Brg1 is essential for BRCT5 repression, the role of the SW1/SNF Brm ATPase subunit was also tested. Similar experiments as those described for Brg1 were carried out using a Brm wild type expression plasmid (Brm (wt)) and a Brm ATPase mutant plasmid (Brm (-/-) Muchardt *et al.* 1993). The result for the luciferase transcription assay in 293T cells using these Brm expression plasmids is shown in Figure 36a. Unlike the results observed using Brg1, the addition of the Brm (-/-) ATPase mutant plasmid does not alleviate repression, and the addition of Brm (wt) does not increase repression by GAL4-TopBP1 amino acids 586-1435 when compared to the empty vector pBIND. These results indicated that although repression by BRCT5 is dependent on Brg1 it is independent of the Brg1 homolog Brm. However in these assays the control plasmid lacking BRCT5 does show an increase in transcriptional repression with the addition of the Brm (wt) and Brm (-/-) plasmids. This is a non-specific effect as it does not depend on the ATPase function of Brm.

The addition of Brm (wt) to C33a also does not restore repression (Figure 36b) unlike the effect previously seen by restoring Brg1 (wt) to this cell line (Figure 32). The addition of Brm (-/-) was tested as a control and has no effect on TopBP1 transcriptional repression in C33a (Figure 36b). It has been shown previously that TopBP1 repression of E2F1 is dependent on both Brg1 and Brm when TopBP1 is overexpressed (Liu *et al.* 2004), therefore as a control the effect of Brm on E2F1 transcriptional activity using a p73 luciferase reporter plasmid was tested (Figure 37). In 293T cells E2F1 shows a 20 fold increase in transcriptional activation, which

is increased to 70 fold with the addition of Brm (when normalised against the p73 luciferase reporter (being equivalent to 1). Similar experiments were performed in C33a cells with the addition of Brm (wt). Although the fold increase in transcription by E2F1 when normalised against the p73 luciferase reporter (being equivalent to 1) is lower than that observed in 293T cells (Figure 37a); 5 fold compared to 20 fold. The addition of Brm (wt) has the same effect increasing E2F1 transcription from 5 to 20 fold in C33a cells. These experiments demonstrate that that the Brm (wt) expression plasmid is expressing a functional protein in both cell lines. Although this increase in E2F1 transcriptional activation when Brm is overexpressed is the opposite of the previously published result (Lui *et al.* 2004), showing repression following Brm, different reporter plasmids were used in these experiments which may account for the different result.

**Figure 36**

a)


293T
Fold Effect
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TopBP1 586-1435
TopBP1 596-1435ΔBRCT5
Untreated
Brm (wt)
Brm (-/-)

b)

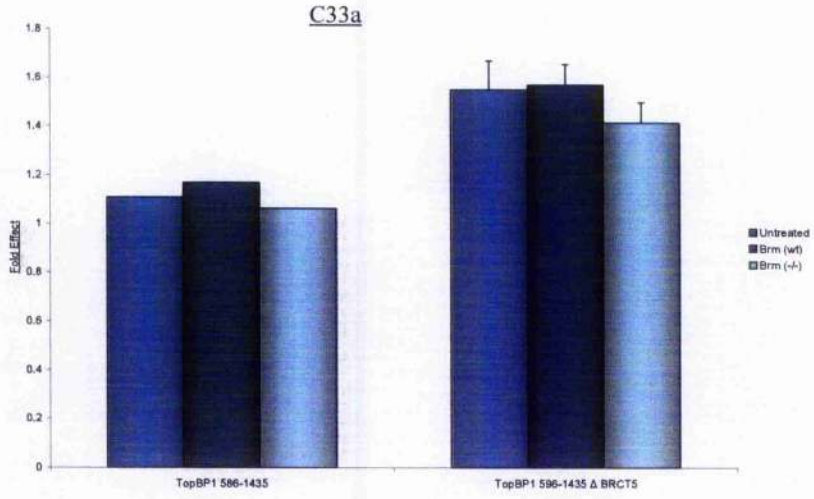
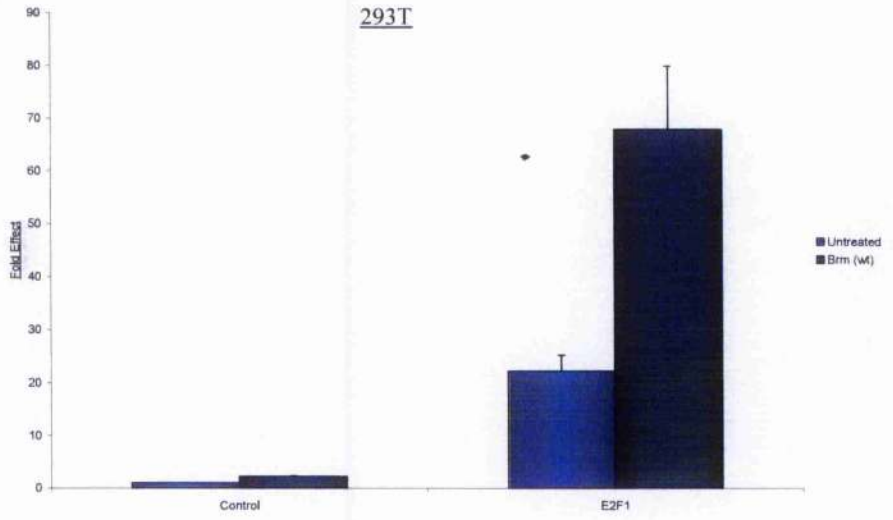

C33a
Fold Effect
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TopBP1 586-1435
TopBP1 596-1435 Δ BRCT5
Untreated
Brm (wt)
Brm (-/-)

**Figure 36: Brm is not Essential for Transcriptional Repression in 293T cells.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of Brm (wt) and Brm (-/-) in 293T cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M. b)Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-1435, and 586-1435 Δ BRCT5, with and without the addition of Brm (wt) or Brm (-/-) in C33a cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 37**

a)


293T
Fold Effect
90
80
70
60
50
40
30
20
10
0
Control
E2F1
Untreated
Brm (wt)

b)

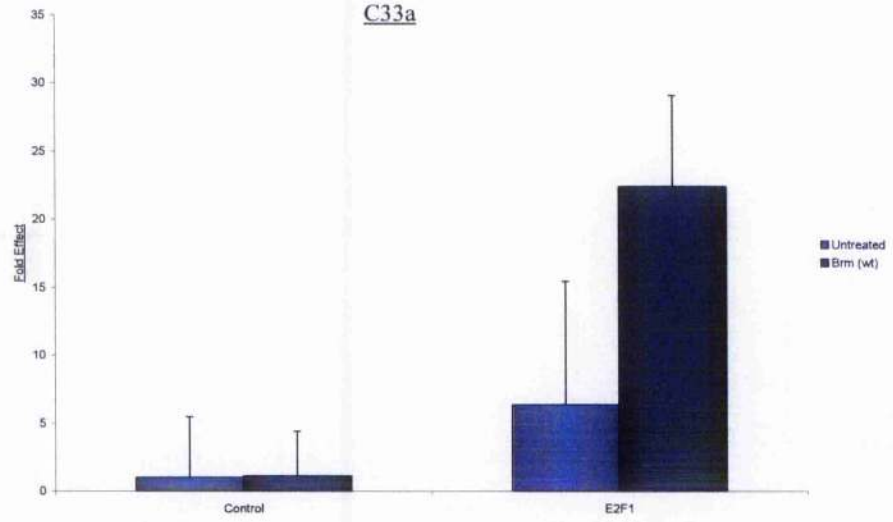

C33a
Fold Effect
35
30
25
20
15
10
5
0
Control
E2F1
Untreated
Brm (wt)

**Figure 37: The Brm (wt) plasmid expresses a functional protein and increases E2F1 Transcriptional Activity in 293T and C33a cell lines.** a) Luciferase assay showing the fold effect against the p73 luciferase reporter (being equivalent to 1), in transcriptional activity of the E2F1 with and without the addition of Brm (wt) in 293T cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M. b) Luciferase assay showing the fold effect against the p73 luciferase reporter (being equivalent to 1), in transcriptional activity of the E2F1 with and without the addition of Brm (wt) in C33a cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

### 3.2.2. Brg1 Physically Interacts with TopBP1 via BRCT5

It has been shown previously that Brg1 can interact with TopBP1 following DNA damage (Liu *et al.* 2004). To test whether TopBP1-Brg1 interaction could be detected in non-damaged cells 293T protein extracts were prepared and immunoprecipitated (2.2.2.20.) using anti-TopBP1 antibody (R1180, see materials and methods 2.2.1.) and pre-immune serum as a control (Boner *et al.* 2002). The resultant blot was probed with anti-Brg1 (H-88); and as Figure 38a shows TopBP1 and Brg1 do interact in non-damaged cells, as endogenous TopBP1 can immunoprecipitate endogenous Brg1 in undamaged 293T cell lysates.

To investigate whether the transcriptional modification domains identified in Chapter 3.1 were the regions responsible for the TopBP1-Brg1 interaction, immunoprecipitation experiments were carried out using the GAL4-DBD antibody, the GAL4-TopBP1 fusion proteins (Figure 38b). To narrow down the region responsible for the interaction three GAL4-TopBP1 fusion proteins were chosen; TopBP1 2-591, TopBP1 253-914 and TopBP1 798-1435 (Numbers correspond to TopBP1 amino acids number). Figure 7a shows the regions of TopBP1 covered by these GAL4-TopBP1 fusions, taken together these constructs cover the full length protein. As shown in Figure 38b, all three constructs can immunoprecipitate Brg1 using the anti-GAL4 DBD antibody, and this binding of Brg1 is specific as GAL4 alone (pBIND) poorly immunoprecipitates Brg1. These results suggest there are at

least two Brg1 interactions domains on TopBP1. It was decided to specifically test whether Brg1 can interact with the repressor domain encoded by BRCT5. To do this the GAL4-TopBP1 fusion proteins; GAL4-TopBP1 586-803 and GAL4-TopBP1 586-803 Δ BRCT5 (Figure 22a) were used. GAL4-TopBP1 586-803 shows a 5 fold repression of transcription and the deletion of BRCT5 removes over 80% of this repression (Figure 22b). Figure 39a shows a luciferase transcription assay using these GAL4-TopBP1 fusions, with the addition of either Brg1 (wt) or Brg1 (-/-) in 293T cells. Similar to the results shown in Figure 34 and 35, repression is dependent on BRCT5 and the addition of Brg1 (-/-) removes transcriptional repression. Figure 39b shows that the expression of these fusion proteins is not altered following the addition of either the Brg1 (wt) or Brg1 (-/-) plasmids. An immunoprecipitation reaction using the anti-GAL4 DBD antibody to immunoprecipitate GAL4 (pBIND), GAL4-TopBP1 586-803 and 586-803 Δ BRCT5, was performed in 293T cells to test the Brg1-BRCT5 interaction. The blot was probed with anti-Brg1 antibody (H-88). As Figure 40a shows TopBP1 amino acids 586-803 show an interaction with Brg1, and the removal of BRCT5 completely removes this interaction between TopBP1 and Brg1. However, the GAL4 DBD antibody had too much background when trying to show the GAL4 protein inputs. Therefore, Figure 40b shows a western blot using the same protein lysates used in Figure 40 to show the expression of the GAL4/GAL4-TopBP1 fusion proteins. Although GAL4 alone (pBIND) does bind Brg1, it is clear when comparing the results for GAL4-TopBP1 586-803 and GAL4-TopBP1 586-803 Δ BRCT5 that binding of Brg1 is significantly reduced with the deletion of BRCT5, suggesting BRCT5 is essential for the Brg1-TopBP1 interaction.

**Figure 38**

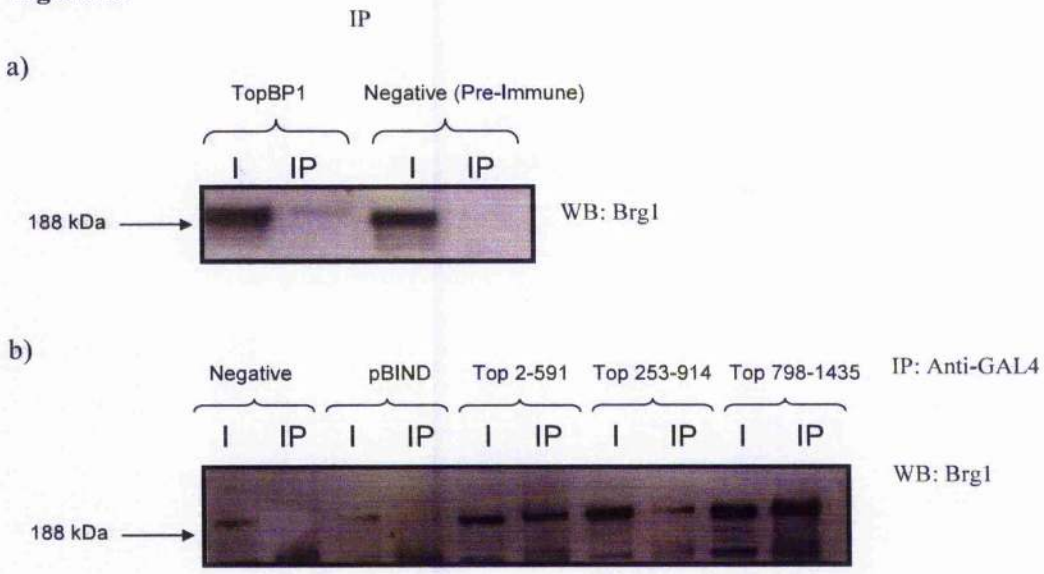
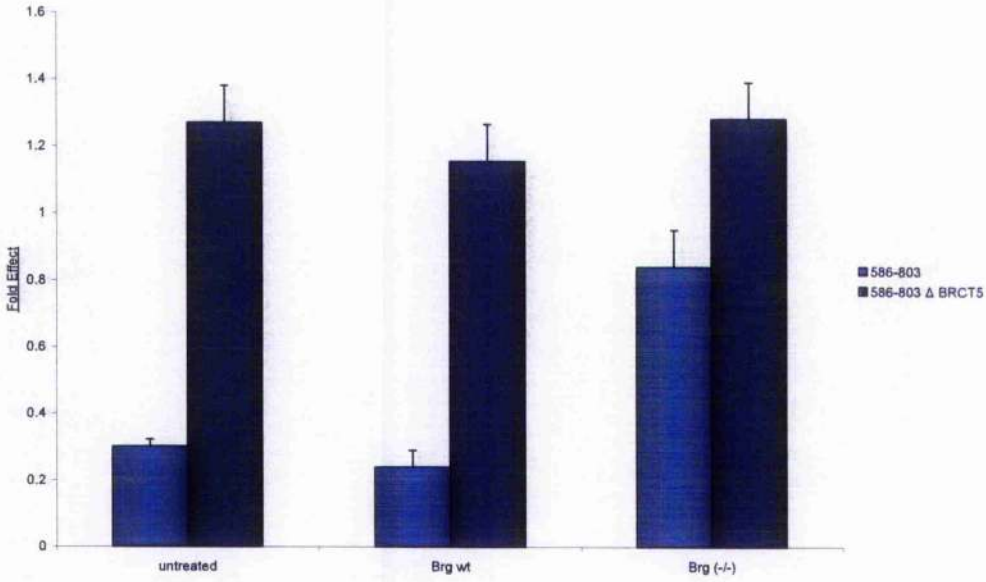


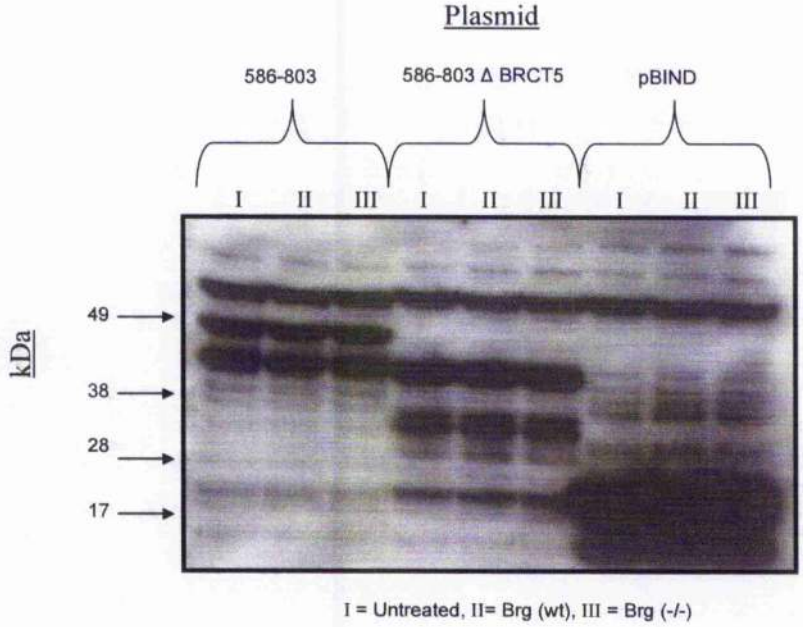

**Figure 38: TopBP1 interacts with Brg1.** a) 293T cells were harvested and immunoprecipitated with anti-TopBP1 antibody (R1180) and a pre-immune serum (2.2.1.20.); this lysate was separated on an SDS-PAGE gel and probed with anti-Brg1 antibody (H-88). b) 293T cells were transfected with the GAL4-TopBP1 constructs indicated above the blot as described (2.2.2.2.) harvested and immunoprecipitated with the anti-GAL4-DBD antibody (2.2.1.20.); this lysate was separated on an SDS-PAGE gel and probed with anti-Brg1 antibody (H-88). The molecular weight marker is shown on the left of the each blot (kDa). I and IP indicate the lanes in which the inputs and immunoprecipitation were separated respectively.

**Figure 39**

a)




b)

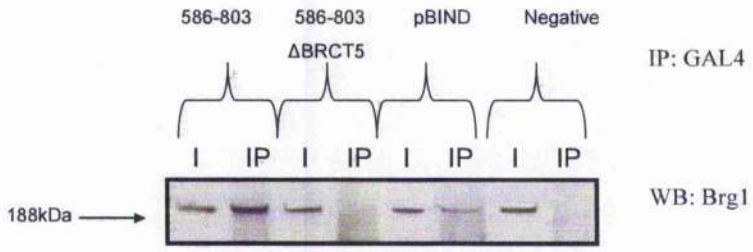



I = Untreated, II= Brg (wt), III = Brg (-/-)

**Figure 39: GAL4-TopBP1 586-803 ± BRCT5 Transcriptional Repression is Dependent on Brg1.** a) Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-803, and 586-803 Δ BRCT5, with and without the addition either the Brg1 (wt) or the Brg1 (-/-) plasmids. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M. b) Western blot showing the expression of the GAL4-TopBP1 fusion proteins Top 586-803 and Top 586-803 Δ BRCT5, and pBIND in either untreated 293T cells or cells co-transfected with Brg1 (wt) or Brg1(-/-). This blot was probed with the anti-GAL4-DBD antibody, and the location of the molecular weight markers (kDa) is shown in the left of the blot.

**Figure 40**

a)


586-803
586-803
ΔBRCT5
pBIND
Negative
IP: GAL4
I
IP
I
IP
I
IP
I
IP
188kDa
WB: Brg1

b)

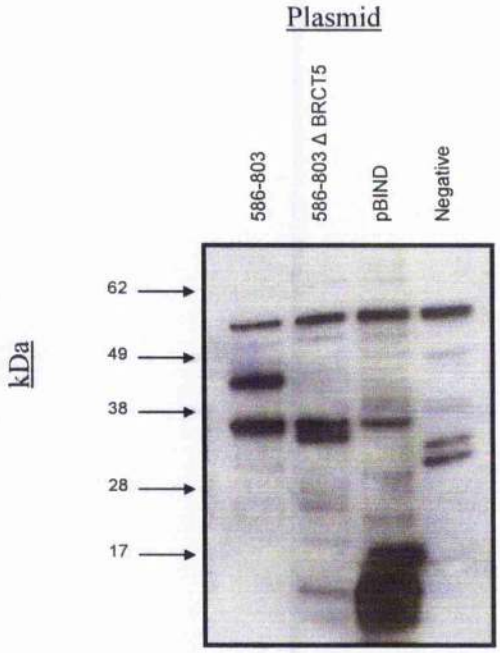

Plasmid
586-803
586-803 Δ BRCT5
pBIND
Negative
kDa
62
49
38
28
17

**Figure 40: Brg1 interacts directly with TopBP1 via BRCT5.** a) 293T cells were transfected as described (2.2.2.2.) with the GAL4 constructs indicated above the blot, harvested and immunoprecipitated with anti-GAL4-DBD antibody (2.2.1.20.); this lysate was separated on an SDS-PAGE gel and probed with anti-Brg1 antibody (H-88); the molecular weight marker is shown on the left of the blot. I and IP indicate the lanes in which the inputs and immunoprecipitation were separated respectively. b) The lysates used in the immunoprecipitation experiment in a) were separated on a SDS-PAGE gel and probed with anti-GAL4-DBD antibody to show expression of the input GAL4-TopBP1 fusion proteins, the molecular weight marker is shown on the left of the blot.

To further investigate this endogenous interaction between TopBP1 and Brg1 chromatin immunoprecipitation (ChIP) assays using 293T cell extracts were carried out as described (2.2.1.22.) to determine the co-occupancy of TopBP1 and Brg1 on the p73 promoter, an E2F1 responsive promoter (Pediconi *et al.* 2003). As shown in Figure 41 p73 promoter sequences (A), but not β-actin (B) promoter sequences, were detected in TopBP1 and Brg1 immunoprecipitates, confirming that not only do TopBP1 and Brg1 interact via BRCT5 but that these proteins are recruited to the same promoters.

**Figure 41**

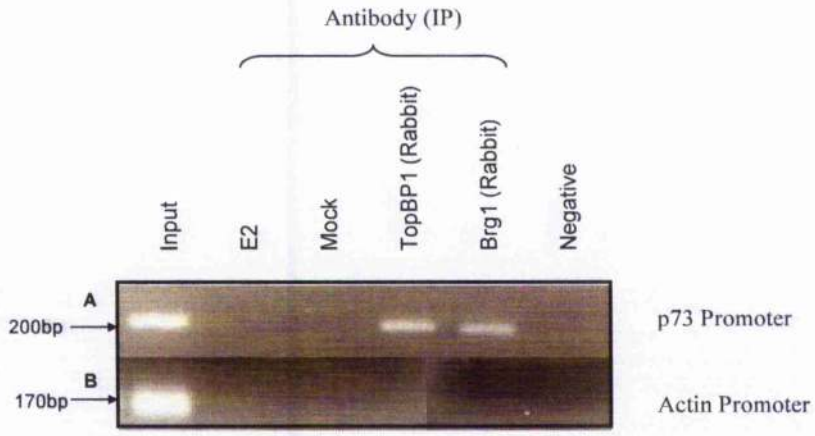



**Figure 41: TopBP1 and Brg1 are recruited to the same Promoter.** Chromatin Immunoprecipitation (ChIP) was performed (2.2.1.22.) using antibodies against TopBP1 (R1180) and Brg1 (H-88) as indicated. The HPV16 E2 antibody (TVG261), and a mock immunoprecipitation were used as negative controls. A negative PCR reaction is also shown. The precipitated DNA was amplified using two primers derived from the p73 promoter (A) and the β-actin promoter (B). The input represented 10% of the total chromatin added to each immunoprecipitation reaction.

### 3.2.3. DNA Damage Regulation of Brg1 Dependent Transcriptional Repression via BRCT5

TopBP1 is involved in all several areas of the DNA damage response; DNA damage recognition (Makinemi *et al.* 2001), signalling (Yamane *et al.* 2002) and repair (Lee *et al.* 2007). Brg1 is also implicated in regulating the DNA damage response and having shown a direct interaction between BRCT5 and Brg1 (Figure 40a) the ability of DNA damage to regulate the Brg1-BRCT5 interaction was investigated. To do this the effect of DNA damage on BRCT5 transcriptional repression was tested. 293T cells were transfected with the GAL4-TopBP1 repressor construct Top 586-803 ± BRCT5 as described (2.2.2.2.). The cells were then damaged with 20Gy from a $Co^{60}$ source four hours prior to harvesting and the luciferase activity was assayed as previously described (2.2.2.8.). As Figure 42 shows the transcriptional repressive activity of TopBP1 amino acids 586-803 is significantly alleviated following DNA damage ($p = 0.031$), when normalised against the empty vector pBIND being equivalent to 1. This alleviation of repression is specific to this repressor domain within BRCT5 as the BRCT5 deletion construct (GAL4-TopBP1 amino acids 586-803 Δ BRCT5), shows no change in activity following DNA damage. The transcriptional activation domain (Chapter 3.1.1) is not effected the same way by DNA damage; GAL4-TopBP1 460-591 shows a 35 fold increase in transcription in untreated cells when compared to the empty vector pBIND and shows no change in transcriptional activity following DNA damage ($p = 0.865$) (Figure 43).

**Figure 42**

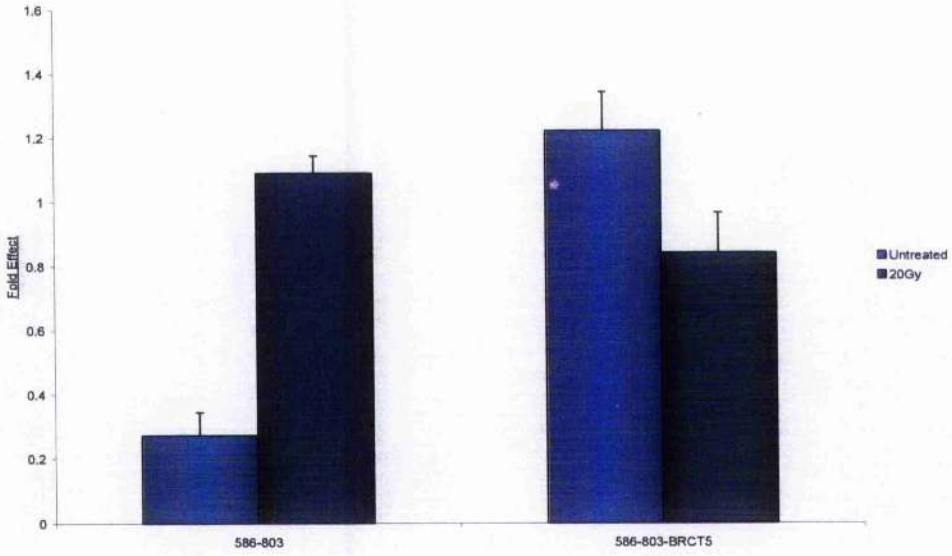



**Figure 42: Transcriptional Repression by BRCT5 is Alleviated following DNA Damage.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 constructs amino acids 586-803, and 586-803 Δ BRCT5, ± 20Gy gamma irradiation. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 43**

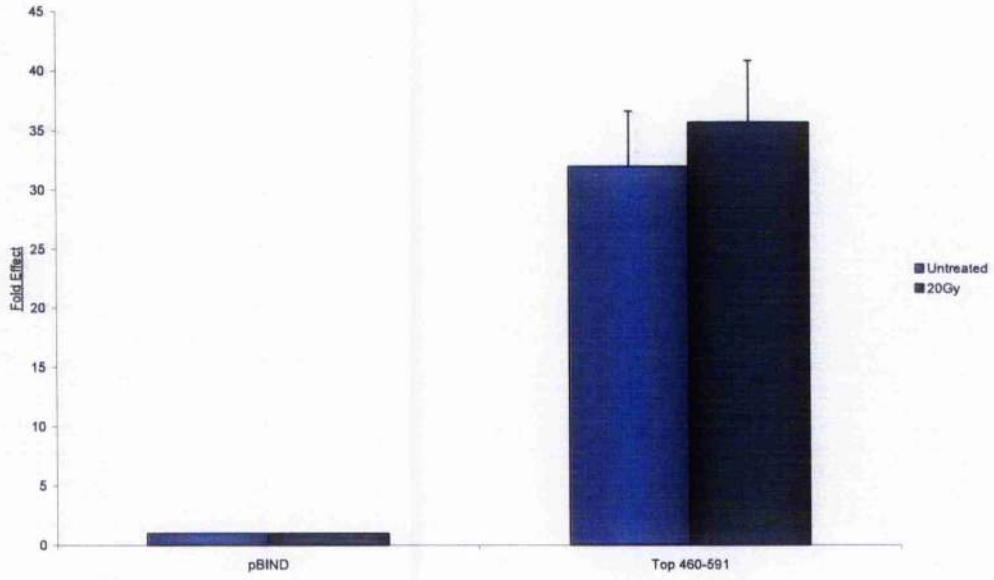



**Figure 43: The Activity of the Activation Domain is not Affected by DNA Damage.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 construct amino acids 460-591 which encompasses the transcriptional activation domain previously identified (3.1.1), with and without a 20Gy gamma irradiation dose. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

To investigate the role of Brg1 in this response by BRCT5 following DNA damage similar experiments were performed in C33a cells. As Figure 44 shows C33a cells were transfected with 586-803 ± the addition of the Brg1 (wt) plasmid. In untreated cells similar to previous observations the addition of Brg1 restores transcriptional repression by BRCT5. Following DNA damage this transcriptional repression in the presence of Brg1 is alleviated mimicking the results seen in 293T following DNA damage.

**Figure 44**

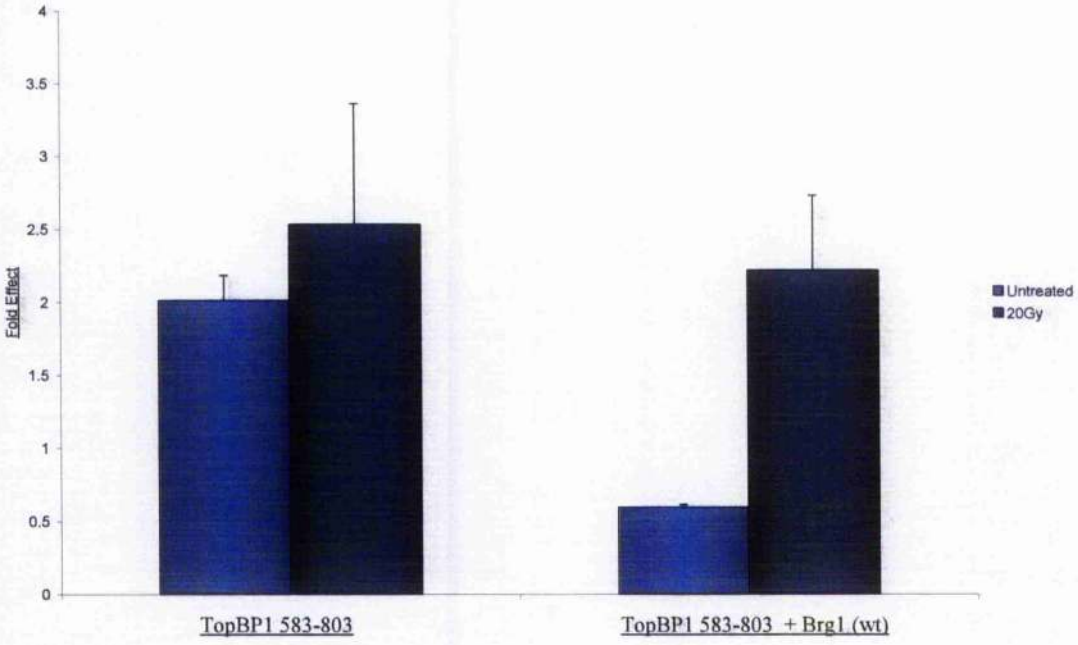



**Figure 44: Alleviation of Repression Following DNA Damage is Dependent on Brg1.** Luciferase assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of GAL4-TopBP1 amino acids 586-803, ± 20Gy gamma irradiation ± Brg (wt) in C33a cells. This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

### 3.2.4 Investigating which DNA Damage kinase mediates the regulation of BRCT5-Brg1 interaction.

In an attempt to determine the mechanism of Brg1 dependent repression via BRCT5 the PIKK inhibitors; caffeine, wortmannin and LY294002 were used (2.2.2.8.) in transcription assays. These drugs are general inhibitors of the DNA damage kinases ATM, ATR and DNA-PK.

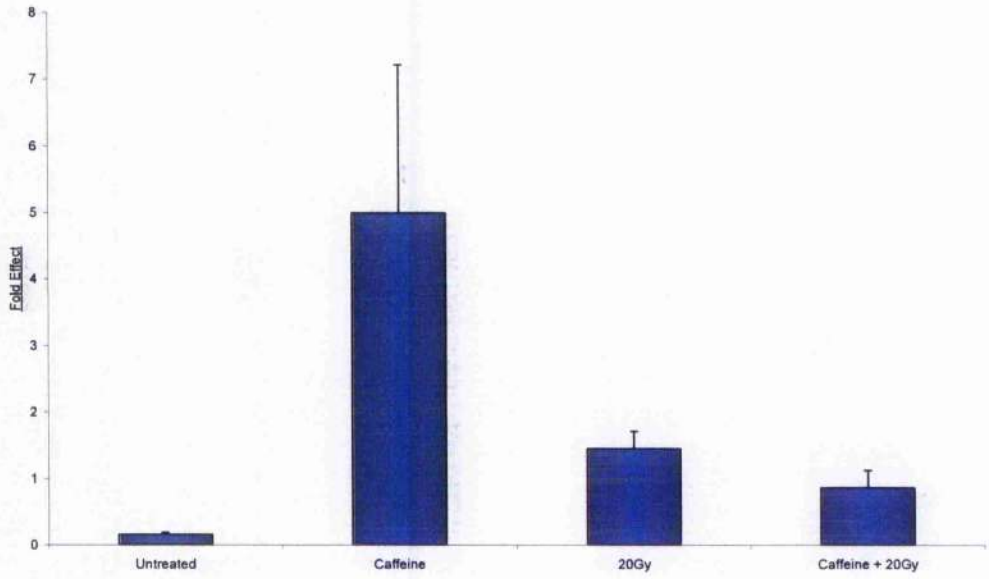
Figure 45 shows the results using caffeine (10mM) in 293T cells ± 20Gy. The treatment of these cells with caffeine alleviates repression by GAL4-TopBP1 586-803 even in undamaged cells (Figure 45a). Following irradiation the repression by BRCT5 is alleviated in cells not treated with caffeine, although this alleviation following damage is reduced in the presence of caffeine. These results suggested that repression by BRCT5 is mediated by ATM, ATR or DNA-PK in undamaged cells. GAL4-TopBP1 586-803 Δ BRCT5, is not significantly effected by gamma irradiation or caffeine (Figure 45b).

Similar experiments were carried out in 293T cells treated with either wortmannin (100mM) or LY294002 (100μM) (2.2.2.8.). The results are shown in Figure 46; this experiment shows the mean of at least three independent experiments carried out in duplicate ± S.E.M. Transcriptional repression is alleviated following the addition of both of these inhibitors, irrespective of DNA damage or not although not to the same

extent as with caffeine where activation is observed. The fungal metabolite wortmannin inhibits ATM and DNA-PK but at the concentration used in this experiment, ATR is not inhibited. The inhibitory effects of LY294002 are less well understood, phosphorylation of S139 on γ-H2AX which is dependent on ATM is inhibited following treatment with LY294002.

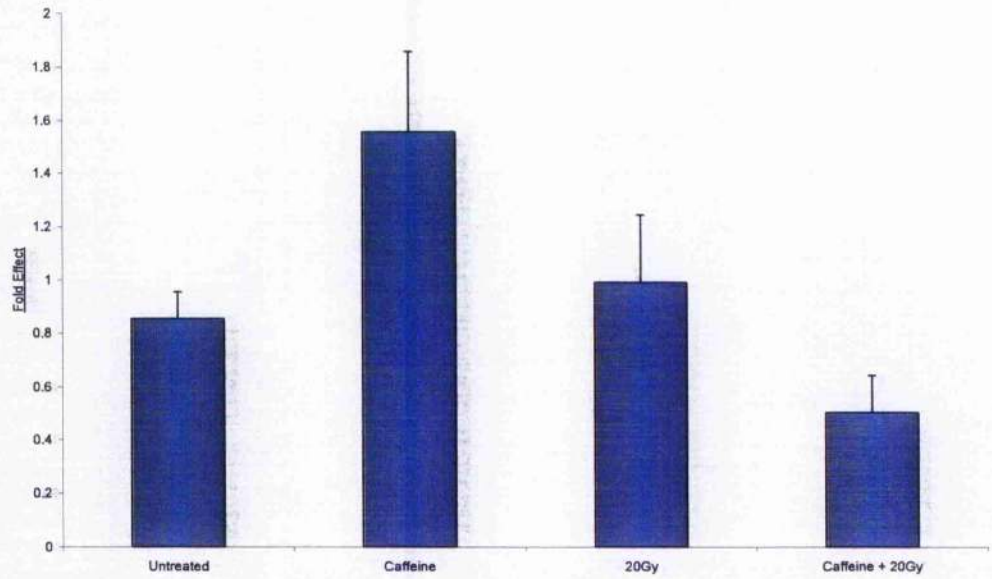
**Figure 45**

a)




TopBP1 586-803

b)




TopBP1 586-803 ΔBRCT5

**Figure 45: Caffeine Alleviates Transcriptional Repression by BRCT5 in Undamaged Cells.** a) Luciferase transcription assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 repressor construct amino acids 586-803, in 293T cells treated with 10mM caffeine 2 hours prior to damage (20Gy gamma irradiation).This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M. b) Luciferase transcription assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 construct amino acids 586-803 Δ BRCT5, in 293T cells treated with 10mM caffeine 2 hours prior to damage (20Gy gamma irradiation).This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

**Figure 46**

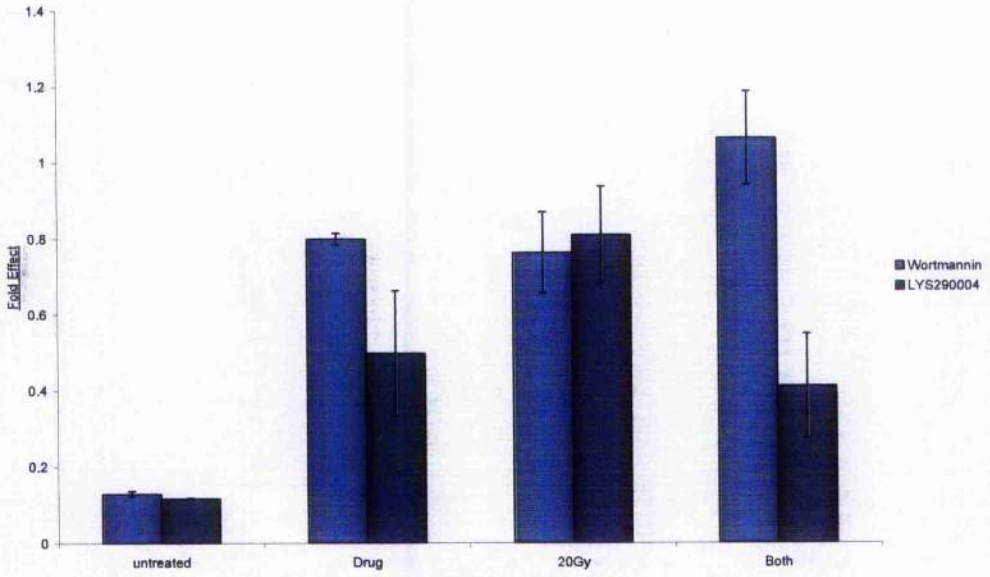



**Figure 46: Wortmannin and LY294002 Alleviate Transcriptional Repression by BRCT5 in Undamaged Cells.** Luciferase transcription assay showing the fold effect against the empty vector pBIND (being equivalent to 1), in transcriptional activity of the GAL4-TopBP1 repressor construct amino acids 586-1435, in 293T cells treated with either 20μM LY294002, or 5μM wortmannin 2 hours prior to damage (20Gy). This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

### 3.2.5. Alleviation of Transcriptional Repression is not a Universal DNA Damage Response

Gamma irradiation causes distinct double strand breaks in DNA however, ultra violet radiation and the repair of this type of damage proceeds via a distinctly different mechanism involving different signal and repair proteins (Introduction 1.1.3). UV forms pyrimidine dimers in DNA which are repaired by NER. To investigate if BRCT5 transcriptional repression and the alleviation of this repression following DNA damage was mediated by a specific type of DNA damage, transcription assays were performed following UV damage.

293T cells were transfected with the GAL4-TopBP1 repressor construct TopBP1 amino acids 586-1435 and pBIND. 36 hours post transfection the cells were treated with 0.02kJ/m$^2$ UVB and harvested at various time points thereafter. As Figure 47 shows unlike gamma irradiation, DNA damage induced by UVB does not result in alleviation of TopBP1 BRCT5 transcriptional repression, indicating that alleviation of repression shows some selectivity depending upon the type of DNA damage induced.

**Figure 47**

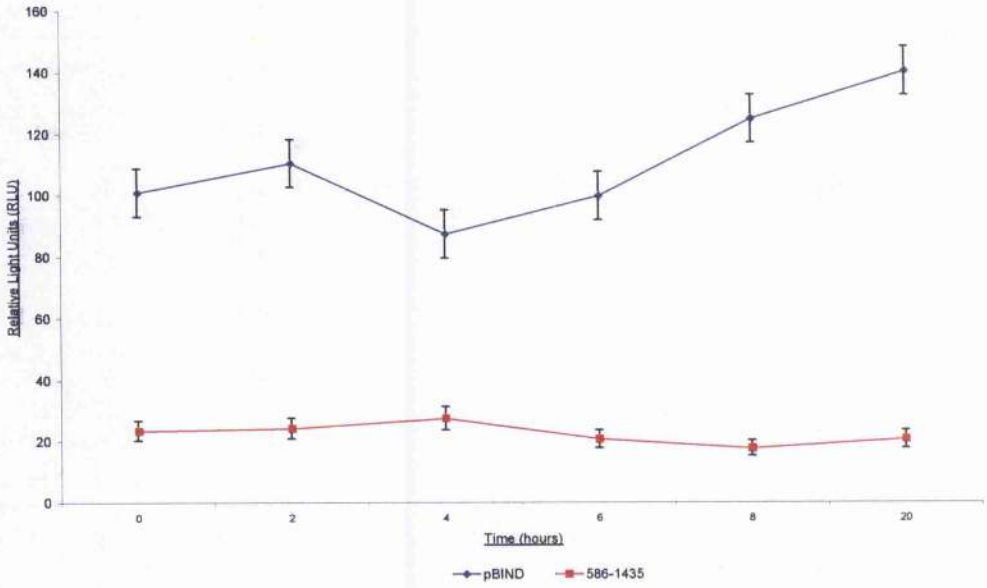



**Figure 47: Alleviation of Repression is not a Universal DNA Damage Response, and is not present in UVB Damaged Cells.** Luciferase transcription assay following UVB irradiation. Transcriptional activity of the GAL4-TopBP1 repressor construct (amino acids 586-1435) was assayed following 0.02kJ/m2 UVB. Cells were harvested following damage at the time points indicated. The results are shown as relative light units (RLU). This experiment was carried out at least three times in duplicate; this graph shows the mean of the triplicate experiments, ± S.E.M.

### 3.2.6 Summary Chapter 3.2.

The modification of chromatin is essential for DNA replication, chromosome recombination, gene expression and DNA damage repair. This chapter has identified that the transcriptional repression domain contained within BRCT5 (3.1.3) and the transcriptional repressive function of this region is dependent on the chromatin remodeller Brg1; the addition the dominant negative Brg1 (-/-) in Brg1 positive 293T cells results in a loss of transcriptional repression (Figure 34), and the reconstitution of Brg (wt) to the Brg1 compromised C33a cell line results in transcriptional repression by Top 586-1435 (Figure 32). The results have also shown that this effect of Brg1 is specific to BRCT5 (Figures 34 and 35) and that the levels of the GAL4-TopBP1 fusion proteins is not altered with the addition of either Brg1 (wt) or Brg1 (-/-) (Figure 39b).

Although SWI/SNF can contain either Brg1 or Brm as the central catalytic ATPase core of the multi-protein, Figure 36 showed that although transcriptional repression by BRCT5 is dependent on Brg1 it is independent of Brm. Although Brg1 and Brm were historically associated with transcriptional activation (Muchardt *et al.* 2001) there is increasing evidence for their role in transcriptional repression (Battaglioli *et al.* 2002). The interaction between Brg1 and TopBP1 following DNA damage has been shown previously (Liu *et al.* 2004) however in Figure 38, it is clear that

endogenous TopBP1 can immunoprecipitate endogenous Brg1 in undamaged cell lysates. Brg1 is involved in mediating transcription repression by BRCT5 and as Figure 40 demonstrated BRCT5 and Brg1 physically interact, the GAL4-TopBP1 586-803 repressor construct can immunoprecipitate Brg1, but this interaction is abrogated in the BRCT5 GAL4-TopBP1 deletion construct. Repression by BRCT5 is alleviated following gamma irradiation (Figure 42) but not following ultra violet irradiation (Figure 47).

Chromatin remodelling enzymes have an essential role to play in the DNA damage response allowing access for repair proteins to the site of damage (Park *et al.* 2006). The results shown in C33a cells suggested that alleviation of BRCT5 repression is dependent on Brg1 (Figure 44). This change in transcriptional activity following gamma irradiation is not a generic effect on TopBP1 transcriptional modification domains, as the activation domain within BRCT4 previously identified (3.1.1) shows no change in activity following gamma irradiation (Figure 43). To investigate the mechanism of this Brg1-BRCT5 response to DNA damage, general PIKK inhibitors were used (Figures 45, and 46) to inhibit the checkpoint kinases ATM, ATR and DNA-PK in transcription assays. These inhibitors alleviated repression in the absence of DNA damage, suggesting that repression in untreated cells and the alleviation of repression following DNA damage is dependent on one of these kinases. More specific kinase inhibitors are required to determine which of these enzymes are required for mediating repression and then alleviating repression following DNA damage.

## 3.3 Investigation of the Genes Regulated by TopBP1

So far the results have shown that TopBP1 is a potential transcription factor, therefore we wanted to identify transcriptional targets by running a gene expression microarray in TopBP1 knockdown cells. To do this we needed to use a cell line in which the knockdown of TopBP1 did not immediately affect the cell cycle, to allow comparable gene expression analysis. This chapter describes the results obtained to do this.

### 3.3.1 TopBP1 is not always essential for Cell Growth and Replication

TopBP1 is involved in DNA replication; physically interacting with the primary leading stand DNA polymerase, DNA polymerase ε (Makinemi *et al.* 2001) and directly associating with chromatin during DNA replication (Kim *et al.* 2005). Topoisomerase IIß, which TopBP1 was originally identified in association with, is also essential for replication, catalysing essential topological changes in DNA during this process. This evidence suggests that TopBP1 may play an essential role in DNA replication. To investigate the role of TopBP1 in DNA replication the ability of different cell lines to replicate in the absence of TopBP1 was tested by knocking out

TopBP1 in cultured cells using specific small interfering RNA (siRNA) targeted against TopBP1 (2.2.2.3.). Cell growth in these cells was measured using a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (2.2.2.6.). For all experiments using siRNA targeted against TopBP1, siRNA oligonucleotides against luciferase, and a mock transfection (lipofectamine only) were also used as experimental controls. The MTT assay provides a direct measure of metabolically active cells in culture, the yellow MTT substrate is reduced in the mitochondria of metabolically active cells form insoluble purple formazan crystals, which are then solubilised in DMSO and the absorbance reading at 570nm gives a direct measure of cell viability.

Five cell lines were chosen to test; HEK293T, U2OS, MRC5, MCF7 and HeLa. Figure 48 shows the MTT assay results for MRC5 and HeLa cells where TopBP1 expression was knocked down. MRC5 (a) and HeLa (b) cells treated with siTopBP1 stopped growing at 24hours and 36hours respectively. This stunted growth curve in the TopBP1 knockdowns is significantly different from the siluciferase growth curves for both cell lines (MRC5, $p=0.031$, and HeLa, $p = 0.022$) indicating that the arrest of cell growth in these cells is due to the knockdown of TopBP1 and not an effect of the siRNA transfection.

However not all cell lines seem to require TopBP1 for replication as some cell lines tested showed no change in cell growth in the absence of TopBP1. As Figure 49 shows MCF7 (a), 293T (b) and U2OS (c) cell lines showed no reduction in cell

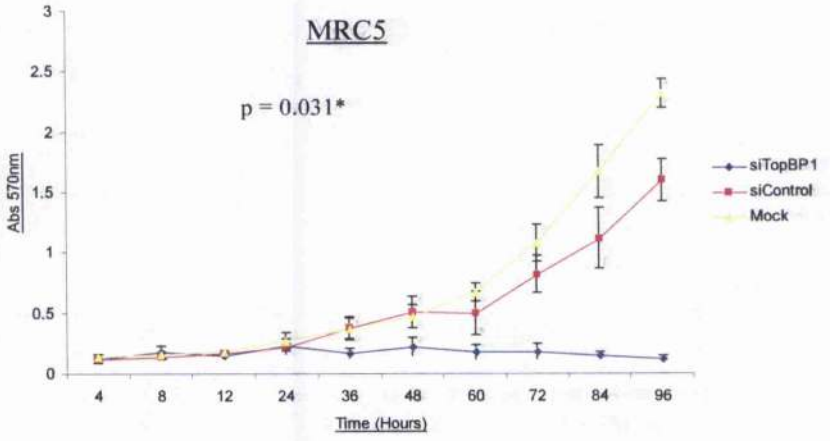
growth when TopBP1 was knocked down compared to the growth curves of the siluciferase and mock controls.

As Figure 48 and 49 show these assays were carried out over four days; the expression of TopBP1 during these four days following siRNA treatment (either siTopBP1 or siluciferase) is shown in Figure 50. These cell lysates were harvested at the same time points as the MTT assay readings were taken, and were transfected using the same siRNA/lipofectamine cocktails. The TopBP1 siRNA knockdown is more effective in certain cell lines; noticeably more efficient in 293T (Figure 50a) and MCF7 (b) cells and to a lesser extent U2OS (c). In contrast the effect of the siRNA in MRC5 (d) and HeLa (e) cells is less efficient but as Figure 48 shows this these cells are more sensitive to the knockdown of TopBP1 as it has a more profound effect their growth. In this experiment the Hela and MRC5 cell lines have a lower level of TopBP1, and even though the knockdown is less efficient it is possible that as the level of TopBP1 in these cell is lincs is already low, the knockdown results in a critically low level of TopBP1 with which the cells cannot survive. From the data presented here it is unclear why certain cell lines can grow in the absence of TopBP1 and others cannot although this will be discussed later. These experiments seemingly contradict previous observations, which demonstrated that TopBP1 was essential for DNA replication, as siTopBP1 treated cells arrested at G1 phase (Jeon *et al.* 2007), however different cell lines and siRNA's were used in these experiments which may account for different results.

To analyse the cell cycle effect of the TopBP1 knockout further FACs analysis was carried out in cells either treated with siTopBP1, siluciferase or a mock (Lipofectamine only 2.2.2.3.). The results are shown in Figure 51. MCF7 cells were siRNA treated and synchronised with aphidicolin for 16 hours to induce an early S-phase arrest (2.2.1.21.). The cells were then either left arrested or released and harvested for FACs analysis four hours following release. As panel A shows the knockdown of TopBP1 has no effect on the cell cycle compared to either the mock or luciferase siRNA in unsynchronized cells. The aphidicolin induced an S-phase arrest in all three samples (Panel B) and following release, the TopBP1 knockout cells show no significant change in cell cycle from the controls and still progress (Panel C) although perhaps at a slower rate.

**Figure 48**

a)


MRC5
p = 0.031*
Abs 570nm
Time (Hours)
siTopBP1
siControl
Mock

b)

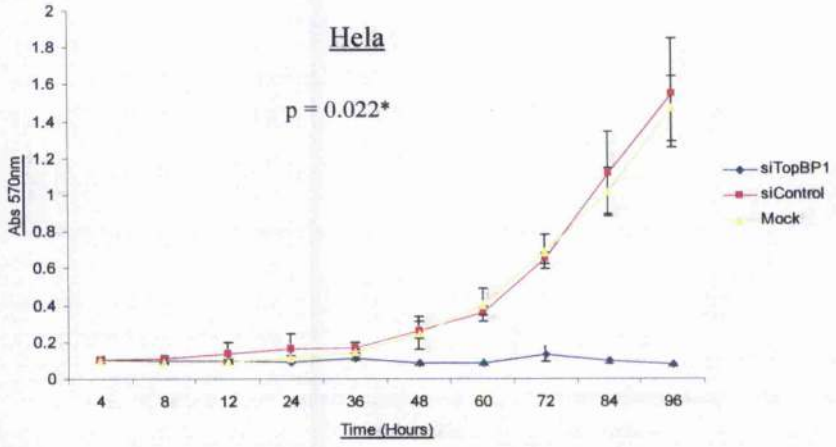
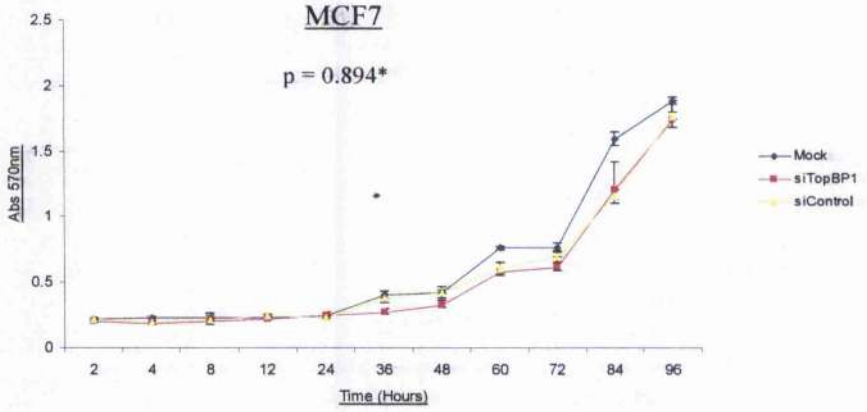

Hela
p = 0.022*
Abs 570nm
Time (Hours)
siTopBP1
siControl
Mock

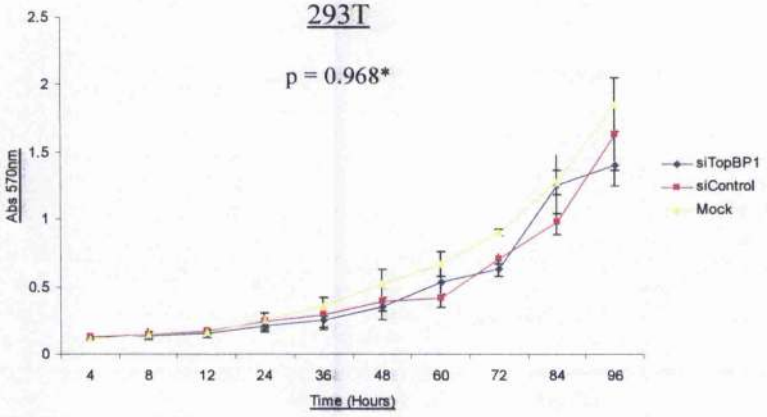
**Figure 48: MCR5 and Hela Cells Cannot Grow in the absence of TopBP1.** a) MRC5 cells were treated with either siTopBP1, siluciferase or a mock siRNA, as described (2.2.2.3.). The cell viability was measured using the MTT assay (as described 2.2.2.6.) at the time points indicated. This experiment was carried out three times in duplicate and this data shows the mean ± S.E.M. for the repeat experiments. b) Hela cells were treated with either siTopBP1, siluciferase or a mock siRNA, as described (2.2.2.3.). The cell viability was then measured using the MTT assay (2.2.2.6.) at the time points indicated. This experiment was carried out three times in duplicate and this data shows the mean ± S.E.M. for the repeat experiments. * Students paired T Test, siluciferase against siTopBP1.

**Figure 49**

a)




b)




c)

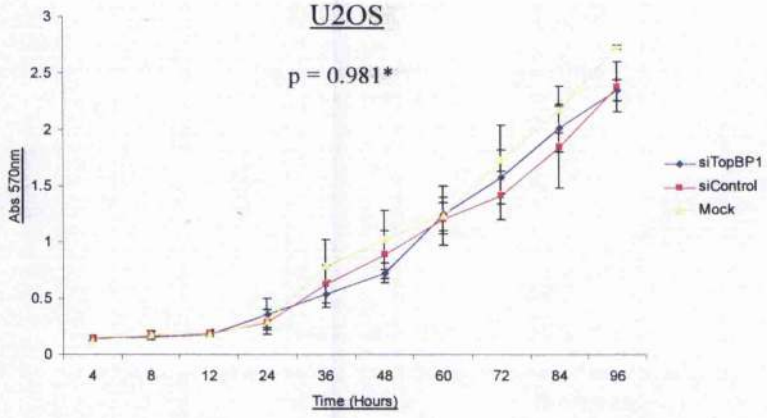


**Figure 49: TopBP1 is not Essential for Cell Growth in all Cell Lines.** a) MCF7 cells were treated with either siTopBP1, siluciferase or a mock siRNA, as described (2.2.2.3.). The cell viability was measured using the MTT assay (2.2.2.6.) at the time points indicated. This experiment was carried out three times in duplicate and this data shows the mean ± S.E.M. for the repeat experiments. b) 293T cells were treated with either siTopBP1, siluciferase or a mock siRNA, as described (2.2.2.3.). The cell viability was measured using the MTT assay (2.2.2.6.) at the time points indicated. This experiment was carried out three times in duplicate and this data shows the mean ± S.E.M. for the repeat experiments. a) U2OS cells were treated with either siTopBP1, siluciferase or a mock siRNA, as described (2.2.2.3.). The cell viability was measured using the MTT assay (2.2.2.6.) at the time points indicated. This experiment was carried out three times in duplicate and this data shows the mean ± S.E.M. for the repeat experiments. * Student paired T-Test results for siluciferase against siTopBP1, there is no significant difference between the growth of the siluciferase and siTopBP1 treated cells in 293T ($p= 0.968$), MCF7 ($p= 0.894$) or U2OS ($p=0.981$) in this assay, indicating that TopBP1 is not essential for replication in these cell lines.

**Figure 50**

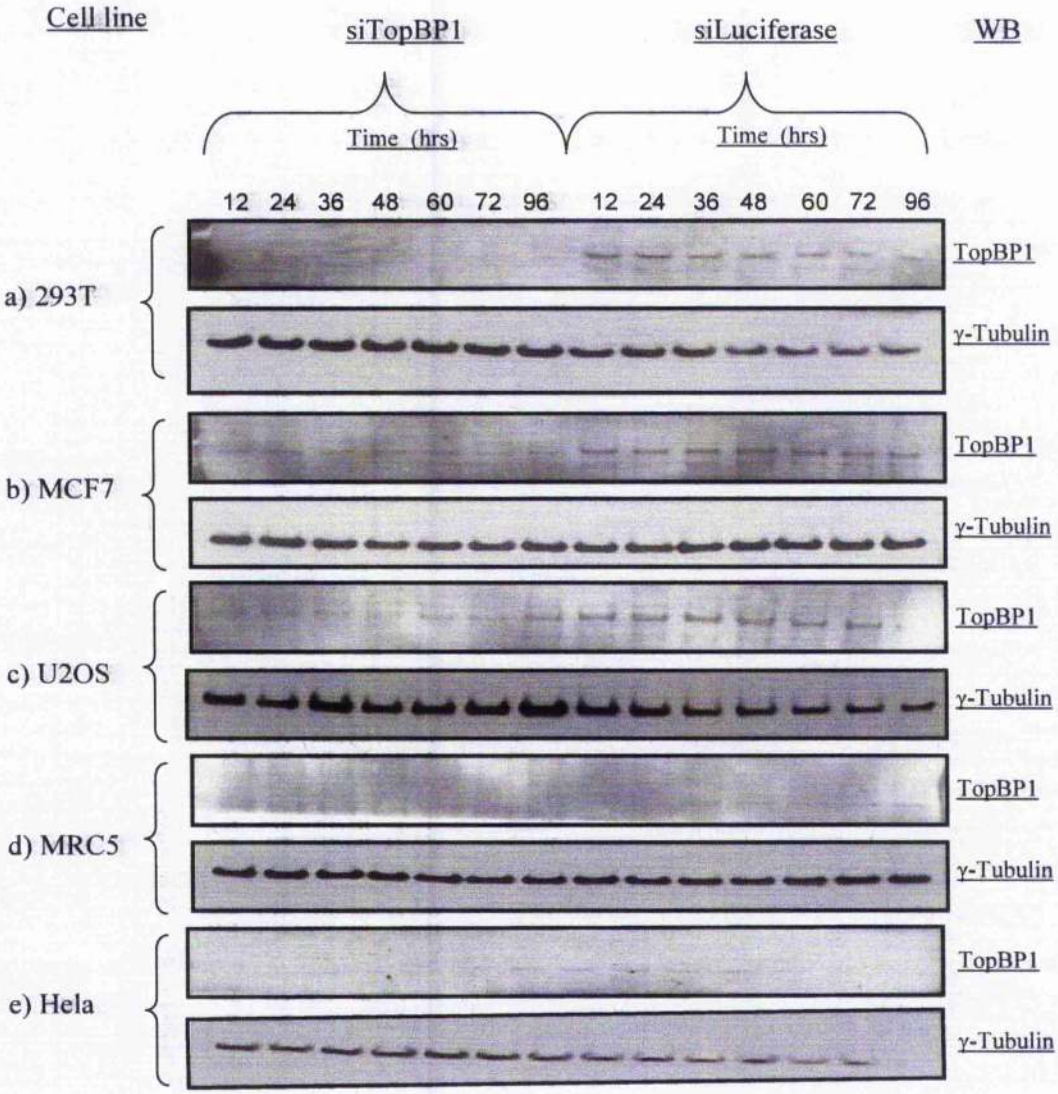

Cell line
siTopBP1
siLuciferase
WB
Time (hrs)
Time (hrs)
12 24 36 48 60 72 96 12 24 36 48 60 72 96
a) 293T
b) MCF7
c) U2OS
d) MRC5
e) Hela
TopBP1
γ-Tubulin

**Figure 50: TopBP1 Expression Levels following siRNA Treatment.** Total Cell lysates were harvested at the time points indicated and equal amounts of protein were separated on a SDS-PAGE gel (2.2.1.17.), the resultant blot was probed for the presence of TopBP1 and γ-tubulin as a loading control. The expression of these proteins following either siTopBP1 or siluciferase transfection over the time course (hours) are shown. Expression of these two proteins was examined from each cell line previously used in the MTT assays (Figure 48 and 49); a) 293T, b) MCF7, c) U2OS, d) MRC5 and e) Hela cells.

**Figure 51**

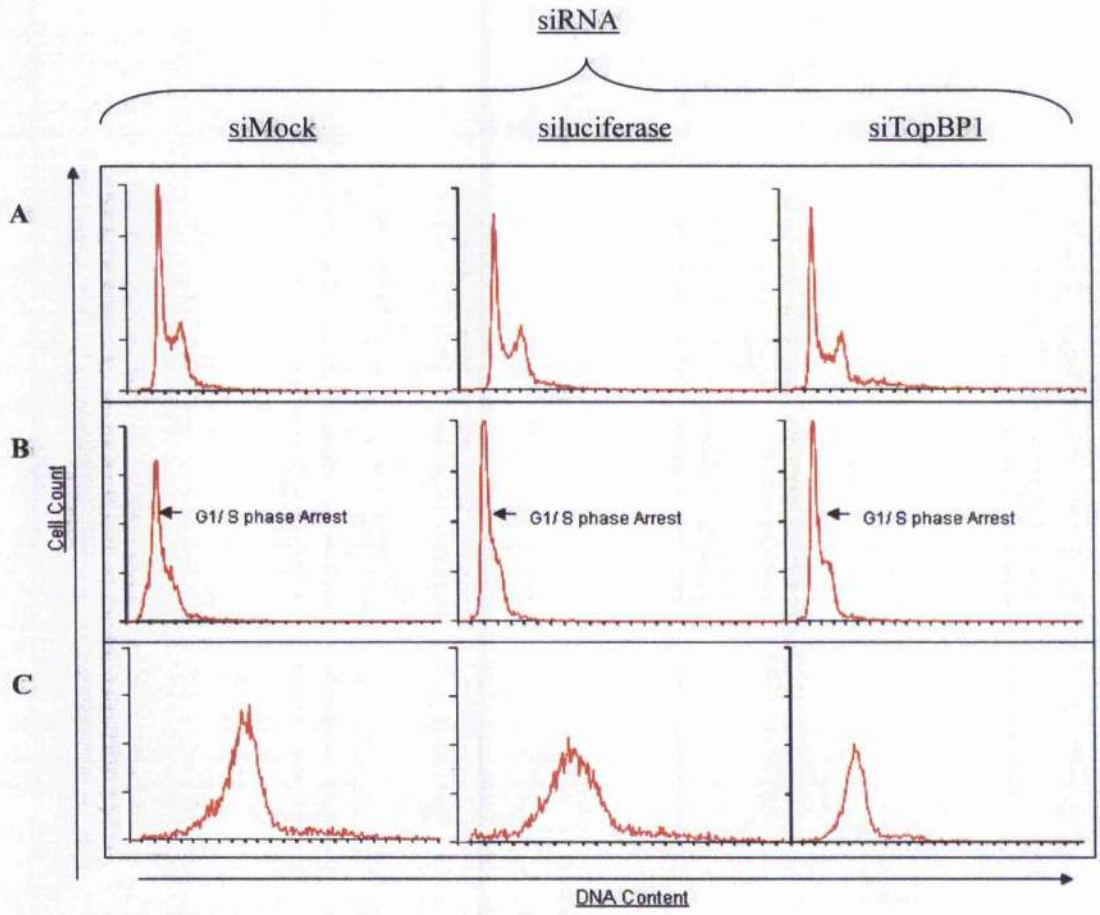

siRNA
siMock
siluciferase
siTopBP1
A
B
C
Cell Count
G1/ S phase Arrest
G1/ S phase Arrest
G1/ S phase Arrest
DNA Content

**Figure 51: The Cell Cycle is not effected by TopBP1 knock down.** FACS analysis of synchronized and unsynchronized MCF7 cells. The siRNA transfected into the cells is indicated at the top of the figure, either siTopBP1, siluciferase or a mock (lipofectamine only as a control). A) Cells were left untreated, no synchronization. B) Cells treated with aphidicholin for 16 hours but not released. A clear early S phase arrest can be observed the siRNA samples following aphidicolin treatment. C) Cells treated with aphidicolin for 16 hours and released for 4 hours before harvesting for FACS analysis. Both siTopBP1 and siluciferase treated cells proceed through S-phase and are not arrested. This experiment was performed twice in triplicate and the profiles shown here are representative of those obtained for all repeats.

### 3.3.2 Long Term Survival is Abolished when TopBP1 is knocked down

Although MCF7, 293T and U2OS cell lines showed no change in cell viability following TopBP1 knockdown (3.3.1), these MTT assays were carried out over 96 hours. It is possible that although cells might replicate for this length of time, a longer period in the absence of TopBP1 might result in cell death; perhaps by accumulation of damage.

To assay if TopBP1 is essential for replication over a longer period of time, crystal violet colony survival assays were carried out over 14 days (2.2.2.5.). MCF7 cells were chosen because they were originally derived from a breast adenocarcinoma and therefore any results would show more biological significance in the context of TopBP1 and breast cancer. In these assays TopBP1 was knocked down using TopBP1 targeted siRNA and similarly to the previous MTT assays; siluciferase and mock (lipofectamine only) controls were also used. The results for the colony survival assay are shown in Figure 52. Photographs of the stained colonies which remained at Day 14 are shown in Figure 52. This experiment was carried out three times in duplicate; this figure shows one of the duplicate plates from the same repeat experiment. It is visually very clear that knocking down TopBP1 expression has a profound effect on MCF7 cell growth over longer time periods than those previously investigated using the MTT assay (3.3.1).

Table 8 shows the mean colony survival numbers ± S.E.M. for the triplicate experiments, following siRNA treatment. There is no significant change in colony survival when comparing the mock and siluciferase transfected cells ($p=0.08$), indicating as a control the siluciferase has no effect on the cell survival. Compared to the mock transfected cells only 10% survived in the TopBP1 knocked down samples ($p = 0.00004$) and 13% compared to the siluciferase ($p = 0.0004$). It is possible that the 10% of cells which do survive were not affected by the TopBP1 targeted siRNA, as shown in Figure 50b the TopBP1 knockout is not complete, therefore the percentage of cells which do survive may contain TopBP1.

**Figure 52**

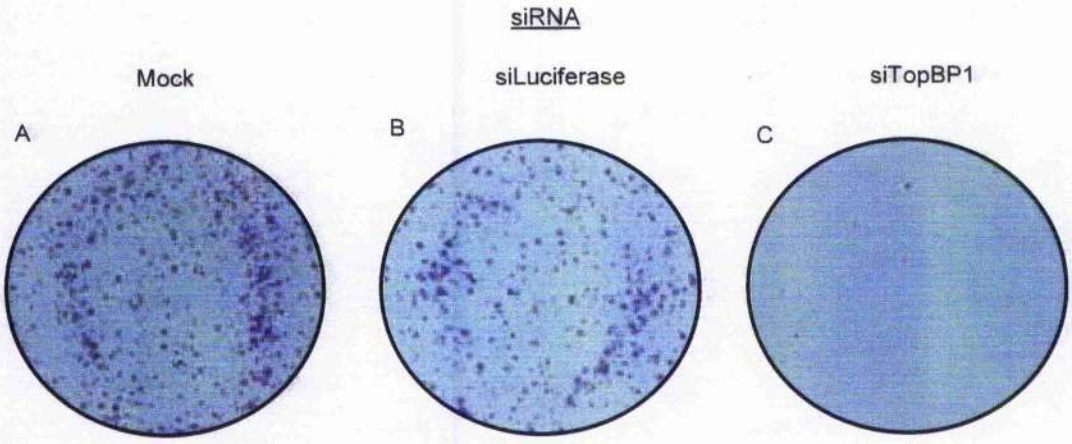



**Figure 52: TopBP1 is Essential for the Long Term survival of MCF7 cells.** Photographs from MCF7 colony survival assay. These experiments were carried out three times in duplicate. The plates shown here are single duplicates from the same repeat experiment and are representative of those obtained for the three experiments. A) MCF7 cells were plated out and mock transfected (lipofectamine only control). B) MCF7 cells transfected with anti-luciferase siRNA. C) MCF7 cells transfected with anti-TopBP1 siRNA. All colonies were visualized 14 days following siRNA treatment using a 0.5% crystal violet solution (2.2.2.5.).

**Table 8**

| | Mock | siRNA Luciferase | siRNA TopBP1 |
|---|---|---|---|
| Mean Colony Number Day 14 | 541 | 429 | 57 |
| Standard Error | 27.94 | 40.09 | 4.80 |
| T-Test* | | 0.0892 | 4.081E-06 |
| T-Test** | | | 0.0004 |

**Table 8: Mean colony Survival number following siRNA treatment in MCF7 cells.** This data shows the mean colony number/plate ± S.E.M for each siRNA oligo knockdown from the triplicate experiments. MCF7 cells were either transfected with a mock siRNA, anti-TopBP1 siRNA or anti-luciferase siRNA as described (2.2.2.3.), and the colonies were visualised at day 14 using a 0.5% crystal violet solution. * indicates a paired T-Test between the siluciferase or siTopBP1 and the mock data. ** indicates a paired T-Test between the siTopBP1 and siluciferase data.

### 3.3.3. Experimental Design and Controls

In order to investigate the genes regulated by TopBP1, microarray experiment was performed; in which TopBP1 was knocked down using siRNA. The experimental design is shown in Figure 53. MCF7 cells were chosen for this experiment as they are a breast carcinoma cell line and therefore provide a model for studying the role of TopBP1 and breast cancer than other cell lines and as shown in Chapter 3.3.2. these cells still grow following TopBP1 knockdown and the cell cycle is not effected, allowing a comparable gene expression profile. TopBP1 was knocked out in these cells using small interfering RNA (as described 2.2.2.3.); and similar to the previous experiments a mock siRNA targeted against luciferase was used as a siRNA control. Figure 54a shows the reduction in TopBP1 protein expression which results following siTopBP1 transfection. TopBP1 expression is reduced compared to the siluciferase control (Blot A). The luciferase control siRNA had no adverse effect on TopBP1 expression compared to the mock siRNA (lipofectamine only). The total cell lysates used in this experiment were harvested 48hours following siRNA treatment; the same time point at which the RNA was extracted for the microarray, therefore serving as a control for the microarray samples, confirming that TopBP1 expression was knocked down when the RNA was harvested. The same blot was probed with anti-γ-tubulin as a loading control (Figure 54a, blot B).

As TopBP1 is a DNA damage response protein, three separate gene expression profiles were determined from the experimental data; 1) Genes regulated by TopBP1, 2) Genes regulated by DNA damage and 3) Genes regulated by TopBP1 following DNA damage. To test if MCF7 cells respond and signal a DNA damage response following the dose of gamma irradiation given in this experiment (1Gy) the phosphorylation of γ-H2AX was determined using immunofluorescence (2.2.4.1. /2.). γ-H2AX is phosphorylated at S139 in response to DNA damage (1.5.1.). Therefore MCF7 cells were stained with a phospho S139 specific anti-γ-H2AX antibody. Cells were plated out on coverslips, and either damaged (1Gy) or left untreated (Control) and stained with DAPI and phosphorylated γ-H2AX. The images are shown in Figure 54b; following DNA damage MCF7 cells do show the induction of γ-H2AX foci demonstrating a DNA damage response.

**Figure 53**

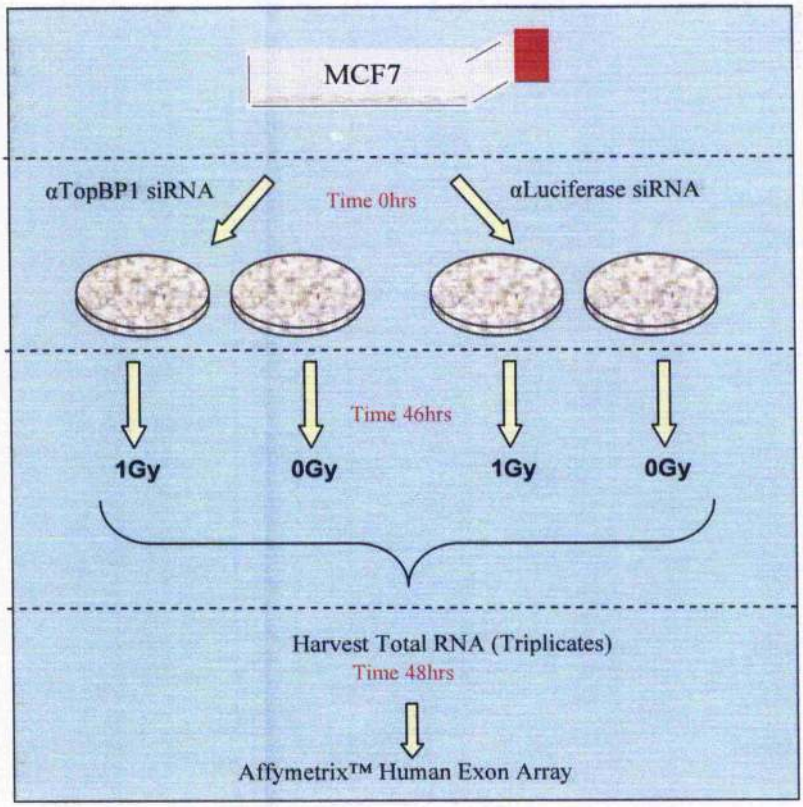
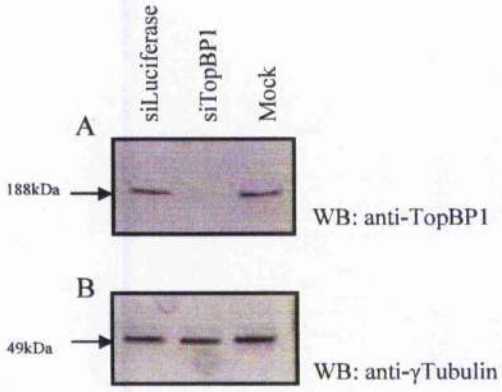



**Figure 53: Experimental Design of Microarray**. Diagrammatic representation showing the treatments of each microarray sample; MCF7 cells were either transfected with siTopBP1 or siluciferase, and damaged (1Gy) or left untreated (0Gy). These four RNA samples represent one repeat. Three repeats were taken for this experiment resulting in 12 RNA samples and 12 arrays in total.

**Figure 54**

a)


siLuciferase
siTopBP1
Mock
A
188kDa
WB: anti-TopBP1
B
49kDa
WB: anti-γTubulin

b)

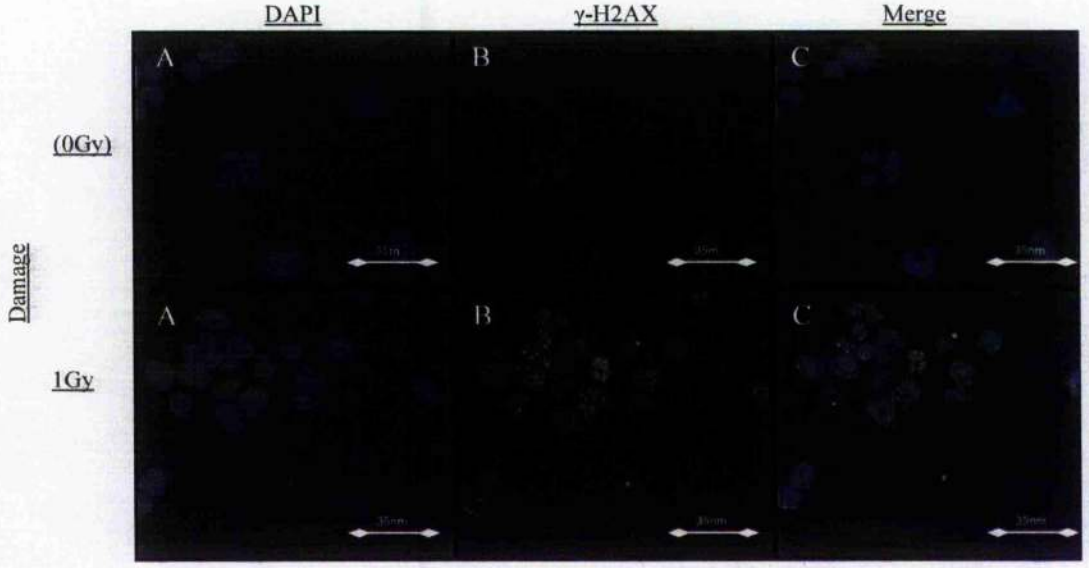

DAPI
γ-H2AX
Merge
A
B
C
(0Gy)
Damage
A
B
C
1Gy

**Figure 54: MCF7 controls for Microarray.** a) Western blot showing TopBP1 expression knock down; MCF7 cells were transfected with anti-TopBP1, anti-luciferase siRNA or mock (lipofectamine only) (2.2.2.3.). Cell lysates were harvested at day 3 (the same time RNA was harvested), and the proteins separated using SDS-PAGE gel and the resultant blot was probed for the presence of TopBP1 using a monoclonal TopBP1 antibody (BD Transduction Laboratories), Blot A. Blot B shows the expression of γ-tubulin (GTU-88, Abcam) as a loading control. b) MCF7 immunofluorescence staining following DNA damage. MCF7 cells were plated out onto glass coverslips; 3 days later the cells were either damaged (1Gy) or untreated (Control, 0Gy), fixed and stained (as described 2.2.4.1/2.). Coverslips were stained with the DNA damage marker γ-H2AX and DAPI to stain the nuclei. Panels A: DAPI only, B: γ-H2AX, C: Merge of two images. The staining of both control (undamaged) and damaged MCF7 is shown, showing that following DNA damage MCF7 form γ-H2AX foci.

To extract the RNA MCF7 cells were plated out and either siTopBP1 or siluciferase treated. 48 hours later, the cells were either left untreated or damaged (1Gy), and the RNA was harvested 4 hours later using Qiagen RNAeasy RNA extraction kit (as described 2.2.1.23.). This experiment was carried out three times, resulting in 3 repeat RNA samples for each condition (Figure 53). The concentration of RNA in each sample was measured (2.2.1.2.) as a minimum of 1.5µg RNA was required per sample to perform the array. The quantity and quality of the 12 RNA samples was further analysed by Jing Wang (Sir Henry Welcome Functional Genomics Facility (SHWFGF), University of Glasgow) using a 21000 Agilent Bioanalyser. The DNA was prepared using GeneChip cDNA synthesis and amplification kit (Affymetrix) also performed by Jing Wang (SHWFGF) and analysed using Affymetrix Exon Array software by Dr. Pawel Herzyk (SHWFGF).

### 3.3.4. Data Analysis

Analysis of the microarray core data was performed using ArrayAssist® software (Affymetrix). ArrayAssist® software is specifically designed for the analysis of Affymetrix Exon microarray chip data, and in this section data analysis was performed only to the level of gene expression. The core data was log transformed, instead of using linear data in order to perform the analysis using this software. Log transformation of the data makes the distribution of the data more symmetrical around the mean of the population, which reduces the influence of outliers and reduces the variance within the replicates.

#### 3.3.4.1. Quality Controls

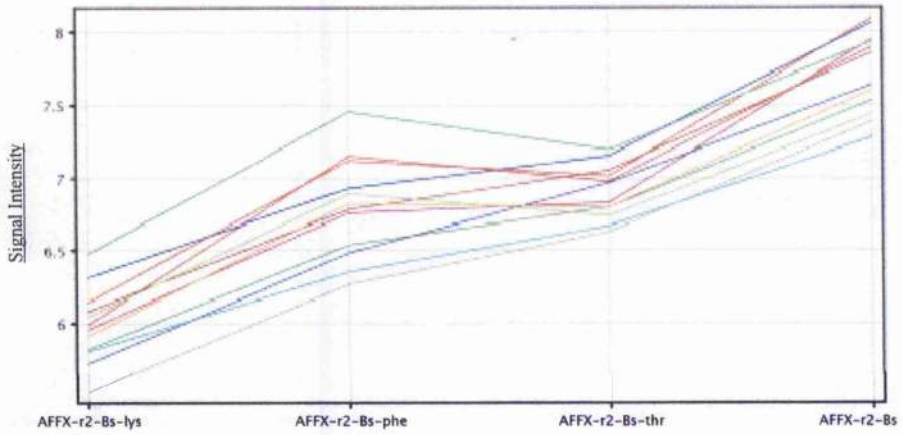
Before any gene expression analysis (3.4.5) was performed, the quality of the 12 arrays was tested using the ArrayAssist® software. These quality controls are an essential process in the analysis of the data; an array which fails these controls might alter the gene expression levels giving rise to false results. The first quality control run on this software was the poly-A control which monitors the entire labeling process. These controls were incorporated into the array at the start of the sample preparation process (Performed Jing Wang, SHWFGF). The GeneChip® Poly-A RNA Control kit (Affymetrix) contains a mixture of *lys, phe, thr* and *trp* (*Bacillus*

*subtilis* genes cloned into pBluescript vectors), at increasing concentrations 1:100,000, 1:50,000, 1:25,000, and 1:7,500 respectively relative to the total RNA in sample. Each of the 12 arrays is represented on the plot (Figure 55a) and the legend shows the array information (siTopBP1 or siluciferase), untreated or damaged (1Gy) and the repeat number (01, 02 or 03) for each array. These Poly-A controls provide exogenous positive controls to monitor the handling of the samples during preparation and as Figure 55a shows these internal controls all show the same trend (increasing concentration) on the plot therefore the samples were all treated in the same way and the gene expression profiles can be compared with confidence.

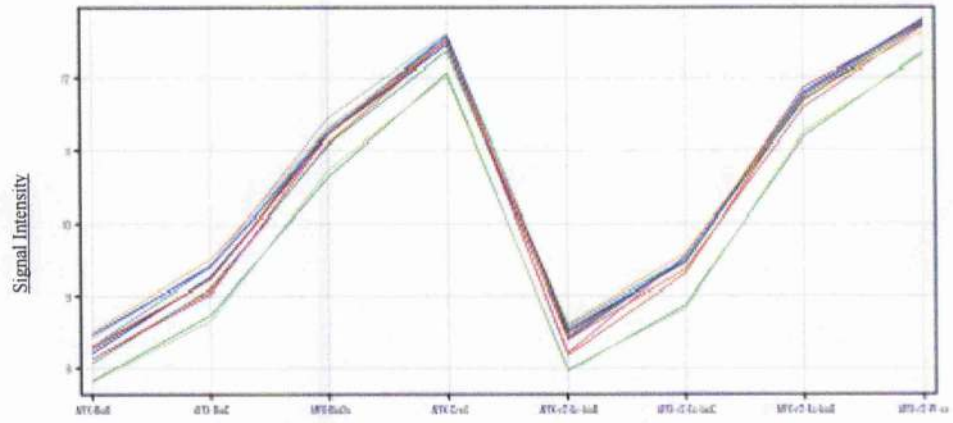
The second quality control performed was the hybridisation control; this plot gives a representation of the quality of hybridisation, measuring the intensities helps to monitor the entire labeling process independently of the starting RNA. The GeneChip® Eukaryotic Hybridisation control kit (Affymetrix) contains a mixture of biotin-labelled cRNA transcripts [bioB (1.5pM), bioC (5pM), bioD (25pM) and cre (10pM)]. These controls are added to hybridisation cocktail of the 12 arrays, and provide a method of comparing the replicates within the experiment for example, a degraded RNA sample or a sample that has not amplified correctly will show a lower intensity compared to the other repeats. All 12 arrays are represented in the plot (Figure 55b) and there are no arrays within this experiment, which do not follow the same hybridisation profile as the other repeats. These quality controls confirmed that further analysis of the arrays could take place without any repeat arrays being performed.

**Figure 55**

a)


Signal Intensity
8
7.5
7
6.5
6
AFFX-r2-Bs-lys
AFFX-r2-Bs-phe
AFFX-r2-Bs-thr
AFFX-r2-Bs

b)


Signal Intensity


Legend
Colour by array
α-Luciferase 01
α-Luciferase 02
α-Luciferase 03
α-Luciferase 1Gy01
α-Luciferase 1Gy02
α-Luciferase 1Gy03
α-TopBP1 01
α-TopBP102
α-TopBP1 03
α-TopBP1 1Gy01
α-TopBP1 1Gy02
α-TopBP1 1Gy03

**Figure 55: Microarray Quality Controls.** a) Poly-A internal control plot showing the signal intensities for each of the poly-A controls, from each of the 12 arrays. The information for each array is shown in the legend below; siRNA (TopBP1/Luciferase), DNA damage (untreated/ 1Gy) and repeat number (01, 02 and 03). b) Hybridisation plot for all 12 arrays. Array information is given in the legend as in a).

### 3.3.5. Genes Regulated by TopBP1

Analysis of the genes regulated by TopBP1 was performed using two Affymetrix Exon Microarray Software packages; ArrayAssist and Rank Products (RP (Breitlang *et al.* 2004), carried out by Pawel Herzyck (SHWFGF). Both analysis methods used a low level normalisation using Robust Multichip Average (RMA), described by Irizary *et al.* 2003, to correct the background levels for all probe sets, reducing non-specific binding signals. These two methods of analysis resulted in two sets of significant genes which were regulated by TopBP1. Only those genes which were significantly changed following both methods of analysis are shown in this chapter.

Table 9 shows the genes which are down regulated in the TopBP1 knock down cells by 1.5 fold or more, and those which have a p value of 0.05 or below. TopBP1 has the highest fold change, as would be expected and serves as a control for the siRNA. Table 10 shows the genes which are up regulated in the absence of TopBP1. Considering the roles of TopBP1 in the DNA damage response, transcription and cell cycle control, there are some genes within these lists which participate in these processes and are significantly regulated by TopBP1. For example; proteins involved in transcriptional regulation include; p300/CBP-associated factor ((PCAF), Reid *et al.* 1998), RAD23 homolog B (Yokoi *et al.* 2000) and three inhibitors of DNA binding (ID) homologues are significantly down regulated in the absence of TopBP1 ((DB2, IDI, and ID3).

There are also several genes regulated by TopBP1 which are involved in cell cycle control these include; S-phase kinase-associated protein 2 (SKP2), MAX dimerisation protein 1 (MXD1), SET domain containing protein 7 and 8 ((SETD7) Couture *et al.* 2005) and the death associated protein 3 (DAP3) which plays a role in both transcriptional activation and apoptosis (Hulkko *et al.* 2000).

## Table 9

| Transcript ID | Gene ID | Gene | p-value | Fold |
|---|---|---|---|---|
| 2695941 | NM_007027 | TOPBP1 // topoisomerase (DNA) II binding protein 1 | 2.33E-07 | -4.855 |
| 3151943 | NM_032026 | TATDN1 // TatD DNase domain containing 1 | 2.08E-02 | -4.280 |
| 2363074 | NR_002190 | SUMO1 pseudogene 3 | 4.77E-02 | -3.634 |
| 2947040 | NM_021066 | HIST1H2AJ // histone 1, H2aj | 3.81E-02 | -3.426 |
| 3991885 | NM_003928 | CXX1 // CAAX box 1 | 3.85E-05 | -2.831 |
| 2468622 | NM_001039 | D2B // inhibitor of DNA binding 2B, dominant negative helix-loop-helix | 1.08E-03 | -2.517 |
| 3568603 | NM_002083 | GPX2 // glutathione peroxidase 2 (gastrointestinal) | 1.67E-04 | -2.385 |
| 3273484 | NM_015155 | LARP5 // La ribonucleoprotein domain family, member 5 | 1.32E-03 | -2.287 |
| 2781813 | NM_024090 | ELOVL6 // ELOVL family member 6, elongation of long chain fatty acids | 6.05E-04 | -2.182 |
| 3564919 | NM_006832 | PLEKHC1 // pleckstrin homology domain containing, family C | 5.35E-05 | -2.176 |
| 3815399 | NM_201277 | CNN2 // calponin 2 | 2.92E-04 | -2.096 |
| 2836663 | NM_024632 | SAP30L // SAP30-like | 4.94E-06 | -2.064 |
| 3627076 | NM_004330 | BNIP2 // BCL2/adenovirus E1B 19kDa interacting protein 2 | 4.86E-05 | -2.057 |
| 3136413 | NM_017813 | IMPAD1 // inositol monophosphatase domain containing 1 | 9.82E-08 | -2.040 |
| 2931683 | NM_024573 | C6orf211 // chromosome 6 open reading frame 211 | 7.10E-06 | -1.989 |
| 2859667 | NM_197941 | ADAMTS6 // ADAM metallopeptidase with thrombospondin type 1 motif | 1.25E-03 | -1.988 |
| 4004819 | NM_006520 | DYNLT3 // dynein, light chain, Tctex-type 3 | 3.07E-04 | -1.976 |
| 2660030 | NM_033029 | Leishmanolysin-like (metallopeptidase M8 family) | 4.23E-02 | -1.966 |
| 2487549 | NM_002357 | MXD1 // MAX dimerization protein 1 | 9.79E-05 | -1.963 |
| 3543756 | NM_031427 | C14orf168 // chromosome 14 open reading frame 168 | 1.03E-02 | -1.899 |
| 2401493 | NM_002167 | ID3 // inhibitor of DNA binding 3, dominant negative helix-loop-helix | 7.18E-04 | -1.826 |
| 3034027 | NM_005494 | DNAJB6 // DnaJ (Hsp40) homolog, subfamily B, member 6 | 8.10E-03 | -1.755 |
| 3316344 | NM_173584 | EFCAB4A // EF-hand calcium binding domain 4A | 4.27E-03 | -1.746 |
| 2613441 | NM_003884 | PCAF // p300/CBP-associated factor | 2.98E-04 | -1.742 |
| 2817053 | NM_004866 | SCAMP1 // secretory carrier membrane protein 1 | 3.73E-04 | -1.722 |
| 2400373 | NM_003760 | EIF4G3 // eukaryotic translation initiation factor 4 gamma, 3 | 1.87E-03 | -1.711 |
| 2777412 | NM_032906 | PIGY // phosphatidylinositol glycan, class Y | 2.45E-04 | -1.703 |
| 3268274 | NM_021622 | PLEKHA1 // pleckstrin homology domain containing, family A | 2.71E-03 | -1.681 |
| 2866704 | NM_020801 | ARRDC3 // arrestin domain containing 3 | 1.17E-02 | -1.679 |
| 3535922 | NM_145251 | STYX // serine/threonine/tyrosine interacting protein | 4.72E-04 | -1.670 |
| 2777070 | NM_016245 | DHRS8 // dehydrogenase/reductase (SDR family) member 8 | 5.61E-03 | -1.670 |
| 3237088 | NM_003473 | STAM // signal transducing adaptor molecule (SH3 domain and ITAM motif) | 1.23E-05 | -1.669 |
| 3185498 | NM_001860 | SLC31A2 // solute carrier family 31 (copper transporters), member 2 | 4.05E-04 | -1.667 |
| 2361036 | NM_033657 | DAP3 // death associated protein 3 | 6.20E-05 | -1.664 |
| 3473331 | NM_024738 | C12orf49 // chromosome 12 open reading frame 49 | 2.82E-06 | -1.657 |
| 2694786 | NM_003925 | Methyl-CpG binding domain protein 4 (MBD4) | 4.63E-02 | -1.650 |
| 4045665 | NM_020672 | S100A14 // S100 calcium binding protein A14 | 1.87E-02 | -1.644 |
| 2722291 | NM_018317 | TBC1D19 // TBC1 domain family, member 19 | 6.92E-03 | -1.626 |
| 3881391 | NM_002165 | ID1 // inhibitor of DNA binding 1, dominant negative helix-loop-helix | 1.31E-02 | -1.615 |
| 2548274 | NM_003162 | STRN // striatin, calmodulin binding protein | 1.59E-04 | -1.605 |
| 2910477 | NM_033481 | FBXO9 // F-box protein 9 | 8.44E-04 | -1.599 |
| 2736750 | NM_001865 | Cytochrome c oxidase subunit VIIa COX7A2 | 2.55E-02 | -1.596 |
| 2786658 | NM_030648 | SET domain containing (lysine methyltransferase) 7 | 3.03E-02 | -1.594 |
| 2423017 | XM_930999 | LOC642939 // hypothetical protein LOC642939 | 3.04E-03 | -1.593 |
| 2876257 | NM_001033 | SAR1B // SAR1 gene homolog B (S. cerevisiae) | 1.67E-02 | -1.593 |
| 2356425 | XM_942453 | LOC652793 // similar to PDZ domain containing 1 | 4.70E-03 | -1.589 |
| 3791996 | NM_198833 | SERPINB8 // serpin peptidase inhibitor, clade B (ovalbumin) | 2.43E-03 | -1.585 |
| 3232944 | NM_001040 | AKR1CL2 // aldo-keto reductase family 1, member C-like 2 | 6.96E-03 | -1.581 |
| 2793346 | NM_000958 | Prostaglandin E synthase 3 | 4.93E-02 | -1.575 |
| 4000456 | NM_024087 | ASB9 // ankyrin repeat and SOCS box-containing 9 | 3.27E-02 | -1.575 |
| 2940929 | NM_004280 | EEF1E1 // eukaryotic translation elongation factor 1 epsilon 1 | 3.74E-02 | -1.559 |
| 3299469 | NM_144590 | ANKRD22 // ankyrin repeat domain 22 | 1.26E-03 | -1.558 |
| 2957462 | NM_001512 | GSTA4 // glutathione S-transferase A4 | 7.51E-03 | -1.555 |
| 3845620 | NM_017797 | BTBD2 // BTB (POZ) domain containing 2 | 3.87E-04 | -1.549 |
| 2921338 | NM_014891 | PDGFA associated protein 1 (PDAP1) | 4.67E-02 | -1.540 |
| 2450855 | NM_012396 | PHLDA3 // pleckstrin homology-like domain, family A, member 3 | 7.26E-04 | -1.540 |
| 3183757 | NM_002874 | RAD23B // RAD23 homolog B | 1.43E-04 | -1.529 |
| 2442103 | NM_000696 | ALDH9A1 // aldehyde dehydrogenase 9 family, member A1 | 1.49E-05 | -1.521 |
| 2457988 | NM_016096 | ZNF706 // zinc finger protein 706 | 6.02E-03 | -1.515 |
| 3012677 | NM_032120 | DKFZP564O0523 // hypothetical protein DKFZp564O0523 | 7.72E-03 | -1.512 |
| 2786657 | NM_030648 | SETD7 // SET domain containing (lysine methyltransferase) 7 | 2.54E-03 | -1.508 |
| 2806520 | NM_005983 | S-phase kinase-associated protein 2 (p45) (SKP2) | 3.35E-02 | -1.505 |
| 2926476 | NM_004865 | TBPL1 // TBP-like 1 | 1.31E-02 | -1.500 |

**Table 9: Genes Down Regulated following siTopBP1** This table shows the genes which are down regulated in the siTopBP1 samples. These transcripts were obtained by retaining those with a p value of 0.05 or below, and those with a fold change of 1.5 or above. Analysis of significant genes was performed using two exon microarray software packages; ArrayAssist and RP, and as discussed earlier the data presented here represents transcripts which were significantly changed following analysis with both software programs.

## Table 10

| Transcript ID | Gene ID | Gene | p-value | Fold |
|---|---|---|---|---|
| 2359352 | NM_178430 | LCE2D // late cornified envelope 2D | 9.6300E-05 | 5.6772 |
| 3403414 | NM_199286 | DPPA3 // developmental pluripotency associated 3 | 1.9338E-03 | 2.7132 |
| 3416834 | NM_054104 | OR6C3 // olfactory receptor, family 6, subfamily C, member 3 | 3.8087E-03 | 2.6674 |
| 2814154 | AF073520 | SERF1A // small EDRK-rich factor 1A (telomeric) | 3.5893E-02 | 2.1977 |
| 3583362 | NM_001005 | OR4M1// olfactory receptor, family 4, subfamily M, member 1 | 4.5222E-02 | 2.0657 |
| 3318253 | NM_001004 | OR51L1 // olfactory receptor, family 51, subfamily L, member 1 | 1.8145E-02 | 2.0439 |
| 3881045 | XM_945478 | RP13-401N8.2 // hypothetical gene supported by BC042812 | 1.7967E-02 | 1.9337 |
| 3860831 | NM_019855 | CABP5 // calcium binding protein 5 | 5.2304E-03 | 1.9305 |
| 2828146 | NM_020240 | CDC42 small effector 2 | 1.8561E-04 | 1.9052 |
| 3316987 | NM_001012 | KRTAP5-6 // keratin associated protein 5-6 | 2.0196E-02 | 1.8709 |
| 3360333 | NM_001005 | OR51A4 // olfactory receptor, family 51, subfamily A, member 4 | 1.9039E-03 | 1.8435 |
| 3360486 | NM_033179 | OR51B4// olfactory receptor, family 51, subfamily B, member 4 | 9.8707E-03 | 1.8288 |
| 3865867 | NM_001002 | IGFL4 // insulin-growth factor-like family member 4 | 1.6376E-02 | 1.8240 |
| 4047607 | NM_024850 | BTND8 // butyrophilin-like 8 | 4.8262E-02 | 1.7976 |
| 3928477 | NM_203405 | KRTAP26-1 // keratin-associated protein | 1.7762E-02 | 1.7887 |
| 3684548 | AK126166 // | LOC94431 // RNA polymerase I transcription factor RRN3-like | 4.3336E-02 | 1.7761 |
| 3713539 | XM_942620 | LOC652894 // similar to RIKEN cDNA 1810036I24 isoform 1 | 2.9916E-02 | 1.7670 |
| 3288482 | NM_001006 | LRRC18 // leucine rich repeat containing 18 | 8.1088E-03 | 1.7511 |
| 3195238 | NM_000832 | GRIN1 // glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 1.9991E-02 | 1.7317 |
| 3977686 | NM_174961 | SSX8 // synovial sarcoma, X breakpoint 8 | 1.0934E-02 | 1.7289 |
| 3883542 | NM_032194 | BXDC1 // brix domain containing 1 | 3.4389E-02 | 1.7261 |
| 3330551 | XM_941859 | LOC652421 // similar to tripartite motif protein 39 | 1.7836E-02 | 1.7068 |
| 3361238 | NM_003703 | OR2D2 // olfactory receptor, family 2, subfamily D, member 2 | 3.3984E-02 | 1.7043 |
| 3386038 | XM_930197 | LOC399937 // similar to RING finger protein 18 | 1.8292E-02 | 1.7003 |
| 3250650 | NM_001743 | CALM2 // calmodulin 2 (phosphorylase kinase, delta) | 4.8102E-02 | 1.6981 |
| 3919033 | NM_006933 | SLC5A3 // solute carrier family 5 (inositol transporters), member 3 | 1.9072E-04 | 1.6969 |
| 3662086 | NM_032935 | MT4 // metallothionein IV | 3.8902E-02 | 1.6810 |
| 3330786 | NM_001004 | OR5L2 // olfactory receptor, family 5, subfamily L, member 2 | 4.5987E-02 | 1.6716 |
| 3765848 | XM_927734 | LOC653498 // similar to TBC1 domain family, member 3B | 1.1066E-02 | 1.6627 |
| 2792459 | BC066960 | GKP3 // glycerol kinase pseudogene 3 | 2.5149E-02 | 1.6582 |
| 3955483 | NM_001001 | hA9F11.1 // hypothetical LOC255349 | 8.8643E-03 | 1.6537 |
| 2887930 | NM_001004 | FLJ16171 // FLJ16171 protein | 3.0117E-02 | 1.6508 |
| 2362230 | NM_030893 | CD1E // CD1e molecule | 3.4151E-03 | 1.6395 |
| 3369755 | NM_207428 | C11orf55 // chromosome 11 open reading frame 55 | 2.7072E-02 | 1.6258 |
| 2547090 | NM_020382 | SET domain containing (lysine methyltransferase) 8 (SETD8), | 3.2100E-02 | 1.6123 |
| 3160658 | NM_004170 | SLC1A1 // solute carrier family 1 (neuronal/epithelial high affinity) | 2.0587E-02 | 1.6034 |
| 3558145 | NM_014430 | CIDEB // cell death-inducing DFFA-like effector b | 1.8421E-02 | 1.6011 |
| 3595594 | NM_020980 | AQP9 // aquaporin 9 | 1.9613E-02 | 1.5981 |
| 3399922 | XM_942369 | IQSEC3 // IQ motif and Sec7 domain 3 | 3.4510E-02 | 1.5877 |
| 3815082 | NM_020637 | FGF22 // fibroblast growth factor 22 | 1.3786E-02 | 1.5787 |
| 3318141 | NM_001004 | OR52M1 // olfactory receptor, family 52, subfamily M, member 1 | 2.2401E-02 | 1.5778 |
| 3568485 | XM_929192 | LOC653716 // similar to spectrin, beta, erythrocytic | 4.2987E-02 | 1.5731 |
| 3721251 | NM_031962 | KRTAP9-3 // keratin-associated protein 9-3 | 1.2684E-02 | 1.5667 |
| 3904858 | NM_021081 | GHRH // growth hormone releasing hormone | 2.4480E-02 | 1.5649 |
| 3923896 | NM_198699 | KRTAP10-12 // keratin associated protein 10-12 | 4.1818E-03 | 1.5633 |
| 3957207 | NM_001037 | LOC652968 // hypothetical protein LOC652968 | 1.3500E-05 | 1.5594 |
| 3373046 | NM_001005 | OR4C12 // olfactory receptor, family 4, subfamily C, member 12 | 2.8680E-02 | 1.5580 |
| 3535780 | NM_000956 | PTGER2 // prostaglandin E receptor 2 (subtype EP2), 53kDa | 3.2035E-02 | 1.5531 |
| 3013255 | XM_928928 | PEG10 // paternally expressed 10 | 2.6448E-03 | 1.5527 |
| 3474787 | AK096009 // | AK096009 // C12orf27 // chromosome 12 open reading frame 27 | 1.8024E-02 | 1.5522 |
| 3361523 | NM_001004 | OR10A6 // olfactory receptor, family 10, subfamily A, member 6 | 2.9964E-03 | 1.5480 |
| 3399379 | NM_174927 | SPATA19 // spermatogenesis associated 19 | 1.3472E-02 | 1.5468 |
| 3472389 | NM_022363 | LHX5 // LIM homeobox 5 | 1.0772E-02 | 1.5445 |
| 3699390 | NM_001025 | CTRB2 // chymotrypsinogen B2 | 1.2053E-02 | 1.5439 |
| 3299578 | NM_003956 | CH25H // cholesterol 25-hydroxylase | 2.4815E-02 | 1.5374 |
| 3815710 | NM_001405 | EFNA2 // ephrin-A2 | 1.1037E-04 | 1.5324 |
| 3523978 | NM_000452 | SLC10A2 // solute carrier family 10 | 2.1421E-02 | 1.5315 |
| 3680249 | NM_021247 | PRM3 // protamine 3 | 3.2606E-02 | 1.5295 |
| 3882823 | NM_001672 | ASIP // agouti signaling protein, nonagouti homolog (mouse) | 1.9534E-02 | 1.5216 |
| 2939232 | NM_001069 | TUBB2A // tubulin, beta 2A | 1.6548E-02 | 1.5210 |
| 3989062 | NM_032498 | PEPP-2 // PEPP subfamily gene 2 | 2.0921E-02 | 1.5198 |
| 3331150 | NM_001005 | LRRC55 // leucine rich repeat containing 55 | 1.2855E-02 | 1.5160 |
| 3527641 | NM_006683 | FAM12A // family with sequence similarity 12, member A | 2.5245E-02 | 1.5085 |
| 3487824 | NM_001010 | C13orf21 // chromosome 13 open reading frame 21 | 4.6915E-02 | 1.5004 |

**Table 10: Genes Up Regulated following siTopBP1.** This table shows the genes which are up regulated in the siTopBP1 samples. These transcripts were obtained by retaining those with a p value of 0.05 or below, and those with a fold change of 1.5 or above. Analysis of significant genes was performed using two exon microarray software packages; ArrayAssist and RP, and as discussed earlier the data presented here represents transcripts which were significantly changed following analysis with both software programs.

### 3.3.6. Genes Regulated by DNA Damage

The second gene list which was extracted from the microarray data was the genes which were either down regulated or up regulated by DNA Damage in the presence of TopBP1. As described earlier (3.3.5.) this analysis was performed using two Affymetrix Exon Microarray Software packages; ArrayAssist and Rank Products (RP), carried out by SHWFGF), and only those genes which were significantly changed in both methods are shown in this chapter. Table 11 shows the genes which are down regulated following DNA damage by -1.5 fold or more, and those which have a p value of 0.05 or below. Table 12 shows the genes which are up regulated following DNA damage, under the same restrictions. Although these DNA damage regulated genes will be discussed in more detail later (Chapter 4.3.3.) it is reassuring that genes involved in the DNA damage response are present in the significant transcript lists. For example; N-myc interactor (involved in apoptosis and interacts with BRCA1 (Li *et al.* 2002)), excision repair cross complementing 8 (Groisman *et al.* 2003), inhibitor of growth family member 2 (Nagashima *et al.* 2001) and programmed cell death 10 (Plummer *et al.* 2005).

## Table 11

| Transcript ID: | Gene ID: | Gene | p-value | Fold |
|---|---|---|---|---|
| 2947040 | NM_021066 | HIST1H2AJ // histone 1, H2aj | 3.14E-02 | -3.64366 |
| 2461717 | NR_002855 | SNORA14A // small nucleolar RNA, H/ACA box 14A | 5.83E-06 | -2.45154 |
| 3348911 | XM_499315 | LOC442573 // similar to postmeiotic segregation increased 2-like 2 | 1.35E-02 | -2.1656 |
| 2979246 | NM_130900 | RAET1L // retinoic acid early transcript 1L | 1.29E-02 | -2.13007 |
| 3038617 | NM_002489 | NDUFA4 // NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 | 5.74E-06 | -2.09816 |
| 2468522 | NM_001039 | ID2B // inhibitor of DNA binding 2B, dominant negative helix-loop-helix | 4.87E-03 | -2.04186 |
| 3208355 | NM_199244 | FOXD4L4 // forkhead box D4-like 4 | 2.16E-02 | -1.99427 |
| 2944025 | NM_000367 | TPMT // thiopurine S-methyltransferase | 2.68E-02 | -1.94849 |
| 3717034 | XR_000654 | LOC400590 // hypothetical LOC400590 | 9.50E-03 | -1.93935 |
| 2707859 | NM_201261 | DNAJC19 // DnaJ (Hsp40) homolog, subfamily C, member 19 | 1.41E-02 | -1.93282 |
| 3025433 | XM_935707 | LOC441262 // similar to aldo-keto reductase family 1, member B10 | 1.93E-02 | -1.90769 |
| 3063380 | NM_024498 | ZNF117 // zinc finger protein 117 (HPF9) | 1.26E-02 | -1.90524 |
| 2788145 | NM_017645 | COMMD8 // COMM domain containing 8 | 9.65E-03 | -1.88148 |
| 2403470 | NM_014260 | DNAJC8 // DnaJ (Hsp40) homolog, subfamily C, member 8 | 1.02E-03 | -1.87412 |
| 2382781 | XM_938194 | LOC127602 // hypothetical protein LOC127602 | 1.77E-02 | -1.84088 |
| 2832387 | NM_019120 | PCDHB8 // protocadherin beta 8 | 6.06E-04 | -1.8266 |
| 2566845 | NM_138798 | LOC129531 // hypothetical protein BC018453 | 3.99E-02 | -1.79283 |
| 2363618 | NM_001035 | SDHC // succinate dehydrogenase complex, subunit C | 2.42E-02 | -1.7871 |
| 2815139 | NM_138782 | ECHO2 // ECH domain only 2 | 7.96E-04 | -1.78403 |
| 2580965 | NM_004688 | NMI // N-myc (and STAT) interactor | 4.99E-02 | -1.78748 |
| 2380785 | NM_138794 | LYPLAL1 // lysophospholipase-like 1 | 2.45E-02 | -1.75313 |
| 2522588 | NM_002491 | NDUFB3 // NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3 | 1.87E-02 | -1.74339 |
| 2466493 | NM_033213 | ZNF670 // zinc finger protein 670 | 3.21E-04 | -1.73009 |
| 2587747 | NM_004882 | CIR // CBF1 interacting corepressor | 6.86E-03 | -1.72636 |
| 3826504 | NM_133473 | ZNF431 // zinc finger protein 431 | 7.12E-04 | -1.7217 |
| 2418461 | NM_001889 | CRYZ // crystallin, zeta (quinone reductase) | 8.37E-03 | -1.71511 |
| 3107828 | XM_931334 | similar to pleckstrin homology domain containing | 1.55E-02 | -1.69534 |
| 2940920 | NM_004280 | EEF1E1 // eukaryotic translation elongation factor 1 epsilon 1 | 1.85E-02 | -1.69128 |
| 2389130 | XM_936697 | hypothetical protein LOC647651 | 1.31E-02 | -1.68908 |
| 2836886 | NM_014180 | MRPL22 // mitochondrial ribosomal protein L22 | 2.68E-02 | -1.68379 |
| 2603887 | NM_145702 | TIGD1 // tigger transposable element derived 1 | 6.95E-04 | -1.67963 |
| 2773545 | NM_001729 | BTC // betacellulin | 4.40E-03 | -1.67331 |
| 2966232 | NM_017421 | COQ3 // coenzyme Q3 homolog, methyltransferase | 3.32E-02 | -1.67088 |
| 2424148 | XM_926707 | LOC642617 // hypothetical protein LOC642617 | 3.49E-03 | -1.66509 |
| 2858668 | NM_001007 | ERCC6 // excision repair cross-complementing | 5.71E-03 | -1.66371 |
| 3004665 | XM_927547 | LOC653473 // similar to Zinc finger protein 568 | 2.84E-04 | -1.65677 |
| 2875634 | NM_017665 | ZCCHC10 // zinc finger, CCHC domain containing 10 | 1.49E-02 | -1.64921 |
| 2559690 | NM_016058 | TPRKB // TP53RK binding protein | 1.42E-03 | -1.64637 |
| 2783473 | NM_001001 | LOC401152 // HCV F-transactivated protein 1 | 1.87E-02 | -1.64308 |
| 3504054 | NM_001039 | ZMYM5 // zinc finger, MYM-type 5 | 8.34E-03 | -1.63464 |
| 2512930 | NM_012198 | GCA // grancalcin, EF-hand calcium binding protein | 2.36E-03 | -1.62732 |
| 2589291 | NM_003319 | TTN // titin | 4.92E-02 | -1.6256 |
| 2740005 | NM_016648 | HDGMA18P // HDGMA18P protein | 6.48E-04 | -1.62199 |
| 2704188 | NM_145859 | PDCD10 // programmed cell death 10 | 7.11E-03 | -1.6182 |
| 3870135 | NM_032584 | ZNF347 // zinc finger protein 347 | 5.91E-03 | -1.61748 |
| 2361241 | NM_014017 | MAPBP1P // mitogen-activated protein-binding protein-interacting protein | 4.83E-02 | -1.61583 |
| 2810395 | NM_153706 | MGC33648 // hypothetical protein MGC33648 | 2.05E-02 | -1.61307 |
| 2534564 | NM_080678 | UBE2F // ubiquitin-conjugating enzyme E2F (putative) | 3.82E-02 | -1.61292 |
| 2821981 | NM_198507 | TMEM157 // transmembrane protein 157 | 4.04E-03 | -1.60153 |
| 2514497 | NM_144711 | KLHL23 // kelch-like 23 (Drosophila) | 2.73E-02 | -1.59794 |
| 2813481 | NM_001799 | CDK7 // cyclin-dependent kinase 7 | 5.96E-03 | -1.59464 |
| 2788143 | NM_014885 | ANAPC10 // anaphase promoting complex subunit 10 | 5.02E-03 | -1.59362 |
| 2517737 | NM_019091 | PLEKHA3 // pleckstrin homology domain containing, family A | 1.83E-04 | -1.5918 |
| 2603597 | NM_002601 | PDE6D // phosphodiesterase 6D, cGMP-specific, rod, delta | 3.03E-03 | -1.59159 |
| 2761151 | NM_001017 | RAB28 // RAB28, member RAS oncogene family | 2.83E-03 | -1.58786 |
| 3130950 | NM_016027 | LACTB2 // lactamase, beta 2 | 1.13E-02 | -1.5862 |
| 3018535 | XM_935514 | LOC653836 // similar to B-cell receptor-associated protein 29 | 3.48E-03 | -1.58202 |
| 2753952 | NM_001564 | ING2 // inhibitor of growth family, member 2 | 1.82E-02 | -1.5783 |
| 2366132 | NM_001031 | TIPRL // TIP41, TOR signalling pathway regulator-like | 7.42E-03 | -1.57395 |
| 2745067 | NM_153702 | ELMOD2 // ELMO/CED-12 domain containing 2 | 5.73E-03 | -1.57039 |
| 2544179 | NM_016047 | SF3B14 // splicing factor 3B, 14 kDa subunit | 8.66E-03 | -1.56703 |
| 2633631 | NM_020202 | NM_020202 // NIT2 // nitrilase family, member 2 | 1.89E-02 | -1.56602 |
| 2429842 | XM_944043 | LOC148786 // hypothetical LOC148786 | 6.44E-04 | -1.5659 |

**Table 11 continued**

| Transcript ID. | Gene ID | Gene | p-value | Fold |
|---|---|---|---|---|
| 2374956 | NM_006335 | TIMM17A // translocase of inner mitochondrial membrane 17 homolog A | 4.47E-02 | -1.56551 |
| 2423625 | NM_002061 | GCLM // glutamate-cysteine ligase, modifier subunit | 1.44E-02 | -1.56536 |
| 2415728 | NM_032027 | TM2D1 // TM2 domain containing 1 | 2.19E-04 | -1.55812 |
| 2422885 | XM_937148 | LOC646821 // similar to cytoplasmic beta-actin | 2.31E-02 | -1.55796 |
| 3559690 | XM_113763 | C14orf125 // chromosome 14 open reading frame 125 | 1.22E-02 | -1.55633 |
| 2469084 | NM_005680 | TAF1B // TATA box binding protein (TBP)-associated factor, | 6.87E-03 | -1.55569 |
| 2946714 | NM_080593 | HIST1H2BK // histone 1, H2bk | 6.31E-03 | -1.55534 |
| 2960872 | NM_138441 | C6orf150 // chromosome 6 open reading frame 150 | 8.20E-03 | -1.55461 |
| 2702154 | NM_007107 | SSR3 // signal sequence receptor, gamma | 5.83E-03 | -1.55105 |
| 2627080 | NM_020685 | C3orf14 // chromosome 3 open reading frame 14 | 7.36E-04 | -1.55023 |
| 3183219 | NM_207647 | FSD1L // fibronectin type III and SPRY domain containing 1-like // | 2.42E-02 | -1.55 |
| 3391255 | NM_001562 | IL18 // interleukin 18 (interferon-gamma-inducing factor) | 3.46E-03 | -1.54999 |
| 2700500 | NM_016094 | COMMD2 // COMM domain containing 2 | 2.54E-03 | -1.5485 |
| 2691421 | NM_006287 | TFPI // tissue factor pathway inhibitor | 1.15E-02 | -1.54581 |
| 3976450 | NM_205856 | RP11-38O23.2 // RNPK6288 | 9.13E-03 | -1.54525 |
| 2792127 | NM_000909 | NPY1R // neuropeptide Y receptor Y1 | 1.75E-02 | -1.54255 |
| 3857105 | NM_003430 | ZNF91 // zinc finger protein 91 // 19p13.1-p12 // 7644 /// XM_92986 | 4.85E-02 | -1.54189 |
| 2676446 | NM_002488 | NDUFA2 // NADH dehydrogenase | 4.68E-02 | -1.5416 |
| 2474071 | NM_012326 | MAPRE3 // microtubule-associated protein, RP/EB family, member 3 | 1.99E-03 | -1.54123 |
| 2829542 | NM_152409 | C5orf24 // chromosome 5 open reading frame 24 | 1.07E-03 | -1.53748 |
| 2483016 | NM_080667 | CCDC104 // coiled-coil domain containing 104 | 1.84E-02 | -1.53619 |
| 2780656 | NM_020395 | INTS12 // integrator complex subunit 12 | 9.21E-03 | -1.53413 |
| 2711034 | NM_178496 | C3orf59 // chromosome 3 open reading frame 59 | 1.08E-02 | -1.53101 |
| 2898562 | NM_018473 | THEM2 // thioesterase superfamily member 2 | 1.85E-02 | -1.53074 |
| 3721124 | NM_145274 | TMEM99 // transmembrane protein 99 | 6.78E-03 | -1.52994 |
| 2448971 | NM_015984 | UCHL5 // ubiquitin carboxyl-terminal hydrolase L5 | 1.20E-02 | -1.52883 |
| 2924619 | NM_138571 | HINT3 // histidine triad nucleotide binding protein 3 | 2.96E-03 | -1.52864 |
| 2472914 | NM_181713 | UBXD4 // UBX domain containing 4 // | 3.14E-03 | -1.52859 |
| 2746269 | NM_007080 | LSM6 // LSM6 homolog, U6 small nuclear RNA associated | 2.71E-02 | -1.52745 |
| 2871717 | NM_152549 | CCDC112 // coiled-coil domain containing 112 | 1.36E-02 | -1.52676 |
| 2546285 | NM_017910 | FLJ20628 // hypothetical protein FLJ20628 | 1.36E-02 | -1.52613 |
| 3536396 | NM_006568 | CGRRF1 // cell growth regulator with ring finger domain 1 | 1.55E-02 | -1.52489 |
| 2351817 | NM_001688 | ATP5F1 // ATP synthase, H+ transporting, mitochondrial F0 complex, | 3.34E-02 | -1.52054 |
| 3387171 | NM_016403 | HSPC148 // hypothetical protein HSPC148 / | 1.50E-02 | -1.51749 |
| 3790479 | NM_033280 | SEC11L3 // SEC11-like 3 (S. cerevisiae) | 1.38E-02 | -1.51392 |
| 3104323 | NM_016010 | C8orf70 // chromosome 8 open reading frame 70 | 2.71E-02 | -1.51276 |
| 2809423 | NM_003950 | F2RL3 // coagulation factor II (thrombin) receptor-like 3 | 3.62E-02 | -1.51091 |
| 3070658 | NM_005000 | NDUFA5 // NADH dehydrogenase (ubiquinone) | 3.19E-02 | -1.50726 |
| 2875419 | NM_007054 | KIF3A // kinesin family member 3A | 1.24E-02 | -1.50308 |

**Table 11: Genes Down-regulated Following DNA damage:** This table list the genes which were significantly ($p<0.05$) up-regulated (Fold >1.5) following DNA damage. Analysis of significant genes was performed using two exon microarray software packages; ArrayAssist and RP, and as discussed earlier the data presented here represents transcripts which were significantly changed following analysis with both software programs.

## Table 12

| Transcript ID | Gene ID | Gene | p-value | Fold |
|---|---|---|---|---|
| 2329131 | NM_002143 | HPCA // hippocalcin | 2.48E-03 | 1.50407 |
| 2335048 | XM_940223 | LOC654164 // similar to cytochrome P450, family 4, subfamily A | 8.63E-03 | 1.81121 |
| 2359362 | NM_178430 | LCE2D // late cornified envelope 2D | 1.72E-05 | 3.08409 |
| 2360377 | NM_178428 | LCE2A // late cornified envelope 2A | 3.06E-02 | 1.64486 |
| 2359431 | NM_178354 | LCE1F // late cornified envelope 1F | 3.33E-02 | 1.99373 |
| 2359891 | NM_002963 | S100 calcium binding protein A7 (psoriasin 1) | 1.64E-02 | 1.52063 |
| 2362230 | NM_030883 | CD1E // CD1e molecule | 6.36E-03 | 1.55475 |
| 2362537 | NM_002001 | FCER1A //Fc fragment of IgE, high affinity I, receptor | 2.51E-02 | 1.60225 |
| 2367606 | AK056349 // | CHRM3 // cholinergic receptor, muscarinic 3 | 2.10E-03 | 1.90611 |
| 2368146 | NM_199546 | NM_198548 //SPATA21 //spermatogenesis associated 21 // 1p36.13 // 374955 /// B | 1.38E-02 | 1.61583 |
| 2407496 | NM_002699 | NM_002699 //POU3F1 // POU domain, class 3, transcription factor 1 // 1p34.1 // | 5.83E-03 | 1.57182 |
| 2542420 | NM_145260 | OSR1 // odd-skipped related 1 (Drosophila) | 7.28E-03 | 1.50743 |
| 2560881 | XM_942076 | LOC647278 // similar to Leucine-rich repeat transmembrane | 9.13E-03 | 1.62418 |
| 2564816 | XR_000661 | LOC150759 // hypothetical protein LOC150759 | 5.51E-03 | 1.84772 |
| 2578608 | XM_497677 | LOC391444 // hypothetical LOC391444 | 1.47E-02 | 1.51492 |
| 2611546 | NM_001998 | FBLN2 // fibulin 2 | 9.41E-04 | 1.50845 |
| 2672442 | NM_000258 | MYL3 // myosin, light polypeptide 3, | 9.79E-03 | 1.60956 |
| 2748605 | NM_004744 | LRAT // lecithin retinol acyltransferase | 1.31E-02 | 1.54331 |
| 2779199 | NM_000667 | ADH1A // alcohol dehydrogenase 1A (class I), alpha polypeptide | 3.70E-03 | 1.75928 |
| 2792459 | BC068980 // | GKP3 // glycerol kinase pseudogene 3 | 4.22E-02 | 1.55971 |
| 2795904 | NM_153008 | FLJ30277 // hypothetical protein FLJ30277 | 8.43E-03 | 1.9022 |
| 2892734 | AF210650 // | C6orf60 // chromosome 6 open reading frame 60 | 4.83E-02 | 1.51919 |
| 2903777 | AY383627 // | GGNBP1 // gametogenetin binding protein 1 | 3.99E-02 | 1.86137 |
| 2937625 | NM_025002 | C6orf208 // chromosome 6 open reading frame 208 | 2.61E-02 | 1.55386 |
| 2939232 | NM_001069 | TUBB2A // tubulin, beta 2A | 1.54E-02 | 1.53082 |
| 3013952 | NM_020196 | ACN9 // ACN9 homolog (S. cerevisiae) | 1.63E-02 | 1.53382 |
| 3029434 | NM_001004 | OR2F2 // olfactory receptor, family 2, subfamily F, member 2 | 1.93E-02 | 1.60358 |
| 3031573 | NM_018384 | GIMAP5 // GTPase, IMAP family member 5 | 6.01E-03 | 1.51344 |
| 3040465 | NM_152774 | MGC42090 // hypothetical protein MGC42090 | 3.71E-02 | 1.63035 |
| 3086809 | NM_020844 | KIAA1456 // KIAA1456 protein | 2.55E-03 | 1.5009 |
| 3147699 | NM_080651 | THRAP6 // thyroid hormone receptor associated protein 6 | 5.19E-03 | 1.50023 |
| 3185953 | NM_001040 | LOC286334 // hypothetical protein LOC286334 | 5.11E-03 | 1.62499 |
| 3191674 | NM_021619 | PRDM12 // PR domain containing 12 | 2.70E-02 | 1.5813 |
| 3203524 | NR_002823 | AQP7P2 // aquaporin 7 pseudogene 2 | 3.71E-03 | 1.62919 |
| 3218209 | NM_147180 | PPP3R2 // protein phosphatase 3 (formerly 2B), regulatory subunit B | 2.77E-02 | 1.60975 |
| 3218496 | NM_001004 | OR13C2 // olfactory receptor, family 13, subfamily C, member 2 | 2.00E-02 | 1.9015 |
| 3250650 | NM_001743 | CALM2 // calmodulin 2 (phosphorylase kinase, delta) | 1.07E-02 | 2.12128 |
| 3316957 | NM_001012 | KRTAP5-6 // keratin associated protein 5-6 | 7.58E-03 | 2.1555 |
| 3318589 | NM_001005 | OR52W1 // olfactory receptor, family 52, subfamily W, member 1 | 3.49E-02 | 1.82922 |
| 3322638 | NM_054031 | MRGPRX3 // MAS-related GPR, member X3 | 3.51E-03 | 1.57167 |
| 3330693 | NM_001004 | OR4C16 // olfactory receptor, family 4, subfamily C, member 16 | 4.17E-02 | 1.61851 |
| 3335124 | NM_145719 | TIGD3 // tigger transposable element derived 3 | 2.95E-03 | 2.00656 |
| 3360486 | NM_033179 | OR51B4 // olfactory receptor, family 51, subfamily B, member 4 | 2.93E-02 | 1.60866 |
| 3369755 | NM_207426 | C11orf55 // chromosome 11 open reading frame 55 | 2.44E-02 | 1.64566 |
| 3373411 | NM_001004 | OR5R1 // olfactory receptor, family 5, subfamily R, member 1 | 1.73E-02 | 1.52718 |
| 3386038 | XM_930167 | LOC399937 // similar to RING finger protein 18 | 4.02E-02 | 1.55195 |
| 3395691 | NM_001040 | SCN3B // sodium channel, voltage-gated, type III, beta | 1.98E-02 | 1.53521 |
| 3416834 | NM_054104 | OR6C3 // olfactory receptor, family 6, subfamily C, member 3 | 2.42E-02 | 1.96689 |
| 3420316 | NM_003483 | HMGA2 // high mobility group AT-hook 2 | 4.03E-02 | 1.61217 |
| 3437780 | NM_007197 | frizzled homolog 10 (Drosophila) | 4.33E-03 | 1.55502 |
| 3455478 | NM_173086 | KRT6E // keratin 6E | 9.21E-03 | 1.51382 |
| 3487824 | NM_001010 | C13orf21 // chromosome 13 open reading frame 21 | 3.44E-02 | 1.55284 |
| 3527831 | NM_032572 | ribonuclease, RNase A family, 7 | 7.69E-03 | 1.56213 |
| 3595694 | NM_020980 | AQP9 // aquaporin 9 | 3.14E-02 | 1.52149 |
| 3652749 | NM_006043 | HS3ST2 // heparan sulfate (glucosamine) 3-O-sulfotransferase 2 | 7.03E-03 | 1.51991 |
| 3680223 | NM_002761 | PRM1 // protamine 1 | 2.24E-02 | 1.59504 |
| 3680248 | NM_021247 | PRM3 // protamine 3 | 3.86E-02 | 1.5026 |
| 3715476 | NR_002181 | PPY2 // pancreatic polypeptide 2 | 7.50E-04 | 1.51708 |
| 3756668 | XM_927544 | LOC644398 // similar to keratin associated protein 3.1 | 8.44E-04 | 1.6271 |
| 3815005 | NM_005317 | GZMM // granzyme M (lymphocyte met-ase 1) | 2.89E-03 | 1.54881 |
| 3817651 | BC090884 // | C19orf30 // chromosome 19 open reading frame 30 | 4.56E-03 | 1.5287 |
| 3866831 | NM_019856 | CABP5 // calcium binding protein 5 | 2.65E-02 | 1.59926 |
| 3867660 | NM_006179 | NTF5 // neurotrophin 5 (neurotrophin 4/5) | 7.08E-03 | 1.71782 |
| 3882367 | NM_130852 | PLUNC // palate, lung and nasal epithelium carcinoma associated | 2.22E-02 | 1.56414 |

**Table 12 continued**

| Transcript ID | Gene-ID | Gene | p-value | Fold |
|---|---|---|---|---|
| 3886994 | NM_080763 | WFDC10A // WAP four-disulfide core domain 10A | 2.08E-02 | 1.51182 |
| 3904858 | NM_021081 | GHRH // growth hormone releasing hormone | 2.48E-02 | 1.56304 |
| 3923850 | AJ566385 // | KRTAP10-7 // keratin associated protein 10-7 | 2.13E-02 | 1.93746 |
| 3923896 | NM_198699 | KRTAP10-12 // keratin associated protein 10-12 | 2.94E-03 | 1.60882 |
| 3934573 | NM_198698 | KRTAP10-6 // keratin associated protein 10-6 | 1.64E-02 | 1.54809 |
| 3944210 | NM_014310 | RASD2 // RASD family, member 2 | 1.18E-02 | 1.96583 |
| 3960605 | NM_004981 | KCNJ4 // potassium inwardly-rectifying channel, subfamily J | 8.15E-03 | 1.59144 |
| 3963913 | BC009368 // | LOC554174 // hypothetical LOC554174 | 1.22E-02 | 1.59612 |
| 3977886 | NM_174961 | SSX8 // synovial sarcoma, X breakpoint 8 | 7.86E-03 | 1.7943 |
| 4007899 | NM_003179 | SYP // synaptophysin | 2.85E-03 | 1.55649 |
| 4010768 | NM_018684 | KIAA1166 // KIAA1166 // Xq11.1 | 3.08E-04 | 1.63738 |
| 4016398 | NM_001006 | TCEAL8 // transcription elongation factor A (SII)-like 8 | 4.81E-02 | 1.52951 |
| 4046481 | XM_930939 | LOC653066 // similar to inhibitor of growth family, member 5 | 7.76E-03 | 1.60823 |
| 4047607 | NM_024850 | BTNL8 // butyrophilin-like 8 | 3.54E-02 | 1.89014 |
| 4052962 | XM_942429 | LOC552779 // similar to Killer-cell immunoglobulin-like receptor 2D | 3.30E-02 | 1.58969 |

**Table 12: Genes Up-regulated Following DNA damage:** This table list the genes which were significantly ($p<0.05$) up-regulated (Fold >1.5) following DNA damage. Analysis of significant genes was performed using two exon microarray software packages; ArrayAssist and RP, and as discussed earlier the data presented here represents transcripts which were significantly changed following analysis with both software programs.

### 3.3.7. Genes Regulated by TopBP1 following DNA Damage

The final gene list which was extracted from the microarray data was the genes which were either down regulated or up regulated by DNA Damage in the absence of TopBP1. Table 13a shows the genes which are upregulated following DNA damage by (>1.5 fold) with a p value of <0.05. Table 13b shows the genes which are down regulated following DNA damage, under the same restrictions. Figure 56a shows a Venn diagram linking the three datasets A) Genes regulated by TopBP1 B) Genes regulated by DNA damage and C) Genes regulated by TopBP1 following DNA damage. The overlap between the gene sets is of interest; for example there are 5 genes which appear in both damage sets (TopBP1/siTopBP1) but the expression of these five genes changes significantly. This data is shown in Figure 56b. Olfactory receptor family6, calcium binding protein 5, calmodulin 2, tigger transposable element 2 and tubulin beta 2A are all upregulated following DNA damage in the presence of TopBP1 however in the absence of TopBP1 these genes are significantly downregulated. Calmodulin and calcium signalling are both associated with estrogen receeptor signalling in breast cancer (Li *et al.* 2007) and the possible role of TopBP1 will be discussed later.

**Table 13**

a)

| Gene-ID | Gene | p value | Fold |
|---|---|---|---|
| XM_375007 | LOC400093 // hypothetical gene supported by AK123815 | 0.013887 | 2.31487 |
| NM_176890 | TAS2R50 // taste receptor, type 2, member 50 | 0.007936 | 2.12575 |
| NM_181600 | KRTAP13-4 // keratin associated protein 13-4 | 0.028409 | 1.74752 |
| NM_021992 | TMSL8 // thymosin-like 8 | 0.013781 | 1.71973 |
| NM_003654 | CHST1 // carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 | 0.020645 | 1.61119 |
| NM_017445 | H2BFS // H2B histone family, member S | 0.004945 | 1.60915 |
| NM_080817 | GPR82 // G protein-coupled receptor 82 | 0.006616 | 1.58825 |
| NM_198089 | ZNF155 // zinc finger protein 155 (pHZ-96) | 0.029217 | 1.58628 |
| NM_178547 | ZBTB8OS // zinc finger and BTB domain containing 8 opposite strand | 0.025251 | 1.57371 |
| XM_294521 | FLJ43950 // FLJ43950 protein | 0.032546 | 1.56334 |
| XM_939596 | LOC646996 // similar to RAB42, member RAS homolog family | 0.044272 | 1.54958 |
| NM_001008397 | LOC493869 // similar to RIKEN cDNA 2310016C16 | 0.047753 | 1.54602 |
| NM_171998 | RAB39B // RAB39B, member RAS oncogene family | 0.043521 | 1.54206 |
| NM_002723 | PRB4 // proline-rich protein BstNI subfamily 4 | 0.016626 | 1.52426 |
| NM_001355 | DDT // D-dopachrome tautomerase | 0.01409 | 1.51348 |

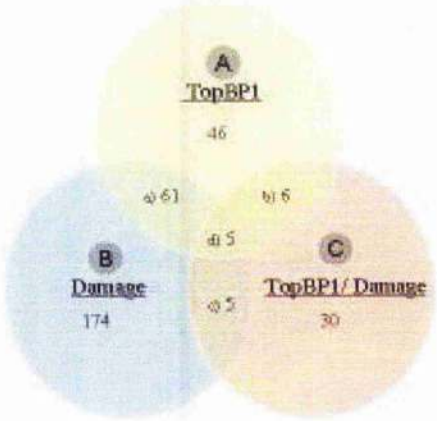
b)

| Gene-ID | Gene | p value | Fold |
|---|---|---|---|
| NM_003552 | OR1D4 // olfactory receptor, family 1, subfamily D, member 4 | 0.006111 | -2.75981 |
| XM_943969 | KRTAP2-2 // keratin associated protein 2-2 | 0.01689 | -2.32292 |
| XM_929003 | LOC646043 // similar to CG32346-PB, isoform B | 0.013304 | -2.20422 |
| XM_927258 | LOC644005 // hypothetical protein LOC644005 | 0.012403 | -2.00166 |
| AK023372 | FLJ13310 // hypothetical protein FLJ13310 | 0.005105 | -1.7843 |
| NM_176882 | TAS2R40 // taste receptor, type 2, member 40 | 0.025566 | -1.77994 |
| NM_014743 | KIAA0232 // KIAA0232 gene product | 0.013125 | -1.74686 |
| NM_199286 | DPPA3 // developmental pluripotency associated 3 | 0.04122 | -1.7088 |
| NM_032498 | PEPP-2 // PEPP subfamily gene 2 | 0.006436 | -1.70539 |
| NM_002667 | PLN // phospholamban | 0.002106 | -1.63511 |
| NM_00103938 | C8orf77 // chromosome 8 open reading frame 77 | 0.000755 | -1.6188 |
| NM_203405 | KRTAP26-1 // keratin associated protein | 0.042714 | -1.60093 |
| AK075204 | FLJ90723 // hypothetical protein FLJ90723 | 0.020559 | -1.57087 |
| NM_00100166 | bA9F11.1 // hypothetical LOC255349 | 0.015491 | -1.56567 |
| NM_014474 | SMPDL3B // sphingomyelin phosphodiesterase, acid-like 3B | 0.003049 | -1.55337 |

**Table 13: Genes Regulated by TopBP1 following DNA damage.** a) This table lists the genes which were significantly ($p<0.05$) up-regulated (Fold>1.5) by TopBP1 following DNA damage. b) This table lists the genes which were significantly ($p<0.05$) down-regulated (Fold>1.5) by TopBP1 following DNA damage.

**Figure 56**

a)




b)

| | | Damage (TopBP1) | | Damage (siTopBP1) | |
|---|---|---|---|---|---|
| Gene ID | Gene | p-value | Fold | p-value | Fold |
| NM_054104 | OR6C3 // olfactory receptor, family 6, subfamily C, member 3 | 0.02415 | 1.96589 | 0.00265 | -2.84460 |
| NM_019855 | CABP5 // calcium binding protein 5 | 0.02653 | 1.59926 | 0.00885 | -1.81294 |
| NM_001743 | CALM2 // calmodulin 2 (phosphorylase kinase, delta) | 0.01070 | 2.12126 | 0.04365 | -1.72230 |
| NM_145719 | TIGD3 // tigger transposable element derived 3 | 0.00295 | 2.00658 | 0.03244 | -1.53303 |
| NM_001069 | TUBB2A // tubulin, beta 2A | 0.01542 | 1.53082 | 0.01699 | -1.51736 |

**Figure 56: Venn Diagram of Three Gene Expression Data Sets.** This diagram shows the number of genes in each data set, A) TopBP1, B) Damage and C) TopBP1 following Damage. The overlaps between these sets are also shown, a-d. b) This table shows the genes which change in TopBP1 knockout cells following damage. ($p<0.05$) fold change-<-1.5, >1.5).

## 3.3.8. Pathway Analysis

Pathway analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery 5th Edition), a pathway analysis program freely available from DAVID Bioinformatics Resources 2007, National Institute of Allergy and Infectious Diseases (NIAID), NIH (http://david.abcc.ncifcrf.gov). DAVID was first published in 2003 (Dennis *et al.* 2003) and since then has been cited in over 900 scientific publications.

In order to perform the pathway analysis for the genes regulated by TopBP1 and the genes regulated by DNA damage, significant genes ($p < 0.05$) were uploaded into the DAVID functional annotation tool. This tool uses the human genome as a background reading (30,000 total background genes). The effect of the significant genes uploaded is then tested against this background to return a significance value for pathways effected within this gene list. An example of this pathway analysis is as follows; 40 genes are involved in the p53 signalling pathway. The loaded gene list contains 300 genes, 3 of which are involved in the p53 pathway. Therefore the tool analyses if 3 genes /300 (Sample) is random compared to 40 genes/30,000 (Background). This gives a p value of 0.001, therefore it is not random and the p53 pathway is significantly altered.

### 3.3.8.1. Pathways predicted to be significantly effected by TopBP1

This pathway analysis was performed using the genes which were significantly changed (both up-regulated and down-regulated) following siTopBP1 treatment. Table 14 shows the pathways which were identified as being significantly ($p<0.05$) effected by the TopBP1 knock down. As the table shows there are eight pathways effected, the percentage of significant genes involved in this pathway is given as well as the p-value for each pathway. The possible implication of TopBP1 and these pathways will be discussed later (4.3.2.1.), however, there are several interesting results considering the roles of TopBP1 discussed earlier in breast cancer, replication and transcription. The MAPK kinase pathway is significantly altered; as this pathway is affected in a wide range of cancers including breast cancer (Dhillon *et al.* 2007).

**Table 14**

| | Pathway | % Genes | P-value |
|---|---|---|---|
| 1 | Sprouty Regulation of Tyrosine Kinase Signals | 0.2 | 0.0041 |
| 2 | p38 MAPK Signaling Pathway | 0.3 | 0.0042 |
| 3 | Gap Junction | 0.8 | 0.0043 |
| 4 | Inositol Phosphate Metabolism | 0.6 | 0.0053 |
| 5 | MAPK Signaling Pathway | 1.7 | 0.0059 |
| 6 | Cell Cycle | 0.9 | 0.0095 |
| 7 | Estrogen-responsive Protein Efp controls Cell Cycle and Breast Tumour Growth | 0.2 | 0.0096 |
| 8 | Lysine Degradation | 0.5 | 0.0321 |

**Table 14: Pathways Effected by TopBP1 knockdown:** This table shows the pathways which are significantly changed following the TopBP1 knockdown. The percentage of genes from the total significant gene population is given. p= Fischer Exact, significant pathways were identified as those with a significance value of 0.05 or less.

The following tables in this section list the genes which are changed in each of the eight identified pathways following the TopBP1 knockdown; Table 15 lists the genes involved in Sprouty regulation of tyrosine kinase signals (Pathway 1), Table 16; genes altered in p38 MAPK signaling pathway (Pathway 2), Table 17; genes changed within gap junctions (3), Table 18; genes altered in the metabolism of Inositol phosphate (4) following TopBP1 knockdown. Table 19 lists the genes which are effected by the knockdown of TopBP1 and that are involved in the MAPK signaling pathway (5). Table 20 lists the genes changed within the cell cycle, Table 21 lists genes involved in estrogen-responsive Efp cell cycle control and breast cancer and Table 22 list the genes involved in lysine degradation which are significantly altered in siTopBP1 treated samples.

**Table 15**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | Cas-Br-M (murine) ecotropic retroviral transforming sequence | 0.01572 | 1.26455 |
| 2 | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.00572 | 1.12195 |
| 3 | mitogen-activated protein kinase kinase 1 | 0.03719 | -1.15154 |
| 4 | v-raf-1 murine leukemia viral oncogene homolog 1 | 0.00098 | -1.20107 |

**Table 15. Genes changed within the Sprouty Regulation of Tyrosine Kinase Signals Pathway (1).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the sprouty regulation of the tyrosine kinase signaling pathway. The p-value and fold change for each gene are also given.

**Table 16**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | mitogen-activated protein kinase kinase kinase 5 | 0.00693 | -1.21284 |
| 2 | mitogen-activated protein kinase kinase kinase 7 | 0.00030 | -1.14143 |
| 3 | ribosomal protein S6 kinase, 90kDa, polypeptide 5 | 0.00794 | -1.36597 |
| 4 | v-death-associated protein 6 | 0.04135 | -1.18875 |
| 5 | TNF receptor-associated factor 2 | 0.01145 | 1.0887 |
| 6 | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.00572 | 1.12195 |
| 7 | cAMP responsive element binding protein 1 | 0.0111 | -1.1037 |
| 8 | potassium voltage-gated channel, subfamily H | 0.0140 | 1.196 |
| 9 | transforming growth factor, beta receptor I | 0.00017 | 1.47149 |

**Table 16. Genes changed within the p38 MAPK Signalling Pathway (2).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the p38 MAPK signaling pathway. The p-value and fold change for each gene are also given.

**Table 17.**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | casein kinase 1, delta | 0.0300 | 1.07749 |
| 2 | casein kinase 1, epsilon | 0.03396 | 1.07469 |
| 3 | mitogen-activated protein kinase kinase 5 | 0.03024 | -1.16659 |
| 4 | CDC20 cell division cycle 20 homolog | 0.00482 | 1.28839 |
| 5 | cell division cycle 2, G1 to S and G2 to M | 0.04257 | 1.28291 |
| 6 | protein kinase, cAMP-dependent, catalytic, beta | 0.01016 | -1.16981 |
| 7 | tubulin, beta 3 | 0.00243 | -1.09104 |
| 8 | adenylate cyclase 1 (brain) | 0.03813 | -1.07511 |
| 9 | guanylate cyclase 1, soluble, alpha 2 | 0.02674 | 1.12141 |
| 10 | mitogen-activated protein kinase kinase 1 | 0.03710 | -1.15154 |
| 11 | mitogen-activated protein kinase kinase 2 | 0.01768 | 1.12798 |
| 12 | v-raf-1 murine leukemia viral oncogene homolog 1 | 0.00098 | -1.20107 |
| 13 | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.00572 | 1.12195 |
| 14 | epidermal growth factor (beta-urogastrone) | 0.03069 | -1.18199 |
| 15 | glutamate receptor, metabotropic 4 | 0.01832 | -1.17231 |
| 16 | glutamate receptor, metabotropic 7 | 0.04483 | 1.18655 |
| 17 | phospholipase C, beta 3 (phosphatidylinositol-specific) | 0.00948 | 1.07685 |
| 18 | phospholipase C, beta 4 | 0.01501 | -1.26094 |
| 19 | inositol 1,4,5-triphosphate receptor, type 3 | 0.03460 | -1.10961 |

**Table 17. List of Genes changed following TopBP1 knockout which are involved in Gap Junctions (3).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on GAP junctions. The p-value and fold change for each gene are also given.

**Table 18. Inositol Phosphate Metabolism (4)**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | inositol polyphosphate-5-phosphatase, 72 kDa | 0.00220 | 1.17757 |
| 2 | synaptojanin 2 | 0.00686 | -1.13937 |
| 3 | multiple inositol polyphosphate histidine phosphatase, 1 | 0.00034 | 1.32195 |
| 4 | inositol polyphosphate-5-phosphatase, 40kDa | 0.00014 | 1.3776 |
| 5 | skeletal muscle and kidney enriched inositol phosphatase | 0.03232 | 1.08955 |
| 6 | inositol polyphosphate-1-phosphatase | 0.01674 | -1.27678 |
| 7 | inositol polyphosphate-4-phosphatase, type I, 107kDa | 0.00178 | -1.09695 |
| 8 | inositol(myo)-1(or 4)-monophosphatase 2 | 0.0337 | -1.11766 |
| 9 | phosphatidylinositol-4-phosphate 5-kinase, type I, gamma | 0.00831 | 1.11185 |
| 10 | phospholipase C, beta 3 (phosphatidylinositol-specific) | 0.00948 | 1.07685 |
| 11 | phospholipase C, beta 4 | 0.01501 | -1.26094 |

**Table 18. Genes changed following TopBP1 knockdown which are involved in Inositol Phosphate Metabolism (4).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the metabolism of inositol phosphate. The p-value and fold change for each gene are also given.

**Table 19**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | dual specificity phosphatase 3 | 0.00716 | 1.08341 |
| 2 | stathmin 1/oncoprotein 18 | 0.04877 | -1.10899 |
| 3 | microtubule-associated protein tau | 0.02882 | 1.10373 |
| 4 | casein kinase 1, gamma 2 | 0.00053 | 1.11543 |
| 5 | cyclin G associated kinase | 0.01006 | 1.08053 |
| 6 | homeodomain interacting protein kinase 2 (HIPK2) | 0.00321 | -1.43692 |
| 7 | eukaryotic elongation factor-2 kinase | 0.00757 | -1.12691 |
| 8 | dual-specificity tyrosine-(Y)-phosphorylation regulated | 0.01747 | -1.20236 |
| 9 | mitogen-activated protein kinase kinase kinase 5 | 0.00693 | -1.21284 |
| 10 | mitogen-activated protein kinase kinase kinase 7 | 0.00030 | -1.14143 |
| 11 | nuclear factor of kappa light polypeptide gene | 0.04655 | -1.18042 |
| 12 | mitogen-activated protein kinase kinase 1 | 0.03710 | -1.15154 |
| 13 | mitogen-activated protein kinase kinase 2 | 0.01768 | 1.12798 |
| 14 | v-raf-1 murine leukemia viral oncogene homolog 1 | 0.00098 | -1.20107 |
| 15 | protein kinase, cAMP-dependent, catalytic, beta | 0.01016 | -1.16981 |
| 16 | protein kinase, X-linked | 0.04936 | 1.12373 |
| 17 | v-akt murine thymoma viral oncogene homolog 2 | 0.00352 | 1.48933 |
| 18 | RAS p21 protein activator 2 | 0.00637 | -1.17921 |
| 19 | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.00572 | 1.12195 |
| 20 | RAS guanyl releasing protein 1 | 0.00263 | -1.48832 |
| 21 | guanine nucleotide binding protein (G protein) alpha 12 | 0.02747 | 1.19497 |
| 22 | fibroblast growth factor receptor 3 | 0.00094 | 1.17852 |
| 23 | Epidermal growth factor (beta-urogastrone) | 0.03069 | -1.18199 |
| 24 | mitogen-activated protein kinase kinase 5 | 0.03024 | -1.16659 |
| 25 | nemo-like kinase | 4.85E-05 | -1.20278 |
| 26 | ribosomal protein S6 kinase, 90kDa, polypeptide 5 | 0.00794 | -1.36597 |
| 27 | ELK4, ETS-domain protein (SRF accessory protein 1) | | |
| | | 0.00319 | -1.16125 |

| | | | |
|---|---|---|---|
| 28 | tumor protein p53 (Li-Fraumeni syndrome) | 0.01833 | -1.22855 |
| 29 | growth arrest and DNA-damage-inducible, beta | 0.02431 | 1.23731 |
| 30 | death-associated protein 6 | 0.04135 | -1.18875 |
| 31 | TNF receptor-associated factor 2 | 0.01145 | 1.0887 |
| 32 | caspase 9, apoptosis-related cysteine peptidase | 0.01201 | -1.23739 |
| 33 | transforming growth factor, beta receptor I | 0.00017 | 1.47149 |
| 34 | transforming growth factor, beta receptor II (70/80kDa) | 0.03717 | -1.20264 |
| 35 | activin A receptor, type IB | 0.01647 | 1.14405 |
| 36 | interleukin 1 receptor, type I | 0.00919 | -1.40704 |
| 37 | tumor necrosis factor receptor superfamily, member 1A | 3.36E-05 | -1.27299 |
| 38 | p21 (CDKN1A)-activated kinase 2 | 0.02760 | -1.14114 |
| 39 | arrestin, beta 1 | 0.01827 | -1.1231 |
| 40 | mitogen-activated protein kinase kinase kinase 3 | 0.00047 | -1.31747 |
| 41 | mitogen-activated protein kinase 9 / | 0.00868 | -1.14003 |
| 42 | v-erk sarcoma virus CT10 oncogene homolog (avian) | 0.00151 | 1.23123 |
| 43 | jun D proto-oncogene | 0.00466 | -1.38914 |
| 44 | v-jun sarcoma virus 17 oncogene homolog (avian) | 0.01776 | -1.18901 |
| 45 | nuclear factor of activated T-cells | 0.00153 | -1.22522 |
| 46 | protein phosphatase 3 (formerly 2B), catalytic subunit | 0.01061 | -1.19879 |
| 47 | Filamin A, alpha (actin binding protein 280) | 0.01102 | -1.18805 |
| 48 | v-myc myelocytomatosis viral oncogene homolog (avian) | 0.04363 | -1.29962 |

**Table 19. Genes changed following TopBP1 knockdown which are involved in the MAPK Signaling Pathway (5).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the MAPK signaling pathway. The p-value and fold change for each gene are also given.

**Table 20**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | origin recognition complex, subunit 6 like (yeast) | 0.00534 | 1.25617 |
| 2 | origin recognition complex, subunit 5-like (yeast) | 0.00408 | -1.23075 |
| 3 | MCM7 minichromosome maintenance deficient 7 | 0.03190 | 1.11717 |
| 4 | MCM6 minichromosome maintenance deficient 6 | 0.03255 | -1.1038 |
| 5 | Cell division cycle 2, G1 to S and G2 to M | 0.04257 | 1.28291 |
| 6 | v-abl Abelson murine leukemia viral oncogene homolog 1 | 0.03137 | -1.09566 |
| 7 | Polo-like kinase 1 (Drosophila) | 0.023051 | 1.17961 |
| 8 | E2F transcription factor 2 | 0.00011 | -1.20265 |
| 9 | E2F transcription factor 5, p130-binding | 0.01953 | 1.30047 |
| 10 | E2F transcription factor 6 | 0.03082 | 1.18788 |
| 11 | histone deacetylase 1 | 0.00326 | -1.19688 |
| 12 | histone deacetylase 3 | 2.33E-05 | 1.61364 |
| 13 | CDC14 cell division cycle 14 homolog B (S. cerevisiae) | 0.00605 | -1.2345 |
| 14 | S-phase kinase-associated protein 2 (p45) | 0.00182 | -1.43619 |
| 15 | WEE1 homolog (S. pombe) | 0.01503 | 1.20016 |
| 16 | Extra spindle poles like 1 (S. cerevisiae) | 0.04431 | 1.19417 |
| 17 | Growth arrest and DNA-damage-inducible, beta | 0.02431 | 1.23731 |
| 18 | tumor protein p53 (Li-Fraumeni syndrome) | 0.01835 | -1.22855 |
| 19 | cyclin-dependent kinase inhibitor 1C (p57, Kip2) | 0.04289 | 1.16848 |
| 20 | cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) | 0.04656 | -1.12777 |
| 21 | cyclin-dependent kinase inhibitor 2D(p19,inhibits CDK4) | 0.00290 | 1.17143 |
| 22 | CDC20 cell division cycle 20 homolog (S. cerevisiae) | 0.00482 | 1.28839 |
| 23 | cyclin-dependent kinase 2 | 0.00834 | 1.11116 |
| 24 | cyclin-dependent kinase 6 | 0.01274 | -1.27104 |
| 25 | cyclin D1 | 0.01029 | 1.06348 |

**Table 20 Genes Involved in the Cell Cycle which are altered by the knock down of TopBP1 (6).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the cell cycle. The p-value and fold change for each gene are also given.

**Table 21**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | stratifin | 0.00021 | -1.0095 |
| 2 | tripartite motif-containing 25 | 0.01092 | 1.1249 |
| 3 | tumor protein p53 (Li-Fraumeni syndrome) | 0.01835 | -1.2285 |

**Table 21 Genes whose expression is changed following siTopBP1 that play a role in the estrogen-responsive protein and its control of the cell cycle and breast tumour growth. (7).** This table lists the genes whose expression is significantly changed in the pathway indicated within the TopBP1 knockout. The p-value and fold change for each gene are also given.

**Table 22**

| | Gene | P-Value | Fold |
|---|---|---|---|
| 1 | aldehyde dehydrogenase 9 family, member A1 | 3.65E-06 | -1.43023 |
| 2 | nuclear receptor binding SET domain protein 1 | 0.03532 | -1.18843 |
| 3 | suppressor of variegation 3-9 homolog 1 (Drosophila) | 0.04409 | 1.20376 |
| 4 | SET domain containing (lysine methyltransferase) 7 | 0.00021 | -1.53705 |
| 5 | procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1 | 0.01179 | -1.30573 |
| 6 | procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | 0.04975 | -1.16235 |
| 7 | procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 | 0.00162 | -1.14653 |
| 8 | enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme | 0.04687 | -1.21743 |
| 9 | glutaryl-Coenzyme A dehydrogenase / | 0.00203 | 1.12358 |
| 10 | ARD1 homolog A, N-acetyltransferase (S. cerevisiae) | 0.03030 | 1.13767 |
| 11 | retinol dehydrogenase 11 (all-trans/9-cis/11-cis) | 4.39E-05 | 1.23329 |
| 12 | serine hydroxymethyltransferase 1 (soluble) | 0.01059 | -1.24662 |
| 13 | pipecolic acid oxidase | 0.04554 | 1.12762 |
| 14 | dihydrolipoamide S-succinyltransferase | 0.02315 | 1.14835 |

**Table 22. Genes Involved in the Degradation of Lysine which are altered by the knock down of TopBP1 (8).** This table lists the genes which were significantly changed in the TopBP1 knockout samples and that were identified using DAVID as having a significant effect on the degradation of lysine. The p-value and fold change for each gene are also given.

### 3.3.8.2. Pathways Predicted to be Significantly Effected by DNA damage

Similar to the results described in the previous section in which pathway analysis was performed to predict pathways which might be effected by TopBP1 using the data obtained from the microarray (Chapter 3.3.8.1.), this chapter describes the pathways which are predicted to be effected by DNA damage. This pathway analysis was performed as described previously using only those genes which were significantly ($p<0.05$) changed (either up-regulated or down-regulated) following gamma irradiation. Table 23 shows the pathways which are predicted (using DAVID) to be significantly ($p<0.05$) altered following DNA damage. Five pathways were identified; 1) Oxidative Stress Induced Gene Expression, 2) FAS Signalling Pathway, 3) The Proteosome, 4) Inositol Phosphate Metabolism and 5) Apoptosis. Although these pathways and the genes with altered expression will be discussed in Chapter 4.3.3., it is of interest that pathways associated with DNA damage and cell death were identified using this method of analysis. Tables 24-28 list the genes which were effected in each pathway, their fold change and significance (p-value).

**Table 23**

| | Pathway | % Genes | P-value |
|---|---|---|---|
| 1 | Oxidative Stress Induced Gene Expression via Nrf2 | 0.2 | 0.0021 |
| 2 | FAS Signalling Pathway | 0.3 | 0.0023 |
| 3 | Proteosome | 0.4 | 0.0039 |
| 4 | Inositol Phosphate Metabolism | 0.5 | 0.0044 |
| 5 | Apoptosis | 0.5 | 0.0483 |

**Table 23: Pathways Effected by DNA Damage:** This table shows the pathways which are significantly changed following DNA Damage. The percentage of genes from the total significant gene population is given. p= Fischer Exact, significant pathways were identified as those with a significance value of 0.05 or less.

**Table 24**

| | Gene | Fold | p-value |
|---|---|---|---|
| 1 | crystallin, zeta (quinone reductase) | -1.21427 | 0.00836 |
| 2 | mitogen-activated protein kinase 14 | -1.10558 | 0.01445 |
| 3 | v-maf musculoaponeurotic fibrosarcoma oncogene homolog K | -1.05368 | 0.02529 |
| 4 | cAMP responsive element binding protein 1 | -1.10425 | 0.00042 |
| 5 | kelch-like ECH-associated protein 1 | -1.02067 | 0.04666 |

**Table 24: Genes Involved in Oxidative Stress Induced Gene Expression which are altered following DNA Damage (1).** This table lists the genes which expression was significantly changed following DNA damage and that were identified using DAVID as having a significant effect on the oxidative stress pathways. The fold change for each gene and their significance is also given.

**Table 25**

| | Gene | Fold | p-value |
|---|---|---|---|
| 1 | p21 (CDKN1A)-activated kinase 2 | -1.10004 | 0.04883 |
| 2 | mitogen-activated protein kinase kinase kinase 7 | -1.09507 | 0.00078 |
| 3 | adenomatosis polyposis coli | -1.29148 | 0.00198 |
| 4 | CASP8 and FADD-like apoptosis regulator | 1.00604 | 0.04625 |
| 5 | receptor-interacting serine-threonine kinase 2 | -1.14571 | 0.00203 |

**Table 25: Genes Involved in FAS Signalling Pathway which are altered following DNA Damage (2).** This table lists the genes which expression was significantly changed following DNA damage and that were identified using DAVID as having a significant effect on the FAS signalling pathway. The fold change for each gene and their significance is also given.

**Table 26**

| | Gene | Fold | p-value |
|---|---|---|---|
| 1 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 1 | -1.11386 | 0.0304 |
| 2 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 11 | -1.0348 | 0.04513 |
| 3 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 6 | -1.04948 | 0.01147 |
| 4 | proteasome (prosome, macropain) 26S subunit, ATPase, 2 | -1.04648 | 0.04666 |
| 5 | proteasome (prosome, macropain) subunit, alpha type, 4 | -1.05013 | 0.01599 |
| 6 | proteasome (prosome, macropain) subunit, alpha type, 7 | 1.00605 | 0.03692 |
| 7 | proteasome (prosome, macropain) subunit, alpha type, 5 | -1.10178 | 0.00341 |
| 8 | proteasome (prosome, macropain) subunit, alpha type, 3 | -1.05101 | 0.03508 |
| 9 | proteasome (prosome, macropain) subunit, beta type, 2 | -1.08852 | 0.02477 |
| 10 | proteasome (prosome, macropain) subunit, beta type, 4 | -1.06895 | 0.00716 |
| 11 | proteasome (prosome, macropain) subunit, beta type, 1 | -1.07999 | 0.00327 |

**Table 26. Genes Involved within the Proteosome and whose Gene Expression is altered following DNA Damage (3)** This table lists the genes whose expression was significantly changed following DNA damage and that were identified using DAVID as having a significant effect on the degradation of lysine. The p-value and fold change for each gene are also given.

**Table 27**

| | Gene | Fold | p-value |
|---|---|---|---|
| 1 | multiple inositol polyphosphate histidine phosphatase, 1 | -1.07247 | 0.01330 |
| 2 | inositol polyphosphate-4-phosphatase, type I, 107kDa | -1.03574 | 0.02142 |
| 3 | Inositol (myo)-1(or 4)-monophosphatase 2 | -1.01325 | 0.03994 |
| 4 | Myo-inositol oxygenase | 1.16080 | 0.03455 |
| 5 | Meis1, myeloid ecotropic viral integration site 1 | -1.06155 | 0.02704 |
| 6 | phosphatidylinositol-4-phosphate 5-kinase, type I, gamma | 1.03439 | 0.04006 |
| 7 | phosphatidylinositol-4-phosphate 5-kinase, type I, beta | -1.0819 | 0.01126 |
| 8 | phospholipase C, beta 2 | 1.08451 | 0.04566 |
| 9 | casein kinase 1, gamma 2 | -1.03717 | 0.00205 |
| 10 | casein kinase 1, gamma 3 | -1.17524 | 0.03914 |
| 11 | eukaryotic translation initiation factor 2-alpha kinase | -1.14254 | 0.02699 |
| 12 | phosphoinositide-3-kinase, catalytic, delta polypeptide | 1.06016 | 0.03838 |

**Table 27: Genes changed following DNA Damage which are involved in Inositol Phosphate Metabolism (4).** This table lists the genes which were significantly changed following DNA damage and that were identified using DAVID as having a significant effect on the metabolism of inositol phosphate. The p-value and fold change for each gene are also given.

**Table 28**

| | Gene | Fold | p-value |
|---|---|---|---|
| 1 | protein phosphatase 3 (formerly 2B), catalytic subunit | -1.17010 | 0.00269 |
| 2 | protein phosphatase 3 (formerly 2B), regulatory subunit | 1.24094 | 0.02765 |
| 3 | v-rel reticuloendotheliosis viral oncogene homolog A, | -1.02087 | 0.01646 |
| 4 | protein kinase, cAMP-dependent, regulatory, type II, | -1.03442 | 0.04557 |
| 5 | protein kinase, cAMP-dependent, regulatory, type II beta | -1.13236 | 0.02263 |
| 6 | protein kinase, cAMP-dependent, catalytic, beta | -1.12834 | 0.03045 |
| 7 | caspase 8, apoptosis-related cysteine peptidase | -1.03997 | 0.04801 |
| 8 | caspase 10, apoptosis-related cysteine peptidase | 1.07863 | 0.00027 |
| 9 | calpain 6 | 1.05458 | 0.00181 |
| 10 | CASP8 and FADD-like apoptosis regulator | 1.00604 | 0.04625 |
| 11 | phosphoinositide-3-kinase, catalytic, delta polypeptide | 1.06016 | 0.03838 |
| 12 | phosphoinositide-3-kinase, regulatory subunit 2 | 1.09384 | 0.00788 |
| 13 | TNF receptor-associated factor 2 | 1.06691 | 0.04653 |
| 14 | receptor (TNFRSF)-interacting serine-threonine kinase 1 | -1.13047 | 0.00670 |
| 15 | tumor necrosis factor receptor superfamily, member 10c | 1.13687 | 0.01284 |
| 16 | tumor necrosis factor (TNF superfamily, member 2) | 1.14243 | 0.02448 |

**Table 28: Apoptosis genes that are altered following DNA Damage (5).** This table lists the genes which were significantly changed following DNA damage and that were identified using DAVID as having a significant effect apoptosis. The p-value and fold change for each gene are also given.

### 3.3.8.3. Pathways Predicted to be significantly Effected by DNA damage following TopBP1 knockout

Following the analysis of the pathways altered following DNA damage in the presence of TopBP1, pathway analysis was performed to predict the pathways effected by DNA damage in the TopBP1 knockout samples. This pathway analysis was performed as described previously (3.3.8.) using only those genes which were significantly ($p<0.05$) changed (either up-regulated or down-regulated) following gamma irradiation. Table 29 shows the pathways which are predicted (using DAVID) to be significantly ($p<0.05$) altered following DNA damage in the absence of TopBP1. Seven pathways were identified; 1) Androgen and Estrogen Metabolism, 2) Biosynthesis of Steroids, 3) Aspirin Blocks Signalling involved in Platelet Activation, 4) Regulation of Splicing Through Sam68, 5) Links between Pyk2 and Map Kinases, 6) Roles of β-arrestin Dependent recruitment of Src Kinase and 7) Sprouty Regulation of Tyrosine Kinase Signals. The percentage of genes involved in each pathway and the p value according to the DAVID analysis is also shown. Tables 30 and 31 list the genes which are altered in the Androgen and Estrogen Metabolism, and the Biosynthesis of Steroids pathways respectively. The genes altered in remaining pathways overlap considerably; therefore the genes altered in these pathways are shown as a combined table (Table 32).

**Table 29**

| | Pathway | % Genes | P-value |
|---|---|---|---|
| 1 | Androgen and Estrogen Metabolism | 0.8 | 0.0023 |
| 2 | Biosynthesis of Steroids | 0.4 | 0.0493 |
| 3 | Aspirin Blocks Signalling involved in Platelet Activation | 0.4 | 0.0077 |
| 4 | Regulation of Splicing Through Sam68 | 0.3 | 0.0024 |
| 5 | Links between Pyk2 and Map Kinases | 0.5 | 0.0021 |
| 6 | Roles of β-arrestin Dependent recruitment of Src Kinase | 0.4 | 0.0078 |
| 7 | Sprouty Regulation of Tyrosine Kinase Signals | 0.4 | 0.0028 |

**Table 29: Pathways Effected by TopBP1 following DNA Damage:** This table shows the pathways which are significantly changed following DNA Damage in the absence of TopBP1. The percentage of genes from the total significant gene population is given. p= Fischer Exact, significant pathways were identified as those with a significance value of 0.05 or less.

**Table 30**

| | Gene | P value | Fold |
|---|---|---|---|
| 1 | Williams Beuren syndrome chromosome region 22 | 0.04415 | 1.14487 |
| 2 | HemK methyltransferase family member 2 | 0.04494 | 1.30164 |
| 3 | protein arginine methyltransferase 2 | 0.04116 | 1.13067 |
| 4 | protein arginine methyltransferase 1 | 0.01564 | 1.23129 |
| 5 | protein arginine methyltransferase 7 | 0.02042 | 1.15939 |
| 6 | 3-hydroxybutyrate dehydrogenase, type 1 | 0.03972 | 1.20702 |
| 7 | arylsulfatase E (chondrodysplasia punctata 1) | 0.01823 | -1.25241 |
| 8 | Beta-carotene dioxygenase 2 | 0.04542 | -1.13379 |

**Table 30: Genes involved in Androgen and Estrogen Metabolism that are altered following DNA Damage in TopBP1 knockouts (1).** This table lists the genes which were significantly changed following DNA damage and that were identified using DAVID as having a significant effect apoptosis. The p-value and fold change for each gene are also given.

**Table 31**

| | Gene | P value | Fold |
|---|---|---|---|
| 1 | farnesyl diphosphate synthase | 0.02272 | -1.3128 |
| 2 | mevalonate kinase (mevalonic aciduria) | 0.04403 | 1.1097 |
| 3 | sterol-C5-desaturase (ERG3 delta-5-desaturase homolog | 0.01703 | 1.3112 |
| 4 | cytochrome P450 | 0.02823 | -1.3258 |

**Table 31: Genes involved in the Biosynthesis of Steroids that are altered following DNA Damage in TopBP1 knockouts (2).** This table lists the genes which were significantly changed following DNA damage and that were identified using DAVID as having a significant effect apoptosis. The p-value and fold change for each gene are also given.

| | Gene | P value | Fold | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| 1 | mitogen-activated protein kinase 3 | 0.00958 | 1.2016 | Yes | Yes | Yes | Yes | Yes |
| 2 | v-raf-1 murine leukemia viral oncogene homolog 1 | 0.04788 | -1.1271 | Yes | - | Yes | Yes | Yes |
| 3 | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.01736 | 1.1388 | Yes | Yes | Yes | Yes | Yes |
| 4 | v-src sarcoma viral oncogene homolog | 0.03369 | -1.1640 | Yes | Yes | Yes | Yes | Yes |
| 5 | mitogen-activated protein kinase kinase 3 | 0.02054 | -1.07714 | - | - | Yes | - | - |

**Table 32: Genes effected in pathways 3-7.** This table shows the genes which are significantly altered in the following pathways. 3) Aspirin Blocks Signalling Pathway involved in Platelet Activation.4) Regulation of Splicing through Sam68. 5) Links between Pyks and Map kinases. 6) Roles of β-arrestin dependent recruitment of Src kinases in GPCR and 7) Sprouty Regulation of Tyrosine kinase signalling.

### 3.3.9. Summary chapter 3.3.

The data presented here has shown that contrary to previously published data (Jeon *et al.* 2007) TopBP1 is not essential for cell growth and replication in all cultured cell lines. Figure 48 showed that the primary cell line MRC5 and the immortalised HeLa cell line cannot continue to grow when TopBP1 is knocked down using siRNA, and this is specifically due to the reduction in TopBP1 expression because the control luciferase siRNA did not adversely effect cell growth in these cells. This is not however true of all cell lines tested, as Figure 49 shows 293T, MCF7, and U2OS cell lines can survive when TopBP1 is knocked down as the growth of these cells shows no significant deviation from control cells, over a four day period. TopBP1 is however essential for continued cell growth over longer time periods, as Figure 52 showed; MCF7 cells in which TopBP1 was knocked down could not survive over a 14 day period. As TopBP1 is essential for the DNA damage response and replication, it is possible that cells lacking TopBP1 would be significantly more effected by DNA damage than controls, resulting in lower colony survival numbers in damaged cells.

Following these results, MCF7 cells were chosen for the study of the genes regulated by TopBP1 because in addition to TopBP1 having no immediate effect on cell growth, this cell line is also a breast carcinoma cell line and therefore provides a model for studying TopBP1 in breast cancer. TopBP1 was knockdown in these cells and an exon microarray was carried out. Chapter 3.3.3. described the experimental

plan and controls; the knockdown of TopBP1 was efficient (Figure 54a) and had no effect on the cell cycle profile when compared to the siluciferase controls (Figure 51). The data analysis performed using ArrayAssist software proved that the labeling process and hybridisation of all 12 arrays was of a high standard (Figure 55) and that all sample repeats could be used in further analyses. The microarray data revealed 64 genes which were significantly ($p<0.05$) down-regulated with a fold effect of $<-1.5$ and 65 genes which were up-regulated by TopBP1 resulting in a fold effect of $>1.5$ (Table 9 and 10 respectively). Chapter 3.3.6. described the genes regulated by DNA damage and Chapter 3.3.7., further investigated these two data sets to provide a list of genes which are specifically regulated by TopBP1 following DNA damage. Pathway analysis was performed using DAVID (as described (3.8.3.), this analysis revealed several pathways which are predicted by this software to be significantly effected by the knockdown of TopBP1 (Table 14) and significantly changed following DNA damage in the presence and absence of TopBP1 (Tables 23 and 29 respectively). These pathways and the possible role of TopBP1 will be discussed in more detail later; however these pathways might provide an insight into previously unknown functions of TopBP1.

# Chapter 4- Discussion

As discussed in Chapter 1.6 TopBP1 is involved in transcription, replication and repair. The modification of chromatin is essential in mediating all these functions of TopBP1; the results presented here have identified three transcriptional regulatory domains within TopBP1 which may mediate these functions via the modification of chromatin.

## 4.1 TopBP1 Contains Transcriptional Modification Domains

Chapter 3.1 described the identification of a transcriptional activation domain partly encompassing BRCT4 (Figure 9) which is repressed by two adjacent repression domains within BRCT5 (Figure 24) and BRCT2 (Figure 10). This discussion chapter will focus on the significance of these domains in transcription and discuss the

possible regulation of these domains by sumoylation, phosphorylation, and ubiquitination during transcription. Initiation and elongation of transcription occurs through a complex set of DNA-protein and protein-protein interactions, involving RNA polymerase II, transcription adaptors and regulators. These regulatory transcription factors bind DNA, or are recruited to DNA via protein-protein interactions, and act as transcriptional activators and repressors depending upon the specific complex. TopBP1 is a possible transcription factor, as it has been shown to interact with both single stranded and double stranded DNA (Yamane *et al.* 1999) RNA polymerase II (Yu *et al.* 2003). TopBP1 co-activates transcription of the HPV16 transcription/replication protein E2 (Boner *et al.* 2002) and represses transcription of myc target genes via Miz1 (Herold *et al.* 2002). The data presented here has identified three transcriptional regulatory domains within TopBP1; an activation domain and two repression domains (Chapter 3.1) further supporting the role of TopBP1 as a potential transcription factor.

### 4.1.1 Transcriptional Activation Domains

Transcriptional activation domains are classified into three distinct groups depending upon their amino acids content; 1) rich in acidic amino acids, 2) glutamine (acidic) rich or 3) proline rich. As Figure 11 shows the activation domain identified in TopBP1 (amino acids 460-500 (Figure 10)) is characteristic of an acidic activation domain, interspersed with hydrophobic amino acids, similar to the activation domain of VP16 (Drysdale *et al.* 1995) and BRCA1 (Hayes *et al.* 2000).

To investigate the transcriptional activation domain further, the specific amino acids essential for TopBP1 transcriptional activity could be investigated. Although TopBP1 is an acidic activation domain it is unlikely that the acidic residues are the residues essential for activation. Studies of the VP16 activation domain identified that mutations which removed the acidic amino acids still retained transcriptional activation (Cress and Triezenbery 1991) but mutation of the hydrophobic residues (V, L, M, F and L) are more important for activation function (mutation of the phenylalanine within VP16 abolished transcriptional activation (Drysdale *et al.* 1995)). These hydrophobic residues within the TopBP1 activation domain are conserved between species (Figure 11). Therefore mutation of both the acidic (aspartic acid (D) and glutamic acid (E)) and hydrophobic residues (phenylalanine (F) valine (V) and leucine (L)) within the TopBP1 activation domain might reveal the residues essential for function. The possible significance of the hydrophobic residues within the TopBP1 activation domain is also suggested by previous studies of the BRCA1 activation domain. Mutation of hydrophobic residues L1780P and V1833E within the activation domain resulted in a loss of BRCT structure and transcriptional activation (Hayes *et al.* 2000).

The different classes of activation domains differ in their mechanism of activation. Glutamine and proline rich activation domains only activate transcription from positions close to the TATA box (proximal) in contrast to acidic activation domains such as GAL4 and VP16 which have the ability to activate transcription from both proximal and remote regions (Seipel *et al.* 1992). Chromatin immunoprecipitation experiments could be used to determine if the TopBP1 activation domain can activate

transcription from both proximal and remote sequences as would be expected due to the amino acid content of the domain.

Recent research has shown that TopBP1 may be involved in the development of breast cancer. Karppinen and colleagues screened polymorphisms within TopBP1 to ascertain if similarly to BRCA1, TopBP1 is a breast cancer predisposing gene. They identified a novel variant Arg309Cys which was found at a higher frequency in familial breast cancer patients compared to healthy controls (Karppinen *et al.* 2006). TopBP1 is also aberrantly expressed in breast cancer (Going *et al.* 2007); in normal breast tissue the staining of TopBP1 was nuclear however in breast cancer the staining was cytoplasmic in 15% and absent in 3% of cases. This cytoplasmic staining is similar to that observed for BRCA1, which is often cytoplasmic in breast cancer (Yoshihawa *et al.* 1999). Karihtala *et al.* 2006 also showed that there was an increase in TopBP1 expression levels in breast cancer which correlated with cancer progression.

BRCA1 mutation studies have demonstrated the link between transcriptional activity and breast cancer; mutations within the transcriptional activation domain are often associated with disease-predisposing mutations which abolish transactivation by BRCA1. Figure 57 shows a sequence alignment of BRCA1 (amino acids 1550-1863) and TopBP1 (amino acids 460-500) activation domains. Several of the disease-associating mutations in BRCA1, which lie within the transcriptional activation domain and that result in a loss of transcriptional activity are highlighted (dash boxes). One such mutation; V1668del in BRCA1, introduces a stop codon suggesting

that the sequence following this residue, which encompasses the transcriptional activation domain of BRCA1 is important for the function. This residue and the area surrounding it is conserved in TopBP1 and similarly to BRCA1 the alteration of this residue resulting in the insertion of a stop codon would result in a TopBP1 lacking transcriptional activation domain (red dash box).

Although similar studies have been performed with TopBP1, which identified TopBP1 as a possible breast cancer susceptibility gene (Karppinen *et al.* 2006), this study used 125 familial breast cancer cancers and compared the results to patients with a non-hereditary breast cancer. It would be interesting to study the non-familial group and sequence TopBP1 from the both the lesion and normal tissue from the same patient. Figures 27 and 28 demonstrated that the activation domain and the repression domain within BRCT2 are conserved in yeast, similar to the activation domain within BRCA1 (Monterio *et al.* 1996, Hu *et al.* 2000). This provides a useful genetic tool to identify the residues within each domain essential for transcriptional regulation. Hu and collegues found that the residues within the predicted coil-coil motif of BRCA1 were essential for transcriptional activation. Although the structure of TopBP1 has not yet determined, motifs of this nature could be predicted using PARACOIL program (Berger *et al.* 1995, http:dot.imgen.bcm.tmc.edu:99331seq-search/struc-predict.html), the residues within this region could then be mutated and their transcriptional activity tested in both yeast and mammalian systems, using similar experiments to those described previously.

**Figure 57**

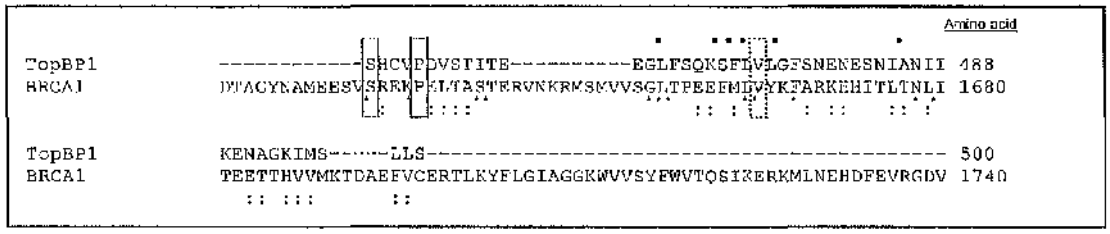



**Figure 57: Disease Predisposing Mutations within BRCA1 Activation Domain.** This diagram shows a sequence alignment of TopBP1 (amino acids 460-500) and BRCA1 (amino acids 1550-1863) transcriptional activation domains. Dashed boxes indicate the cancer pre-disposing residues within BRCA1, of interest is BRCA1 V1668 (Red dashed). A possible serine phosphorylation target is indicated by green box and an adjacent conserved proline is indicated by a red box. * indicates residues which are conserved between the two activation domains. • indicates hydrophobic residues conserved residues and : indicates a position where the charge of the residue is conserved.

### 4.1.2. Regulation of TopBP1 Activation and Repression Domains

Figure 15 suggested the repression domain within BRCT2 repressed the activation domain via the recruitment of a repressor complex and not via an intramolecular interaction, as BRCT2 was able to repress the transcriptional activity of VP16. However to be certain this would need to be confirmed with further experiments as the repression of the activation domain might still be via an intramolecular interaction because of the domain similarity between TopBP1 and VP16. Both domains consist of acidic activation domains with pockets of hydrophobic residues. It is possible that BRCT2 specifically binds to domains of this type. To confirm the recruitment of a repressor complex as the method of repression additional (BRCT2 repressor)-(activation domain X) fusion proteins using different types of activation domain (glutamine, proline rich) would need to be constructed and tested in similar luciferase assays. Nuclear versions of BRCT2 could also be over-expressed to determine if it can inhibit the transcriptional activation domain when placed in trans.

One possible method of TopBP1 transcriptional repression might be via the recruitment of HDAC (histone deacetyltransferase). HDAC complexes are important in the regulation of gene expression. HDAC enzymes deacetylate histone H3 and H4, which results in a tighter chromatin state and the repression of transcription. HDAC directly interact with several transcription factors including p53, E2F and NFκB (Mayo *et al.* 2003). As Figure 16 showed the treatment of cells with the HDAC

inhibitor (TSA) did not alleviate repression of the activation domain by BRCT2, therefore repression of the activation domain is not via histone deacetylation.

A second method of transcriptional regulation tested in this thesis was the involvement of the HAT in transcriptional activation. The acetylation of the core histones results in a more "open" state providing access for DNA damage repair proteins, and transcriptional machinery. Figure 18 shows the BRCT2 repressor and the activation domain show no intrinsic HAT activity in the HAT activity assay tested however to confirm this result the HAT inhibitor garcinol, a polyisoprenylated benzophenone isolated from *Garcinia indica* could be used in the luciferase assays (Balasubramanyam *et al.* 2004).

However there are many post translation modifications of proteins such as phosphorylation, ubiquitination, sumoylation and methylation which play an essential role in the regulation of their target protein. The possible role of these modifications in the context of TopBP1 transcriptional activity will be discussed further in this chapter.

### 4.1.2.1 TopBP1 and Ubiquitination

One possible method of TopBP1 regulation could be ubiquitination. Ubiquitin (Ub) is a small 76 amino acid protein which is tagged to target proteins to signal them for degradation by the 26S proteosome. Ubiquitination involves three enzymes, E1, E2 and E3. Ub is activated by the ubiquitin activating enzyme E1 (a reaction requiring

ATP) (Figure 58). The Ub is then transferred to the Ub-conjugating enzyme E2 which transfers the Ub to the target protein via the Ub-ligase E3 (Varshavsky *et al.* 1997). There are two groups of human Ub ligases; 1) the HECT domain Ub-ligase enzymes and 2) the RING finger Ub-ligase E3 enzymes. The HECT-domain E3 ligases directly interact with Ub and transfer the Ub directly to the substrate, conversely the RING finger E3 ligases form complexes with the E2 conjugating enzyme and Ub, which then as a complex transfers Ub to the target protein (Figure 58).

TopBP1 specifically interacts with the HECT E3 ligase, hHYD (*hyperplastic discs* in *Drosophila melanogaster)* via amino acids 574 to 993 incorporating the BRCT5 transcriptional repression domain and BRCT6 (Honda *et al.* 2002). This is a specific interaction with hHYD and not a general interaction with HECT domain ligases; as NEDD4, another member of the HECT domain E3 ligases does not immunoprecipitate with TopBP1. Honda and colleagues also showed that TopBP1 was ubiquitinated and degraded by the proteosome, under "normal" conditions, however following gamma irradiation ubiquitination of TopBP1 was reduced, implying that the regulation of TopBP1 by ubiquitination is essential for TopBP1 response to DNA damage. hHYD activates Chk2 following DNA damage (Henderson *et al.* 2006) and has shown abnormal expression im breast cancers; both a decrease (Fuja *et al.* 2004) and an increase in expression (Clancy *et al.* 2003) when comparing invasive breast cancers and normal breast tissue.

Many transcription factors are unstable and are constantly destroyed by ubiquitin-mediated proteolysis (Thomas *et al.* 2000) mediated by ubiquitination, as described

TopBP1 is also continually degraded by hHYD until required following DNA damage. Mutational analysis of transcriptional activation domains has revealed a close correlation between transcriptional activation and proteolysis. Salghetti *et al.* 2000 showed that three transcriptional activation domains also acted as degrons, destabilising the LexA Protein in S *cerevisiae*, therefore linking transcriptional activation and ubiquitination. They suggested that Ub "licences" transcription factors by linking their activity to their destruction; E3 ligases interact with the transcriptional activator and simultaneously activate the transcription factor and target it for degradation. The phosphorylation of the target protein is one mechanism which reduces the ability of Ub ligases to ubiquitinated their target protein. TopBP1 is phosphorylated following DNA damage, preventing ubiquitination and therefore TopBP1 degradation following DNA damage.

**Figure 58**

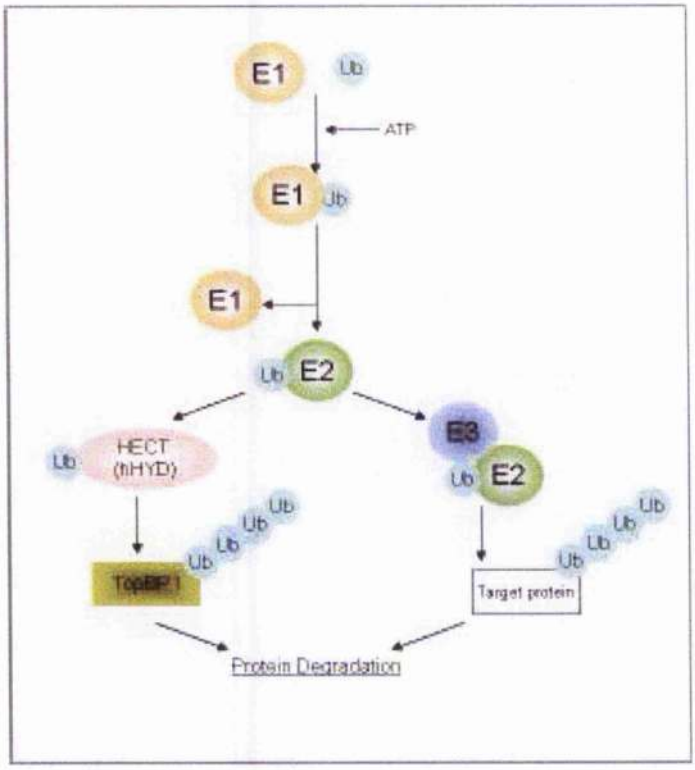



**Figure 58: Ubiquitination TopBP1 by hHYD is essential for TopBP1 regulation under normal conditions.** This diagram shows the method of TopBP1 ubiquitination via the HECT-domain containing E3 ligase hHYD.

### 4.1.2.2 Transcriptional Regulation by Sumoylation

It is possible that TopBP1 activity might be regulated by sumoylation. SUMO (small ubiquitin related modifier) is an 11kDa ubiquitin like protein, but unlike ubiquitination sumoylation does not lead to the degradation of the target protein. Sumolyation does however regulate a wide variety of cellular control processes; protein-protein interactions, nuclear localisation, and protein-DNA interactions (For review see Verger *et al.* 2003). In addition sumolyation has been shown to have both positive and negative effects on the activity of transcription factors; increasing the transcriptional activity of p53 but repressing the activity of c-Jun, and p300. Therefore SUMO may be involved in TopBP1 transcriptional activation and repression (Review Girdwood *et al.* 2003).

SUMO is added to proteins in a similar way to the Ub pathway (Figure 61); it is activated in an ATP dependent manner by an E1 enzyme (Uba2), and transferred to an E2 conjugating enzyme (Ubc9). However the two E3 ligase enzymes differ in the SUMO pathway from those used in the Ub pathway; the first is similar to the RING E3 ligase from the Ub pathway (PIAS, Schmidt *et al.* 2003), the second E3 ligase (RanBP2) is a component of the nuclear pore complex. Although SUMO can be transferred to the target protein without the cooperation of an E3 ligase (Muller *et al.* 2001) the efficacy is greatly enhanced in the presence of the E3 ligases, although the mechanism of action is still unknown.

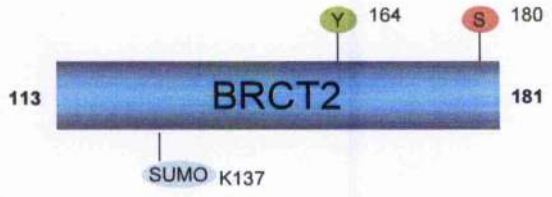
SUMO binds to a specific SUMO recognition sequence on the target protein, binding to a specific lysine residue within the target (Melchior *et al.* 2000). CBP/p300 contains a transcriptional repression domain and this repressor domain contains a SUMO binding motif. The mutation of the lysine residue within this domain removed all repression suggesting that sumolyation has an essential role to play in transcriptional repression.

Sequence analysis of the TopBP1 transcriptional activation and repression domains (using SUMOplot™) identified two possible SUMO sites; one within the BRCT2 repression domain (K137) (Figure 59a) and one site within the transcriptional activation domain (K469), (Figure 59b). It is possible that these domains are regulated by sumolyation and there are several ways in which SUMO might do this; 1) by causing a conformational change in the TopBP1, resulting in alleviation of BRCT4 repression by BRCT2, 2) by changing the surface charge of TopBP1 which might physically mask BRCT2 binding site on BRCT4 or visa versa or 3) by changing the activation and repression domain binding partners, resulting in the recruitment of a repressor/activator complex. Sumoylation also functions to repress transcription by blocking acetylation and by competing for Ub target sites; for example IκBα is sumoylated and ubiquitinated at the same lysine therefore sumolyation stabilises the protein by inhibiting Ub and therefore inhibits the activity of the transcription factor NF-κB. As TopBP1 is ubiquitinated by hHYD and then degraded, it might be possible that SUMO would stabilise TopBP1, preventing ubiquitination. This might be of particular importance following DNA damage (Discussed Chapter 4.2.).

Future work could focus on these potential SUMO sites and investigate if indeed they are SUMO targets, by co-immunoprecipitation using SUMO antibodies. Does mutation of the predicted critical lysine residue (according to prediction Figure 62) within these SUMO motifs result in a loss of transcriptional repression/activation? Is TopBP1 stabilised by sumolyation following DNA damage?

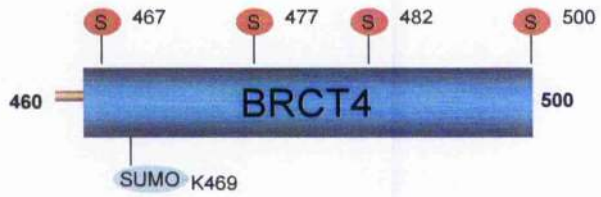
**Figure 59**

a)



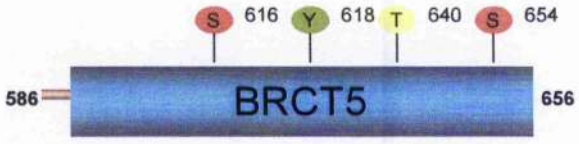

| | Score |
|---|---|
| K137: VQQLTV**K**HGGQYMG | 0.84 |
| Y164: KGQK**Y**ECAK | 0.91 |
| S180: CQDE**S**IYKY | 0.88 |

b)




| | Score |
|---|---|
| S467: EGLF**S**QKSF | 0.88 |
| K469: EGLFSQ**K**SFLVLGF | 0.89 |
| S477:VLGF**S**NENE | 0.93 |
| S482:NENE**S**NIAN | 0.78 |
| S500:MSLL**S**RTVA | 0.77 |

c)




| | Score |
|---|---|
| S616: ERGG**S**KYEA | 0.99 |
| Y618: GGSK**Y**EAAK | 0.95 |
| T640: ETAR**T**GKRA | 0.71 |
| S654: LIEN**S**TKEE | 0.98 |

**Figure 59: Possible phosphorylation sites and Sumolyation sites within the TopBP1 activation and repression domains.** a) Predicted serine (S), threonine (T) and tyrosine (Y) phosphorylation sites and sumolyation sites within the BRCT2 repression domain amino acids 113-181. The number of each amino acid predicted to be phosphorylated is also indicated. b) Predicted serine (S), threonine (T) and tyrosine (Y) phosphorylation and sumolyation sites within the transcriptional activation domain amino acids 460 -500. The number of each amino acid predicted to be phosphorylated is also indicated. c) Predicted serine (S), threonine (T) and tyrosine (Y) phosphorylation sites and sumolyation sites within the BRCT5 repression domain amino acids 586-606. The number of each amino acid predicted to be phosphorylated is also indicated. These predictions were carried out using NetPhos 2.0 phosphorylation prediction tool (Centre for Biological sequence analysis (CBS), University of Denmark). This software predicted the likelihood of phosphorylation of serine, tyrosine and threonine residues within TopBP1 and returned a predicted score result. A higher score result indicates a more significant result, and the cut off for this analysis was taken as 0.75, residues with a score higher than 0.75 indicates a very likely phosphorylation site.

### 4.1.2.3. Transcriptional Regulation by Phosphorylation

The regulation of the domains identified in Chapter 3.1 may be facilitated by phosphorylation. As described previously the interaction of transcription factors with the basal transcription machinery and other transcription factors can be regulated by phosphorylation. There is evidence to support the regulation of both transcriptional activators and repressors by phosphorylation. CREB (CRE response element binding protein) is an example of a transcriptional activator regulated by phosphorylation. CREB is phosphorylated at S133 by PKA, which recruits CREB-binding protein (CBP) to link CREB to the basal transcriptional machinery (De Cesare *et al.* 1999). Similarly the retinoblastoma binding protein (RB) is an example of a transcriptional repressor which is regulated by phosphorylation; de-repression of E2F transcription is dependent on phosphorylation of RB by CDK, and this phosphorylation is a key controller of the cell cycle.

The activation domain and repression domains within TopBP1 might be regulated by phosphorylation, as Figure 59 shows there are several predicted phosphorylation sites (Serine, Tyrosine and Threonine) within these regions. These potential phosphorylation sites were predicted using NetPhos 2.0 phosphorylation prediction tool (Centre for Biological sequence analysis (CBS), University of Denmark). Which predicted the likelihood of phosphorylation of; serine, tyrosine and threonine residues within the TopBP1 activation (Figure 59a) and repression domains (Figure 59b, and 59c) and returned a predicted score result (Cut off for this analysis was taken as 0.75,

residues with a score higher than 0.75 indicates a very likely phosphorylation site). It would be interesting to mutate these residues in future studies in order to determine if the repression of the activation domain is regulated by phosphorylation, and if the transcriptional activity of the domains is altered following phosphorylation. The role of phosphorylation in the activation and amplification of signal transduction pathways is well documented. For example c-Jun is phosphorylated within the transactivation domain by c-Jun $NH_2$-terminal kinase (JNK) increasing its transcriptional activity (Review JNK Bogoyevitch *et al.* 2006).

## 4.2 TopBP1 interaction with Chromatin Remodellers

Chapter 3.1. described the identification of chromatin modification domains by using GAL4 fusion proteins and monitoring transcriptional activity. These domains could be involved in replication, transcription and DNA damage repair. Chapter 3.2 showed that transcriptional repression by BRCT5 and the alleviation of it following DNA damage is dependent on the ATPase subunit of the chromatin remodeller SWISNF, Brg1. The data presented also showed that TopBP1 directly interacts with Brg1 and that this interaction is dependent on BRCT5. The results discussed here focus on this relationship between these two proteins and the possible role their interaction may have in mediating TopBP1 function.

### 4.2.1 SWISNF

The human SW12/SNF2 chromatin remodelling enzymes are a family of remodellers which can be further divided into 8 subtypes: 1) SNF2, 2) ISW1 3) CHD1 4) INO80 5) ERCC6, 6) RAD53, 7) DDM1 and 8) MOT1, according to the structural domains within the complexes for example ISW1 contain SANT-like domains, CHD1 chromatin remodellers contain chromodomains (Lusser *et al.* 2003, Eisen *et al.* 1995) and the SNF2 subfamily contain bromodomains. The chromatin remodellers destabilise the interaction between DNA and histones using ATP which results in the

a more open chromatin state, which reveals binding sites for transcription factors and DNA damage repair proteins (Owen-Hughes *et al.* 1996).

SWISNF a SNF2 chromatin remodeller is a 2MDa complex (Peterson *et al.* 2000, Cote *et al.* 1994, Wang *et al.* 1996), which contains 10-12 subunits (Recht *et al.* 1999). This complex hydrolyses 3 ATPs to displace approximately 52bp of DNA from the nucleosome to create a bulge (Zofall *et al.* 2006). The central catalytic ATPase core is either Brg1 or Brm (Phelan *et al.* 1999, Tyler *et al.* 1999). Brg1 and Brm interact with several cell cycle, transcriptional regulatory and repair proteins including, RB, p53, p107, p130, and BRCA1 (Muchardt *et al.* 2001, Barker *et al.* 2001, Bochar *et al.* 2000), c-myc (Grace *et al.* 1999), Cyclin E (Shanahan *et al.* 1999) CEBPB (CCAAT/enhancer binding protein-beta), HDAC4, LDB1 (LIM Domain Binding 1), MXD4 (Max dimerisation protein 4), NFκB2 (Nuclear factor κ light enhancer in B cells) and TBX2 (T-Box 2). In addition these proteins are both either mutated or down regulated in approximately 10% of human cancer cell lines (DeCristofaro *et al.* 2001) and a variety of human malignancies (Kiochendler *et al.* 2002) and heterozygote Brg1 mice show a tumour phenotype and are susceptible to the formation of mammary tumours (Bultman *et al.* 2000). Therefore Brg1 is implicated in many of the functions attributed to TopBP1; transcription and DNA damage signalling/repair.

### 4.2.2. Brg1-BRCT5 and DNA Damage

Although Brg and Brm are primarily associated with transcriptional activation, there is also evidence to suggest they are also involved in transcriptional repression. The transcriptional repressor REST represses transcription of neuronal genes in non-neuronal cells, by binding specific repressor element 1 (RE1) sites. Repression by REST is dependent HDAC and a co-repressor complex; CoREST. CoREST interacts with the SW1SNF subunit BAF57 (Battaglioli *et al.* 2002) and is recruited along with REST and CoREST to RE1 sites. Brg1 interacts with REST and is recruited to RE1 sites (Ooi *et al.* 2006), and this recruitment of Brg1 is dependent on an interaction between Brg1 and the REST repression domains as expression of a REST dominant negative lacking the repression domain reduces Brg1 recruitment to RE1 sites.

The cell cycle regulator E2F1 is stabilised by phosphorylation by ATM (Lin *et al.* 2001) and Chk2 (Stevens *et al.* 2003) following DNA damage. TopBP1 was identified as an E2F1 interacting protein using a yeast two hybrid assay and was shown to interact with E2F1 via BRCT6 following ATM phosphorylation of E2F1. The function of this interaction was tested in E2F1 luciferase assays and showed that TopBP1 represses the transcriptional activity of E2F1 (Liu *et al.* 2003), however in several cell lines however repression was lost in Brg1 and Brm compromised C33a cell lines (Liu *et al.* 2004). This data suggested that TopBP1 regulates the transcriptional activities of E2F1 during apoptosis and DNA damage via Brg1 and Brm by recruiting Brg1 and Brm to E2F1-responsive promoters.

To investigate the role of Brg1 in TopBP1 BRCT5 transcriptional repression, luciferase transcription assays in the Brg1 and Brm compromised cell line C33a were carried out (Figure 30). The results showed that transcriptional repression by BRCT 5 is dependent on Brg1 but independent of Brm in several ways; 1) BRCT5 repression is absent in C33a cells 2) Addition of Brg (wt) but not Brm (wt) restores repression in C33a cells 3) Addition of Brg (-/-) but not Brm (-/-) alleviates repression in 293T cells and lastly 4) The addition of Brg (wt) to 293T cells increases repression. TopBP1 and Brg1 have been shown previously to interact following DNA damage (Liu *et al.* 2004), but as Figure 38a shows Brg1 and TopBP1 can physically interact in undamaged cells.

Repression by BRCT5 is alleviated following DNA damage (Figure 42) and this alleviation of repression is dependent on Brg1, as this effect is not observed in C33a cells until the addition of Brg (wt) (Figure 44). TopBP1 contains no chromatin remodelling activities but does have eight BRCT domains as potential binding sites, therefore perhaps recruits Brg1 via a direct interaction to the sites of DNA damage to allow access for repair. Immunoprecipitation experiments showed that the interaction between Brg1 and one of the TopBP1 repressor regions is dependent on BRCT5 (Figure 40), suggesting that Brg1 mediated BRCT5 repression is dependent on a direct interaction between the two proteins. However further immunoprecipitation would need to be carried out to ascertain if the alleviation of repression following DNA damage is also dependent on a direct Brg1-TopBP1 interaction. It is possible also that following DNA damage Brg1 no longer binds TopBP1, and TopBP1

changes its binding partners to respond to the damage, interacting with NBS and Rad9 to facilitate the signalling and repair of the damage.

TopBP1 recruitment of Brg1 and repression of E2F1 transcription following DNA damage is dependent on the prior phosphorylation of E2F1 by ATM (Liu *et al.* 2004). TopBP1 is phosphorylated following DNA damage on S345 by ATM, therefore to study the mechanism of Brg1-BRCT5 alleviation of repression following DNA damage the PIKK inhibitors; caffeine LY294002 and wortmannin were used in transcription assays (Figures 45 and 46).

The results suggested that transcriptional repression in un-damaged cells was dependent on one of these three kinases, as it was lost following their addition. This could be via several mechanisms for example 1) One of the kinases may regulate the Brg1 protein to facilitate repression. 2) The phosphorylation of BRCT5 by one of these kinases mediates the BRCT5-Brg1 interaction which is essential for repression or 3) As well as Brg1, there is a PIKK dependent aspect to the BRCT5 repressor domain. It is possible that addition of these inhibitors disrupts the Brg-BRCT5 interaction and that is why repression is alleviated in damaged cells. Immunoprecipitation reactions would need to be performed to test if Brg1 and BRCT5 still directly interact following the inhibition of ATM, ATR and DNA-PK. As Figure 59c shows there are two predicted serine phosphorylation sites within the BRCT repression domain (S616, S654). Perhaps ATM, ATR, or DNA-PK phosphorylates BRCT5 at these residues to facilitate the interaction with Brg1 (Figure 60a). The use of more specific inhibitors such as: NU-66933 and KU55933

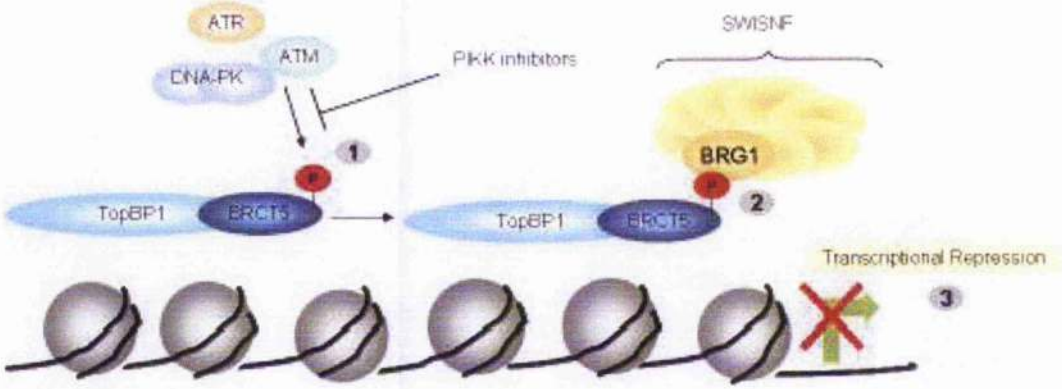
(KuDOS Pharmaceuticals Ltd), which specifically inhibit DNA-PK and ATM respectively which would be beneficial to dissect the kinase responsible repression. Once the specific kinase is identified using these inhibitors, creation of TopBP1 BRCT5 phosphorylation site mutants (S616, S654) followed by immunoprecipitation reactions with Brg1 would confirm if the interaction between BRCT5 and Brg1 is mediated by PIKK BRCT5 phosphorylation.

These series of experiments should be performed over a time course following DNA damage; as the literature suggests that the phosphorylation of H2AX and the signalling of the DNA damage response are dependent on the prior modification of the surrounding chromatin (Ikura *et al.* 2007) by recruited chromatin remodellers. The phosphorylation of H2AX is dependent on Brg1, as Brg1 compromised cell lines show a reduction in γH2AX foci (Park *et al.* 2006). Following DNA damage TopBP1 might initially bind Brg1 and recruit it to the sites of damage. Then following the initiation of the damage signal by phosphorylated H2AX, TopBP1 may then change its binding partners to aid in the activation of the cell cycle checkpoints and DNA damage repair (Figure 60b) and no longer bind Brg1. Immunofluorescence experiments should be performed staining TopBP1 and Brg1 (and γH2AX as a control for damage) following DNA damage. This would be interesting if the cells were fixed and stained at various time points following DNA damage to see if TopBP1 and Brg1 initially co-localise and then perhaps dis-associate later on in the damage response. Although it has been previously shown that Brg1 localises the sites of DNA damage, represented by γH2AX foci (Park *et al.* 2006), the staining was only carried out 1 hour post damage. Although the length it takes for cells to respond to

damage, arrest, repair and then re-enter the cell cycle is varied, MCF7 cells for example show γH2AX foci 2 hours post damage but the repair protein XRCC4 is not recruited until 8 hours following damage.

**Figure 60**

a)


ATR
ATM
DNA-PK
PIKK inhibitors
SWISNF
BRG1
TopBP1
BRCT8
TopBP1
BRCT8
Transcriptional Repression
1
2
3

b)

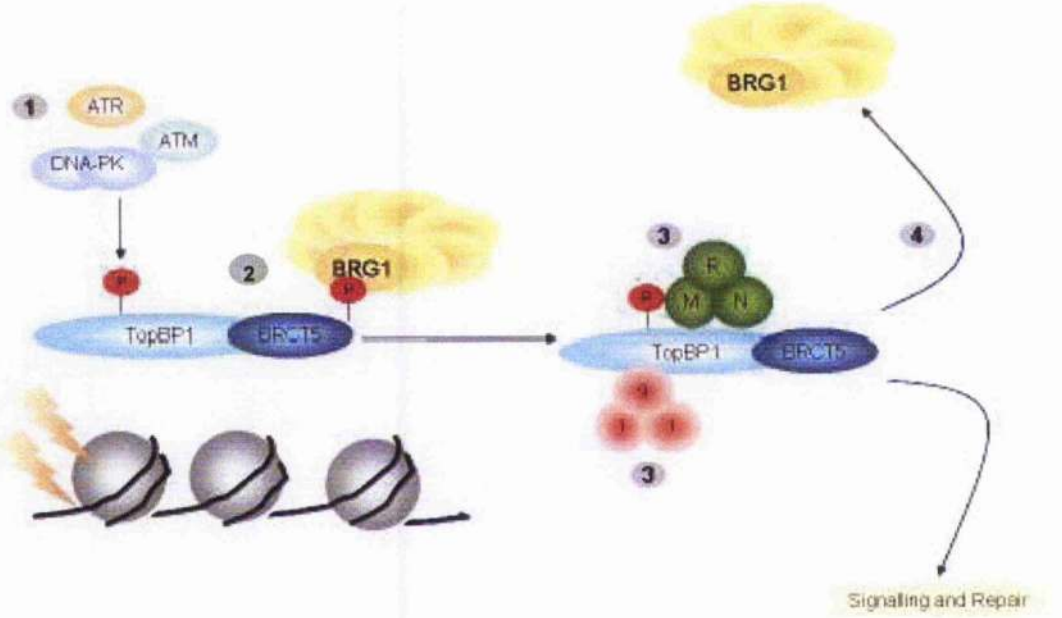

1
ATR
ATM
DNA-PK
2
BRG1
TopBP1
BRCT8
3
R
M
N
TopBP1
BRCT8
BRG1
4
Signalling and Repair

**Figure 60: Model of TopBP1 BRCT5-Brg1 interaction in undamaged and damaged cells.** a) In undamaged cells TopBP1 is phosphorylated by ATR, ATM or DNA-PK within BRCT5 (1) this phosphorylation of BRCT5 then mediates the BRCT5-Brg1 interaction (2), to repress transcription (3). This would support the result observed when repression is alleviated following the addition of the PIKK inhibitors. b) Following DNA damage Brg1 and TopBP1 may still interact, as both proteins localise to γH2AX foci. Then following TopBP1 phosphorylation (1), TopBP1 changes its binding partners (2, 3) to respond to the damage signal and no longer binds Brg1 (4).

### 4.2.3. Repression is Independent of Brm

BRCT5 repression is dependent on Brg1 but independent of Brm; the addition of Brm (wt) to Brm null C33a cells did not restore repression observed in 293T and the addition of Brm (-/-) did not alleviate repression as Brg1 (-/-) showed (Figure 37). Although these two homologues do have both sequence (75% identical and 86% similar) and functional redundancies there are several distinct differences; 1) Brg specifically interacts with zinc finger proteins (Kadam *et al.* 2003) via the N-terminal which is absent in Brm 2) Brm interacts with ankyrin repeats containing proteins (Xue *et al.* 2000). 4) Brg1 null mice are embryonic lethal (Bultman *et al.* 2000) in contrast to Brm null mice which are not, in fact their phenotypic characteristic is a tendency to gain weight (Reyes *et al.* 1998). 5) Brg1 specifically represses transcription from the c-fos promoter (Murphy *et al.* 1999).

A functional Brg1 and Brm are lost in a variety of cancer cell lines (Muchardt *et al.* 2001). Brg1 in these lines is mutated but Brm is not (Wong *et al.* 2000). In fact treatment of these cell lines with the histone deacetylase inhibitor TSA resulted in Brm protein expression demonstrating that Brm is not mutated rather silenced in these cell lines. To confirm that Brm is not required for repression further luciferase transcription assays could be performed in C33a cells treated with TSA. These two proteins also differences in there expression during cell development; oocytes contain both Brg1 and Brm however at the four cell stage when transcription starts the Brg1

gene is expressed but no Brm mRNA was detected until later on in the blastocyst stage (Muchardt *et al.* 2001). Brm is found in cells that are removed from the cell cycle, and downregulated in cells which are actively proliferating where Brg1 is expressed (Muchardt *et al.* 1999, Reyes *et al.* 1998, Machida *et al.* 2001). One explanation for the results observed is that the Brm expression plasmids do not encode a functional protein; however transfection of the Brm (wt) expression plasmid into C33a and 293T increased E2F1 transcription 3 fold demonstrating that a functional Brm protein is present. The preferential selection of Brg1 over Brm could be due to the N-terminal region of Brg1 a region not present in Brm. This N-terminal domain could be the region of Brg1 which interacts with BRCT5. To determine if this is the region within Brg1 which binds TopBP1 radioactively labelled TopBP1 BRCT5 *in vitro* translation (IVT)'s could be incubated against GST-Brg1 truncations, either incorporating or deleting the N-terminal domain.

These results have identified a functional interaction between TopBP1 and Brg1. This interaction could be involved in mediating the DNA damage response and transcriptional regulatory functions of TopBP1. As discussed in the following Chapter, TopBP1 is clearly involved in gene regulation following DNA damage.

# 4.3 Genes Regulated by TopBP1

An investigation into the genes regulated by TopBP1 (Chapter 3) was carried out; identification of these will potentially reveal novel genes involved in the DNA damage response and breast carcinogenesis. This study was composed of two parts, firstly and an investigation into the effect of the TopBP1 knockdown on cell growth, and secondly the microarray and analysis.

## 4.3.1 TopBP1 and Cell Growth

In order to perform the microarray following the knockdown of TopBP1, an investigation into the effect of the TopBP1 knockdown on cell growth was carried out. This was an essential control for the microarray, to ensure that the cell cycle was not adversely affected and therefore the data retrieved from the microarray analysis was comparable. Figures 49 and 51 showed that contrary to previously published data (Jeon *et al.* 2007) TopBP1 is not essential for cell growth in all cultured cell lines for a short period of time (4 days) although TopBP1 is essential for cell growth over longer periods of time (Figure 52).

TopBP1 is essential for signalling the DNA damage response interacting with NBS1 and Rad9, and ultimately the activation of the cell cycle checkpoint and repair. It is

therefore possible that cells lacking TopBP1 cannot initiate these pathways and display a compromised DNA damage response, over time (14 day period) and as the damage accumulates and is not repaired the cells finally undergo apoptosis or senescence. It is also possible that TopBP1 is essential for replication in undamaged cells where difficult DNA structures have to be overcome. The failure to do this over several cell divisions could ultimately result in the accumulation of damage leading to a catastrophic event resulting in cell death or senescence. This could be tested in several ways. 1) Total protein extracts could be prepared from cells siTopBP1 treated at different time points throughout the 14 day period, these extracts could be run on SDS-PAGE gel and the expression of the caspases discussed Chapter 1.5. (Review Rupinder et *al.* 2006) determined by western blotting, TopBP1 expression could also be tested to ensure that the cells are still knocked down. This would determine if and at what point the cells start to undergo apoptosis. 2) Single cell-gel electrophoresis 'Comet' assays could be used to determine if the cells that have TopBP1 knocked down do accumulate damage from endogenous sources over time. As the DNA fragmented "tails" observed in comet assays are directly proportional to the amount of damage, the accumulation of damage over time could be easily measured in this way (Review Collins *et al.* 2004). The knock down of TopBP1 in undamaged cells results in the formation of double strand breaks, characterised by the phosphorylation of H2AX (Kim *et al.* 2005), suggesting that in the absence of TopBP1 cells do start to accumulate DNA damage. Senescent cells express a β-galactosidase enzyme; senescence-associated (SA)-β-Gal), therefore this could be used as a marker (Going *et al.* 2002) in TopBP1 knocked down cells to determine if they are undergoing apoptosis or senescence.

TopBP1 may be involved in replication; interacting with DNA polymerase ε (Makinemi *et al.* 2001), studies of Dpb11 suggest that TopBP1 may be involved in the direct recruitment and loading of Cdc45 to replication origins (Araki *et al.* 1995, Wang *et al.* 2002), and TopBP1 increases the ability of the HPV16 replication factor E2 to activate replication (Boner *et al.* 2002). The results shown in Figure 51 demonstrated that cells do replicate in the absence of TopBP1. The experiments described in Chapter 3 provide a system for studying the role of TopBP1 in replication, as it has identified cell lines which can and those which cannot survive without TopBP1 albeit for a limited amount of time.

### 4.3.2 Genes and Pathways Analysis

This section will focus on the theoretical importance of target pathways of TopBP1 in light of TopBP1 roles in transcription, the DNA damage response, chromatin modification and breast cancer. Although this method of analysis provides possible insights into additional roles of TopBP1, further validation of the microarray results are still required as the fold changes are relatively low (less than 1.5) although statistically significant. The pathway analysis could also be further supported by testing other microarray pathway analysis databases such as GenMAPP 2.0 (www.GenMAPP.org).

### 4.3.2.1 Genes and Pathways Regulated by TopBP1

The data presented in Chapter 3 identified two lists of genes which are regulated by TopBP1; genes which were up-regulated and genes which were downregulated following the knockdown of TopBP1 using siRNA (Table 9 and 10). Importantly TopBP1 itself was the gene most effected by the siRNA showing a downregulation of -4.8 ($p= 2.33 \times 10^{-7}$), serving as a control for the experiment. These lists of genes will be used to further validate the microarray, several significantly up-regulated and down-regulated genes could be chosen and the observed effect validated by running Real Time-PCR. Pathway analysis was performed on these gene lists using DAVID (as described 3.3.4.). This pathway analysis revealed that there were eight pathways that were significantly altered following the knockdown of TopBP1 (Table 14) 1) Sprouty Regulation of Tyrosine kinase signals 2) p38 MAPK signalling pathway, 3) Gap Junction, 4) Inositol Phosphate Metabolism, 5) MAPK Signalling Pathway 6) Cell Cycle, 7) Estrogen-responsive Protein Efp controls Cell Cycle and Breast Tumour Growth and 8) Lysine Degradation.

### 4.3.2.2. MAPK Signalling Pathway

One of the most significantly effected pathways (following DAVID analysis 3.3.4.) by the knockdown of TopBP1 is the mitogen activated protein kinase (MAPK) signalling pathway. The MAPK signalling pathway on the DAVID database has 246

members, the knockdown of TopBP1 significantly affects 48 genes of these genes (Table 19). Recently, Taricani *et al.* (2007) showed that the TopBP1 fission yeast homologue $Rad4^{TopBP1}$ was repressed by srr2 (a stress response gene) and this suppression links the stress response and the MAPK signalling response to promote cell survival. The MAPK pathway can be further divided into three separate pathways; 1) MAPK/ERK, 2) MAPK/SAPK/JNK and 3) p38 MAPK. p38 MAPK signalling pathway which is induced by stress is significantly affected by the knockdown of TopBP1. These pathways have several roles within the cell including the regulation of cell proliferation, differentiation and death. All three pathways are regulated by a hierarchical cascade. The MAPKK kinases (MAPKKK) phosphorylate and activate the MAPK kinases (MAPKK) which activate the MAPK. These MAPK then interact with other protein kinases to activate cell proliferation or apoptosis. A number of studies have demonstrated the potential role of MAPK signalling in the initiation and pathogenesis of breast cancer. Sivarmas and colleagues compared the MAPK activity of breast cancers to benign tissues and found that the MAPK signalling cascade was increased over 6 fold in invasive breast cancers (Migliaccio *et al.* 1998). Breast cancer cell growth can be divided into two groups; oestradiol dependent and independent. Oestradiol independent breast cancers often use MAPK to mediate cell growth. The breast cancer cell line used for this microarray analysis (MCF7) respond to oestradiol, and insulin (Alblas *et al.* 1998) to stimulate the MAPK signalling cascade. Five minutes following the addition of oestradiol, MCF7 cells show a strong increase in MAPK kinase activity (Migliaccio *et al.* 1996). The p38 MAPK pathway induces cell cycle arrest, by targeting stress activated protein 1(SAP1, (Chen *et al.* 2000) and signal transducer and activator of transcription

(STAT1, (Ip *et al.* 1998)). p38 is activated following DNA damage, and activates STAT1 transcription. BRCA1 directly interacts with STAT1 following DNA damage providing a link between the DNA damage response and apoptosis. Expression of BRCA1 in MCF7 cells (by infection of recombinant adenovirus containing BRCA1 cDNA) results in G2/M cell cycle arrest via ERK1/2 signalling. BRCA1 increased the activation of ERK1/2 by phosphorylation and using a kinase assay; Yan and colleagues found that BRCA1 induced cell cycle arrest via the 14-3-3σ and WEE1. The knockdown of TopBP1 in this microarray has shown that 14-3-3σ and WEE1 are affected by TopBP1. Does TopBP1 also regulate ERK1/2 cell cycle control via 14-3-3σ and WEE1? There are antibodies, commercial kits and MAPK specific inhibitors available which would provide a method to investigate if TopBP1 does regulate the MAPK signalling pathway. It would be necessary before proceeding any further to investigate if the knockdown of TopBP1 does adversely effect the MAPK pathway.

### 4.3.2.3. Estrogen-Responsive RING-finger protein (Efp) controls Cell Cycle and Breast Tumour Growth

One of the other pathways significantly affected (p=0.0096) by the knockdown of TopBP1 is the oestrogen responsive protein (Efp) control of the cell cycle during breast tumour growth (Table 21). Efp has recently been linked to the growth of breast cancers; this mechanism of cell growth which is controlled by Efp in breast cancer is shown in Figure 61. The 14-3-3σ protein under the control of p53-independent transcription sequesters CDKs in the cytoplasm. This retention of CDKs blocks entry into mitosis. Efp facilitates the ubiquitination of 14-3-3σ and therefore its degradation

by the proteosome (Hermeking *et al.* 2006). The degradation of 14-3-3σ then releases the CDKs which transport to the nucleus and promote uncontrolled cell division (Urano *et al.* 2000). Suzuki *et al.* stained 151 breast carcinomas for Efp and found that an increase in Efp expression is associated with a decrease in 14-3-3σ expression (58/151 cases), supporting the role of Efp in 14-3-3σ degradation and cell cycle progression (Suzuki *et al.* 2005). Furthermore increased Efp expression is associated with a poor survival prognosis (Suzuki *et al.* 2005) and increased resistance to the cancer treatment tamoxifen (McKenna *et al.* 2000). It is suggested that Efp induces a switch in breast cancer growth from estrogen dependent to independent as Efp stable transfectant MCF7 cells inoculated into nude mice will produce tumours even in the absence of oestrogen (Horie *et al.* 2003). Because of this the inhibition of Efp is being investigated as a possible therapeutic target. It would be necessary to validate if this pathway is affected by the knockdown of TopBP1 and to do this TopBP1 could be knocked down using siRNA and 14-3-3σ protein levels could be detected using western blotting to see if TopBP1 does promote 14-3-3σ degradation.

Estrogen increases the expression of several estrogen responsive genes in breast cancer (Ikeda *et al.* 2004). These estrogen responsive genes include cytochrome c oxidase subunit VIIa related polypeptide (COX7RP), estrogen receptor binding fragment associated antigen 9 (EBAG9) and estrogen responsive protein (Efp). The role of TopBP1 in estrogen responsive transcription could be assayed in MCF7 cells (as they are estrogen receptor positive) using a luciferase reporter containing estrogen responsive elements (EREs) in luciferase transcription assays.

**Figure 61**

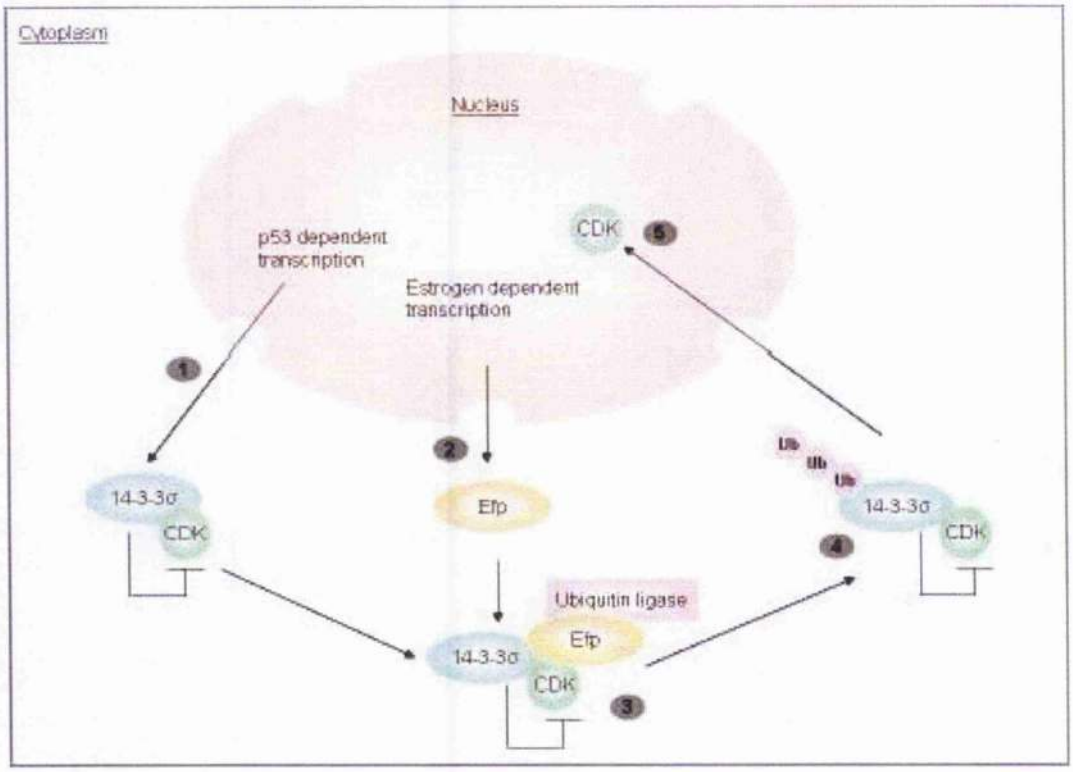



**Figure 61 Cell Cycle control of Breast cancer Cells by Efp.** The order the following step is shown on the diagram (Grey circle). (1) p53 dependent transcription modulates 14-3-3σ activity, which retains CDKs in the cytoplasm. (2) Efp binds 14-3-3σ and targets it for degradation by ubiquitination (3). 14-3-3σ is degraded by the proteosome and CDK are released and travel to the nucleus, (4) and the cell enters mitosis.

### 4.3.2.4. Cell Cycle

TopBP1 is involved replication and cell cycle progression. MCF7 cells in which the expression of TopBP1 is knocked down show no cell cycle arrest, compared to controls (Figure 51). DAVID analysis indicated that the cell cycle is affected by the knockdown of TopBP1 (Table 20). Analysis revealed that several proteins involved in the initiation of replication are disrupted by the knockdown of TopBP1 including the ORC subunits 5 and 6, and the MCM proteins 6 and 7. This analysis also revealed a change in gene expression within genes involved in cell cycle checkpoints and apoptosis; Cell division cycle 2 (G1to S and G2 to M), S phase kinase associated protein 2, inhibitors of the cyclin dependent kinases, p57, p18 and p19 (which inhibit CDK4), CDK2, CDK6 and WEE1, death associated protein 6, caspase 9, caspase 10, p21, arrestin, growth arrest and DNA-damage-inducible-beta. WEE1, like p53, inactivates the CDKs, in particular CDK2, which inhibits cell cycle progression, interestingly WEE1 directly interacts with the Efp targeted protein 14-3-3σ (Honda *et al.* 1997) possibly liking the Efp and WEE1 cell cycle control. In addition, as discussed BRCA1 regulates ERK1/2 G2/M cell cycle arrest via WEE1 and 14-3-3σ (Yan *et al.* 2005).

The gene expression data for the cell cycle pathway shows no bias to either up-regulated or downregulated genes; 40% of the genes are down-regulated and 60% of the genes are up-regulated. There is no increase in CDK inhibitors/decrease in CDKs, or visa versa which might suggest a mechanism of regulation. Further FACS analysis

would need to be performed incorporating cell cycle inhibitors in cells where TopBP1 is knocked down to investigate the role of TopBP1 and the cell cycle further. It is clear however that although cells can survive without TopBP1 for a limited amount of time (4 days) eventually they stop replicating and undergo apoptosis or senescence (3.3.2.).

### 4.3.2.5. Other significantly Affected Pathways

Other pathways which are significantly affected by TopBP1 knockdown are; Sprouty Regulation of Tyrosine Signals pathway, gap junctions, inositol phosphate metabolism, and the degradation of lysine. The receptor tyrosine kinases control a wide variety of cell processes including proliferation, differentiation and cell cycle control. The sprouty proteins are ligand induced inhibitors of the receptor tyrosine kinase pathways (Manson *et al.* 2005) and a downregulation in their expression has been observed in both prostate and breast cancers (McKie *et al.* 2004, Lo *et al.* 2004). Inositol phosphate metabolism is involved in a wide variety of cellular processes including calcium release, trafficking, chemotaxis, regulation of gene expression, DNA repair (York *et al.* 2006) and telomere maintenance (Saiardi *et al.* 2005, York *et al.* 2005). Gap junctions are also affected by the knockdown of TopBP1; scaffolding proteins including casein 1 δ, ε and tubulin as well as second messengers. Currently no connection appears between these pathways and the roles of TopBP1 in transcription and DNA repair. This might be because there is an overlap between the groups of genes affected by the TopBP1 knockdown; for example within the gap junction pathway, CDC2, CDC20, MAPKK1, MAPKK2, and MAPKK5 are

significantly altered but these genes are also significantly altered during the MAPK signalling pathway and cell cycle.

### 4.3.2.2. Genes and Pathways Regulated by DNA Damage

Five pathways are regulated by DNA damage in the presence of TopBP1 (Table 23), these include; Oxidative Stress Induced Gene Expression, FAS Signalling Pathway, apoptosis, the Proteosome and inositol phosphate metabolism. Although this is merely a preliminary result it is reassuring that these pathways are associated with cell death (Oxidative stress, FAS signalling, apoptosis). A comparison of this damage gene dataset with the genes regulated following DNA damage in the absence of TopBP1 was performed, resulting in a list of five genes:1) Olfactory receptor 1, 2) Calcium binding protein 3) 5, Calmodulin 2, 4) Tigger transposable element 3 and 5) Tubulin β2A. These genes were significantly upregulated following DNA damage in the presence of TopBP1 however in the absence of TopBP1 these genes were significantly downregulated following DNA damage (Figure 56b). This indicates that their increase in gene expression following DNA damage is specifically regulated by TopBP1. The increase in the calmodulin-calcium signalling pathway mediates an increase in cell cycle progression and has been associated with several human cancers including breast cancer (Lu *et al.* 1993). Calmodulin forms a complex with calcium, and activates several pathways involved in cell growth (Review Strobl *et al.* 1995), including the estrogen receptor dependent transcription activation pathway also involved in breast cancer.

The TopBP1 up-regulated and down-regulated genes were compared against breast cancer prognosis datasets. This method of analysis derives a prognostic value of the gene lists identified in the TopBP1 knockout samples using the survival procedure in the R-package (Performed by Dr. Keith Vass, Computing Service CR-UK Beatson Laboratories, Beatson Institute, Switchback Rd Glasgow) to determine if the knockdown of TopBP1 and the resultant gene expression profile is diagnostic of poor survival. Following analysis no significant correlation found with either survival or relapse; however in one study - a distinct, but not significant correlation ($p = 0.127$) between the TopBP1 down-regulated genes (Table 9) and relapse in patients with the same phenotype was found. Although this is not significant it is suggestive of a possible poorer prognosis associated with a downregulation of TopBP1, providing another possible role for TopBP1 in breast cancer recurrence. This result has a very real consequence for the cancer sufferers it might possibly affect as it would change the screening and follow -up monitoring of these patients with a high likelihood of cancer recurrence.

## 4.3.3. Conclusions and Future Work

This thesis has identified three transcriptional regulatory domains; one activator characteristic of other acidic activation domains similar to BRCA1, which is repressed by two adjacent repressor domains. These domains have the potential to modify chromatin and in support of this the BRCT5 repressor domain interacts with the chromatin remodelling ATPase enzyme Brg1. Both proteins are required for transcriptional repression and they physically interact; future experiments would focus on identifying the regions where these proteins bind in order to confirm if transcriptional repression is mediated by a direct protein-protein interaction using a purified system. Indeed transcriptional repression is alleviated following the addition of PIKK inhibitors in undamaged cells, suggesting repression is dependent on the phosphorylation of TopBP1, Brg1 or an as yet unidentified protein by these kinases. More targeted inhibitors will determine which PIKK is involved in this regulation. Future work could focus on the identification of the exact location of these domains, identify protein binding partners by proteonomics, and investigate the regulation these domains (As discussed 4.1 for example sumoylation, phosphorylation, ubiquitination) both in untreated cells and following DNA damage. The identification of these transcriptional regulatory domains further reinforces TopBP1 as a transcription factor.

As TopBP1 is a transcription factor, and has been implicated in breast carcinogenesis

(1.6.4.), possible TopBP1 transcriptional targets were investigated by running a gene expression array in MCF7 cells in which TopBP1 expression was knocked down using siRNA. The array studies demonstrate, in combination with previous studies that TopBP1 is involved in breast carcinogenesis as the pathways regulated by TopBP1 been previously implicated in breast cancer for example MAPK signalling, and estrogen signalling. ChIP on Chip experiments would provide a mechanism for identifying TopBP1 regulated promoter regions. Understanding the role that TopBP1 might have in the development of breast cancer would lead to a better understanding of disease and ultimately a more targeted cancer therapy strategy. A comparison between TopBP1 regulated genes identified and breast cancer arrays with survival data showed that the genes downregulated in the TopBP1 knockout samples are associated with recurrence. As TopBP1 is aberrantly expressed in breast cancer, and perhaps associated with recurrence TopBP1 would provide a useful tool as a marker for breast cancer. Future studies should focus on the characterisation of the role of TopBP1 in breast carcinogenesis. Large population studies investigating the effects of cancer drug treatments, against TopBP1 staining and survival could be carried out. This would determine if TopBP1 is associated with poor prognosis, and if TopBP1 could be used as marker for therapy strategies; for example drug X is associated with poor prognosis when TopBP1 is aberrantly expressed but drug Y is associated with a more positive outcome. The identification of TopBP1 as a marker for poor/good cancer prognosis would be of the utmost importance to the women it would ultimately affect, providing a more targeted therapy and follow-up strategy.

# References

**Alblas**, J., Slager-Davidov, R., Steenbergh, P. H., Sussenbach, J. S., & van der, B. B. 1998, "The role of MAP kinase in TPA-mediated cell cycle arrest of human breast cancer cells", *Oncogene*, vol. 16, no. 1, pp. 131-139.

**Alderton**, G. K., Joenje, H., Varon, R., Borglum, A. D., Jeggo, P. A., & O'Driscoll, M. 2004, "Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway", *Hum.Mol.Genet.*, vol. 13, no. 24, pp. 3127-3138.

**Allinen**, M., Huusko, P., Mantyniemi, S., Launonen, V., & Winqvist, R. 2001, "Mutation analysis of the CHK2 gene in families with hereditary breast cancer", *Br.J.Cancer*, vol. 85, no. 2, pp. 209-212.

**Araki**, H., Leem, S. H., Phongdara, A., & Sugino, A. 1995, "Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual

role in S-phase progression and at a cell cycle checkpoint", *Proc.Natl.Acad.Sci.U.S.A*, vol. 92, no. 25, pp. 11791-11795.

**Bai**, M., Papoudou-Bai, A., Kitsoulis, P., Horianopoulos, N., Kamina, S., Agnantis, N. J., & Kanavaros, P. 2005, "Cell cycle and apoptosis deregulation in classical Hodgkin lymphomas", *In Vivo*, vol. 19, no. 2, pp. 439-453.

**Bakkenist**, C. J. & Kastan, M. B. 2003, "DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation", *Nature*, vol. 421, no. 6922, pp. 499-506.

**Balasubramanyam**, K., Altaf, M., Varier, R. A., Swaminathan, V., Ravindran, A., Sadhale, P. P., & Kundu, T. K. 2004, "Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression", *J.Biol.Chem.*, vol. 279, no. 32, pp. 33716-33726.

**Banin**, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., & Ziv, Y. 1998, "Enhanced phosphorylation of p53 by ATM in response to DNA damage", *Science*, vol. 281, no. 5383, pp. 1674-1677.

**Barker**, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., & Clevers, H. 2001, "The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation", *EMBO J.*, vol. 20, no. 17, pp. 4935-4943.

**Bartek**, J. & Lukas, J. 2001, "Mammalian G1- and S-phase checkpoints in response to DNA damage", *Curr.Opin.Cell Biol.*, vol. 13, no. 6, pp. 738-747.

**Bartek**, J. & Lukas, J. 2003, "Chk1 and Chk2 kinases in checkpoint control and cancer", *Cancer Cell*, vol. 3, no. 5, pp. 421-429.

**Bartek**, J. & Lukas, J. 2007, "DNA damage checkpoints: from initiation to recovery or adaptation", *Curr.Opin.Cell Biol.*, vol. 19, no. 2, pp. 238-245.

**Bartek**, J., Lukas, C., & Lukas, J. 2004, "Checking on DNA damage in S phase", *Nat.Rev.Mol.Cell Biol.*, vol. 5, no. 10, pp. 792-804.

**Bassing**, C. H. & Alt, F. W. 2004, "H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity", *Cell Cycle*, vol. 3, no. 2, pp. 149-153.

**Battaglioli**, E., Andres, M. E., Rose, D. W., Chenoweth, J. G., Rosenfeld, M. G., Anderson, M. E., & Mandel, G. 2002, "REST repression of neuronal genes

requires components of the hSWI.SNF complex", *J.Biol.Chem.*, vol. 277, no. 43, pp. 41038-41045.

**Berger**, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., & Kim, P. S. 1995, "Predicting coiled coils by use of pairwise residue correlations", *Proc.Natl.Acad.Sci.U.S.A*, vol. 92, no. 18, pp. 8259-8263.

**Berkovich**, E., Monnat, R. J., Jr., & Kastan, M. B. 2007, "Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair", *Nat.Cell Biol.*, vol. 9, no. 6, pp. 683-690.

**Blattner**, C., Sparks, A., & Lane, D. 1999, "Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53", *Mol.Cell Biol.*, vol. 19, no. 5, pp. 3704-3713.

**Bochar**, D. A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W. S., Wang, W., Kashanchi, F., & Shiekhattar, R. 2000, "BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer", *Cell*, vol. 102, no. 2, pp. 257-265.

**Bogoyevitch**, M. A. & Kobe, B. 2006, "Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases", *Microbiol.Mol.Biol.Rev.*, vol. 70, no. 4, pp. 1061-1095.

**Boner**, W., Taylor, E. R., Tsirimonaki, E., Yamane, K., Campo, M. S., & Morgan, I. M. 2002, "A Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1", *J.Biol.Chem.*, vol. 277, no. 25, pp. 22297-22303.

**Breitling**, R., Armengaud, P., Amtmann, A., & Herzyk, P. 2004, "Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments", *FEBS Lett.*, vol. 573, no. 1-3, pp. 83-92.

**Broeks**, A., de Klein, A., Floore, A. N., Muijtjens, M., Kleijer, W. J., Jaspers, N. G., & 't Veer, L. J. 1998, "ATM germline mutations in classical ataxia-telangiectasia patients in the Dutch population", *Hum.Mutat.*, vol. 12, no. 5, pp. 330-337.

**Broeks**, A., Urbanus, J. H., Floore, A. N., Dahler, E. C., Klijn, J. G., Rutgers, E. J., Devilee, P., Russell, N. S., Van Leeuwen, F. E., & 't Veer, L. J. 2000, "ATM-heterozygous germline mutations contribute to breast cancer-susceptibility", *Am.J.Hum.Genet.*, vol. 66, no. 2, pp. 494-500.

**Bultman**, S., Gebuhr, T., Yee, D., La Mantia, C., Nicholson, J., Gilliam, A., Randazzo, F., Metzger, D., Chambon, P., Crabtree, G., & Magnuson, T. 2000, "A Brg1 null mutation in the mouse reveals functional differences

among mammalian SWI/SNF complexes", *Mol.Cell*, vol. 6, no. 6, pp. 1287-1295.

**Canman**, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., & Siliciano, J. D. 1998, "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53", *Science*, vol. 281, no. 5383, pp. 1677-1679.

**Capponcelli**, S., Pedrini, E., Cerone, M. A., Corti, V., Fontanesi, S., Alessio, M., Bachi, A., Soddu, S., Ribatti, D., Picci, P., Helman, L. J., Cantelli-Forti, G., & Sangiorgi, L. 2005, "Evaluation of the molecular mechanisms involved in the gain of function of a Li-Fraumeni TP53 mutation", *Hum.Mutat.*, vol. 26, no. 2, pp. 94-103.

**Celeste**, A., Difilippantonio, S., Difilippantonio, M. J., Fernandez-Capetillo, O., Pilch, D. R., Sedelnikova, O. A., Eckhaus, M., Ried, T., Bonner, W. M., & Nussenzweig, A. 2003, "H2AX haploinsufficiency modifies genomic stability and tumor susceptibility", *Cell*, vol. 114, no. 3, pp. 371-383.

**Celeste**, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., & Nussenzweig, A. 2003, "Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks", *Nat.Cell Biol.*, vol. 5, no. 7, pp. 675-679.

**Chai**, J., Charboneau, A. L., Betz, B. L., & Weissman, B. E. 2005, "Loss of the hSNF5 gene concomitantly inactivates p21CIP/WAF1 and p16INK4a activity associated with replicative senescence in A204 rhabdoid tumor cells", *Cancer Res.*, vol. 65, no. 22, pp. 10192-10198.

**Chen**, C. C., Chen, J. J., & Chou, C. Y. 2000, "Protein kinase calpha but not p44/42 mitogen-activated protein kinase, p38, or c-Jun NH(2)-terminal kinase is required for intercellular adhesion molecule-1 expression mediated by interleukin-1beta: involvement of sequential activation of tyrosine kinase, nuclear factor-kappaB-inducing kinase, and IkappaB kinase 2", *Mol.Pharmacol.*, vol. 58, no. 6, pp. 1479-1489.

**Cheung**, W. L., Turner, F. B., Krishnamoorthy, T., Wolner, B., Ahn, S. H., Foley, M., Dorsey, J. A., Peterson, C. L., Berger, S. L., & Allis, C. D. 2005, "Phosphorylation of histone H4 serine 1 during DNA damage requires casein kinase II in S. cerevisiae", *Curr.Biol.*, vol. 15, no. 7, pp. 656-660.

**Clancy**, J. L., Henderson, M. J., Russell, A. J., Anderson, D. W., Bova, R. J., Campbell, I. G., Choong, D. Y., Macdonald, G. A., Mann, G. J., Nolan, T., Brady, G., Olopade, O. I., Woollatt, E., Davies, M. J., Segara, D., Hacker, N. F., Henshall, S. M., Sutherland, R. L., & Watts, C. K. 2003, "EDD, the human orthologue of the hyperplastic discs tumour suppressor gene, is

amplified and overexpressed in cancer", *Oncogene*, vol. 22, no. 32, pp. 5070-5081.

**Collins,** A. R. 2004, "The comet assay for DNA damage and repair: principles, applications, and limitations", *Mol.Biotechnol.* , vol. 26, no. 3, pp. 249-261.

**Cortez,** D., Wang, Y., Qin, J., & Elledge, S. J. 1999, "Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks", *Science*, vol. 286, no. 5442, pp. 1162-1166.

**Costelloe**, T., Fitzgerald, J., Murphy, N. J., Flaus, A., & Lowndes, N. F. 2006, "Chromatin modulation and the DNA damage response", *Exp.Cell Res.*, vol. 312, no. 14, pp. 2677-2686.

**Cote**, J., Quinn, J., Workman, J. L., & Peterson, C. L. 1994, "Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex", *Science*, vol. 265, no. 5168, pp. 53-60.

**Couture**, J. F., Collazo, E., Brunzelle, J. S., & Trievel, R. C. 2005, "Structural and functional analysis of SET8, a histone H4 Lys-20 methyltransferase", *Genes Dev.*, vol. 19, no. 12, pp. 1455-1465.

**Cress, A. & Triezenberg**, S. J. 1991, "Nucleotide and deduced amino acid sequences of the gene encoding virion protein 16 of herpes simplex virus type 2", *Gene*, vol. 103, no. 2, pp. 235-238.

**De Cesare** D., Fimia G. M., Sasssone-Corsi P., (1999). "Signaling routes to CREM and CREB: plasticity in transcriptional activation." Trends Biochem 1999 Jul;24(7):pp. 281-5.

**Decristofaro**, M. F., Betz, B. L., Rorie, C. J., Reisman, D. N., Wang, W., & Weissman, B. E. 2001, "Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies", *J.Cell Physiol*, vol. 186, no. 1, pp. 136-145.

**Delacroix**, S., Wagner, J. M., Kobayashi, M., Yamamoto, K., & Karnitz, L. M. 2007, "The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1", *Genes Dev.*, vol. 21, no. 12, pp. 1472-1477.

**Dennis**, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., & Lempicki, R. A. 2003, "DAVID: Database for Annotation, Visualization, and Integrated Discovery", *Genome Biol.*, vol. 4, no. 5, p. 3.

**Dhillon** A. S., Hagan S. Rath O., Kolch W., (2007) "MAP kinase signalling pathways in cancer" Oncogene 2007 May 14;26(22):pp3279-90.

**Dong** X. Wang L. Taniguchi K., 2003 "Mutations in CHEK2 associated with prostate cancer risk." Am J. Human. Genetics. 2003 Feb;72(2):270-80.

**Downey**, M. & Durocher, D. 2006, "Chromatin and DNA repair: the benefits of relaxation", *Nat.Cell Biol.*, vol. 8, no. 1, pp. 9-10.

**Drysdale,** C. M., Duenas, E., Jackson, B. M., Reusser, U., Braus, G. H., & Hinnebusch, A. G. 1995, "The transcriptional activator GCN4 contains multiple activation domains that are critically dependent on hydrophobic amino acids", *Mol.Cell Biol.*, vol. 15, no. 3, pp. 1220-1233.

**Du**, L. L., Nakamura, T. M., & Russell, P. 2006, "Histone modification-dependent and -independent pathways for recruitment of checkpoint protein Crb2 to double-strand breaks", *Genes Dev.*, vol. 20, no. 12, pp. 1583-1596.

**Du**, L. L., Nakamura, T. M., Moser, B. A., & Russell, P. 2003, "Retention but not recruitment of Crb2 at double-strand breaks requires Rad1 and Rad3 complexes", *Mol.Cell Biol.*, vol. 23, no. 17, pp. 6150-6158.

**Durocher**, F., Labrie, Y., Soucy, P., Sinilnikova, O., Labuda, D., Bessette, P., Chiquette, J., Laframboise, R., Lepine, J., Lesperance, B., Ouellette, G., Pichette, R., Plante, M., Tavtigian, S. V., & Simard, J. 2006, "Mutation

analysis and characterization of ATR sequence variants in breast cancer cases from high-risk French Canadian breast/ovarian cancer families", *BMC.Cancer*, vol. 6, p. 230.

**Edwards**, R. J., Bentley, N. J., & Carr, A. M. 1999, "A Rad3-Rad26 complex responds to DNA damage independently of other checkpoint proteins", *Nat.Cell Biol.*, vol. 1, no. 7, pp. 393-398.

**Eisen**, J. A., Sweder, K. S., & Hanawalt, P. C. 1995, "Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions", *Nucleic Acids Res.*, vol. 23, no. 14, pp. 2715-2723.

**Eisenman**, R. N. 2001, "Deconstructing myc", *Genes Dev.*, vol. 15, no. 16, pp. 2023-2030.

**Ekwall**, K. 2005, "Genome-wide analysis of HDAC function", *Trends Genet.*, vol. 21, no. 11, pp. 608-615.

**Fackenthal**, J. D., Cartegni, L., Krainer, A. R., & Olopade, O. I. 2002, "BRCA2 T2722R is a deleterious allele that causes exon skipping", *Am.J.Hum.Genet.*, vol. 71, no. 3, pp. 625-631.

**Falck**, J., Mailand, N., Syljuasen, R. G., Bartek, J., & Lukas, J. 2001, "The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis", *Nature*, vol. 410, no. 6830, pp. 842-847.

**Falck**, J., Petrini, J. H., Williams, B. R., Lukas, J., & Bartek, J. 2002, "The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways", *Nat.Genet.*, vol. 30, no. 3, pp. 290-294.

**Froggatt**, N. J., Green, J., Brassett, C., Evans, D. G., Bishop, D. T., Kolodner, R., & Maher, E. R. 1999, "A common MSH2 mutation in English and North American HNPCC families: origin, phenotypic expression, and sex specific differences in colorectal cancer", *J.Med.Genet.*, vol. 36, no. 2, pp. 97-102.

**Fuja** T. J., Lin F., Osann K. E., 2004 "Somatic mutations and altered expression of the candidate tumor suppressors CSNK1 epsilon, DLG1, and EDD/hHYD in mammary ductal carcinoma." Cancer Res. 2004 Feb 1;64(3):pp942-51.

**Furuya**, K., Poitelea, M., Guo, L., Caspari, T., & Carr, A. M. 2004, "Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of Rad4TOPBP1", *Genes Dev.*, vol. 18, no. 10, pp. 1154-1164.

**Futaki**, M. & Liu, J. M. 2001, "Chromosomal breakage syndromes and the BRCA1 genome surveillance complex", *Trends Mol.Med.*, vol. 7, no. 12, pp. 560-565.

**Futreal**, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., & . 1994, "BRCA1 mutations in primary breast and ovarian carcinomas", *Science*, vol. 266, no. 5182, pp. 120-122.

**Garcia**, V., Furuya, K., & Carr, A. M. 2005, "Identification and functional analysis of TopBP1 and its homologs", *DNA Repair (Amst)*, vol. 4, no. 11, pp. 1227-1239.

**Gatei**, M., Scott, S. P., Filippovitch, I., Soronika, N., Lavin, M. F., Weber, B., & Khanna, K. K. 2000, "Role for ATM in DNA damage-induced phosphorylation of BRCA1", *Cancer Res.*, vol. 60, no. 12, pp. 3299-3304.

**Gatei**, M., Young, D., Cerosaletti, K. M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M. F., Gatti, R. A., Concannon, P., & Khanna, K. 2000, "ATM-dependent phosphorylation of nibrin in response to radiation exposure", *Nat.Genet.*, vol. 25, no. 1, pp. 115-119.

**Girdwood**, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., & Hay, R. T. 2003, "P300 transcriptional repression is mediated by SUMO modification", *Mol.Cell*, vol. 11, no. 4, pp. 1043-1054.

**Glaros**, S., Cirrincione, G. M., Muchardt, C., Kleer, C. G., Michael, C. W., & Reisman, D. 2007, "The reversible epigenetic silencing of BRM: implications for clinical targeted therapy", *Oncogene*.

**Glover**, J. N. 2006, "Insights into the molecular basis of human hereditary breast cancer from studies of the BRCA1 BRCT domain", *Fam.Cancer*, vol. 5, no. 1, pp. 89-93.

**Glover**, J. N., Williams, R. S., & Lee, M. S. 2004, "Interactions between BRCT repeats and phosphoproteins: tangled up in two", *Trends Biochem.Sci.*, vol. 29, no. 11, pp. 579-585.

**Going**, J. J., Nixon, C., Dornan, E. S., Boner, W., Donaldson, M. M., & Morgan, I. M. 2007, "Aberrant expression of TopBP1 in breast cancer", *Histopathology*, vol. 50, no. 4, pp. 418-424.

**Going,** J. J., Stuart, R. C., Downie, M., Fletcher-Monaghan, A. J., & Keith, W. N. 2002, "'Senescence-associated' beta-galactosidase activity in the upper gastrointestinal tract", *J.Pathol.*, vol. 196, no. 4, pp. 394-400.

**Goldberg,** M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., & Jackson, S. P. 2003, "MDC1 is required for the intra-S-phase DNA damage checkpoint", *Nature*, vol. 421, no. 6926, pp. 952-956.

**Grace** C. O., Fink G., Quinn J. P., 1999. "Characterization of potential regulatory elements within the rat arginine vasopressin proximal promoter." Neuropeptides 1999 Feb;33 (1):pp. 81-90.

**Graves,** P. R., Yu, L., Schwarz, J. K., Gales, J., Sausville, E. A., O'Connor, P. M., & Piwnica-Worms, H. 2000, "The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01", *J.Biol.Chem.*, vol. 275, no. 8, pp. 5600-5605.

**Greer,** D. A., Besley, B. D., Kennedy, K. B., & Davey, S. 2003, "hRad9 rapidly binds DNA containing double-strand breaks and is required for damage-dependent topoisomerase II beta binding protein 1 focus formation", *Cancer Res.*, vol. 63, no. 16, pp. 4829-4835.

**Groisman**, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., & Nakatani, Y. 2003, "The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage", *Cell*, vol. 113, no. 3, pp. 357-367.

**Hamilton**, S. R., Liu, B., Parsons, R. E., Papadopoulos, N., Jen, J., Powell, S. M., Krush, A. J., Berk, T., Cohen, Z., Tetu, B., & . 1995, "The molecular basis of Turcot's syndrome", *N.Engl.J.Med.*, vol. 332, no. 13, pp. 839-847.

**Hammet**, A., Magill, C., Heierhorst, J., & Jackson, S. P. 2007, "Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast", *EMBO Rep.*, vol. 8, no. 9, pp. 851-857.

**Hara**, R., Mo, J., & Sancar, A. 2000, "DNA damage in the nucleosome core is refractory to repair by human excision nuclease", *Mol.Cell Biol.*, vol. 20, no. 24, pp. 9173-9181.

**Hartikainen**, J., Kataja, V., Pirskanen, M., Arffman, A., Ristomaa, U., Vahteristo, P., Ryynanen, M., Heinonen, S., Kosma, V. M., & Mannermaa, A. 2007, "Screening for BRCA1 and BRCA2 mutations in Eastern Finnish breast/ovarian cancer families", *Clin.Genet.*, vol. 72, no. 4, pp. 311-320.

**Hashimoto** Y., Tsujimura T., Sugino A., 2003 "The phosphorylated C-terminal domain of Xenopus Cut5 directly mediates ATR-dependent activation of Chk1." Genes Cells. 2006 Sep;11(9):993-1007

**Hashimoto**, Y., Tsujimura, T., Sugino, A., & Takisawa, H. 2006, "The phosphorylated C-terminal domain of Xenopus Cut5 directly mediates ATR-dependent activation of Chk1", *Genes Cells*, vol. 11, no. 9, pp. 993-1007.

**Hayes**, F., Cayanan, C., Barilla, D., & Monteiro, A. N. 2000, "Functional assay for BRCA1: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation", *Cancer Res.*, vol. 60, no. 9, pp. 2411-2418.

**He**, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., & Kinzler, K. W. 1998, "Identification of c-MYC as a target of the APC pathway", *Science*, vol. 281, no. 5382, pp. 1509-1512.

**Henderson**, M. J., Munoz, M. A., Saunders, D. N., Clancy, J. L., Russell, A. J., Williams, B., Pappin, D., Khanna, K. K., Jackson, S. P., Sutherland, R. L., & Watts, C. K. 2006, "EDD mediates DNA damage-induced activation of CHK2", *J.Biol.Chem.*, vol. 281, no. 52, pp. 39990-40000.

**Hermeking**, H. 2006, "14-3-3 proteins and cancer biology", *Semin.Cancer Biol.*, vol. 16, no. 3, p. 161.

**Herold**, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaoja, J., Saluz, H. P., Haenel, F., & Eilers, M. 2002, "Negative regulation of the mammalian UV response by Myc through association with Miz-1", *Mol.Cell*, vol. 10, no. 3, pp. 509-521.

**Honda**, R., Ohba, Y., & Yasuda, H. 1997, "14-3-3 zeta protein binds to the carboxyl half of mouse wee1 kinase", *Biochem.Biophys.Res.Commun.*, vol. 230, no. 2, pp. 262-265.

**Honda**, Y., Tojo, M., Matsuzaki, K., Anan, T., Matsumoto, M., Ando, M., Saya, H., & Nakao, M. 2002, "Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response", *J.Biol.Chem.*, vol. 277, no. 5, pp. 3599-3605.

**Horie**, K., Urano, T., & Inoue, S. 2003, "Efp as a new molecular target for breast cancer therapy", *Anticancer Drugs*, vol. 14, no. 1, pp. 1-2.

**Horie**, K., Urano, T., Ikeda, K., & Inoue, S. 2003, "Estrogen-responsive RING finger protein controls breast cancer growth", *J.Steroid Biochem.Mol.Biol.*, vol. 85, no. 2-5, pp. 101-104.

**Hu**, Y. F., Hao, Z. L., & Li, R. 1999, "Chromatin remodeling and activation of chromosomal DNA replication by an acidic transcriptional activation domain from BRCA1", *Genes Dev.*, vol. 13, no. 6, pp. 637-642.

**Hu**, Y. F., Miyake, T., Ye, Q., & Li, R. 2000, "Characterization of a novel trans-activation domain of BRCA1 that functions in concert with the BRCA1 C-terminal (BRCT) domain", *J.Biol.Chem.*, vol. 275, no. 52, pp. 40910-40915.

**Hulkko**, S. M., Wakui, H., & Zilliacus, J. 2000, "The pro-apoptotic protein death-associated protein 3 (DAP3) interacts with the glucocorticoid receptor and affects the receptor function", *Biochem.J.*, vol. 349 Pt 3, pp. 885-893.

**Huyen**, Y., Zgheib, O., Ditullio, R. A., Jr., Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Sheston, E. A., Mellert, H. S., Stavridi, E. S., & Halazonetis, T. D. 2004, "Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks", *Nature*, vol. 432, no. 7015, pp. 406-411.

**Ikeda**, K. & Inoue, S. 2004, "Estrogen receptors and their downstream targets in cancer", *Arch.Histol.Cytol.*, vol. 67, no. 5, pp. 435-442.

**Ikura**, T., Tashiro, S., Kakino, A., Shima, H., Jacob, N., Amunugama, R., Yoder, K., Izumi, S., Kuraoka, I., Tanaka, K., Kimura, H., Ikura, M., Nishikubo, S.,

Ito, T., Muto, A., Miyagawa, K., Takeda, S., Fishel, R., Igarashi, K., & Kamiya, K. 2007, "DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics", *Mol.Cell Biol.*

**Ip**, Y. T. & Davis, R. J. 1998, "Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development", *Curr.Opin.Cell Biol.*, vol. 10, no. 2, pp. 205-219.

**Irizarry**, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., & Speed, T. P. 2003, "Summaries of Affymetrix GeneChip probe level data", *Nucleic Acids Res.*, vol. 31, no. 4, p. e15.

**Ito**, Y., Yoshida, H., Tomoda, C., Uruno, T., Takamura, Y., Miya, A., Kobayashi, K., Matsuzuka, F., Kuma, K., Nakamura, Y., Kakudo, K., & Miyauchi, A. 2005, "Expression of cdc25B and cdc25A in medullary thyroid carcinoma: cdc25B expression level predicts a poor prognosis", *Cancer Lett.*, vol. 229, no. 2, pp. 291-297.

**Ito**, Y., Yoshida, H., Uruno, T., Takamura, Y., Miya, A., Kuma, K., & Miyauchi, A. 2004, "Expression of cdc25A and cdc25B phosphatase in breast carcinoma", *Breast Cancer*, vol. 11, no. 3, pp. 295-300.

**Jackson** JR, Gilmartin A, Imburgia C, Winkler JD, Marshall LA, Roshak A. 2000 "An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage." Cancer Res. 2000 Feb 1;60(3):566-72

**Jeon**, Y., Lee, K. Y., Ko, M. J., Lee, Y. S., Kang, S., & Hwang, D. S. 2007, "Human TopBP1 participates in cyclin E/CDK2 activation and preinitiation complex assembly during G1/S transition", *J.Biol.Chem.*, vol. 282, no. 20, pp. 14882-14890.

**Jiang**, X., Sun, Y., Chen, S., Roy, K., & Price, B. D. 2006, "The FATC domains of PIKK proteins are functionally equivalent and participate in the Tip60-dependent activation of DNA-PKcs and ATM", *J.Biol.Chem.*, vol. 281, no. 23, pp. 15741-15746.

**Kadam, S. & Emerson, B. M.** 2003, "Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes", *Mol.Cell*, vol. 11, no. 2, pp. 377-389.

**Karihtala**, P., Winqvist, R., Syvaoja, J. E., Kinnula, V. L., & Soini, Y. 2006, "Increasing oxidative damage and loss of mismatch repair enzymes during breast carcinogenesis", *Eur.J.Cancer*, vol. 42, no. 15, pp. 2653-2659.

**Karppinen**, S. M., Erkko, H., Reini, K., Pospiech, H., Heikkinen, K., Rapakko, K., Syvaoja, J. E., & Winqvist, R. 2006, "Identification of a common polymorphism in the TopBP1 gene associated with hereditary susceptibility to breast and ovarian cancer", *Eur.J.Cancer*, vol. 42, no. 15, pp. 2647-2652.

**Kharbanda**, S., Pandey, P., Morris, P. L., Whang, Y., Xu, Y., Sawant, S., Zhu, L. J., Kumar, N., Yuan, Z. M., Weichselbaum, R., Sawyers, C. L., Pandita, T. K., & Kufe, D. 1998, "Functional role for the c-Abl tyrosine kinase in meiosis I", *Oncogene*, vol. 16, no. 14, pp. 1773-1777.

**Kim**, J. E., McAvoy, S. A., Smith, D. I., & Chen, J. 2005, "Human TopBP1 ensures genome integrity during normal S phase", *Mol.Cell Biol.*, vol. 25, no. 24, pp. 10907-10915.

**Kimura**, H. 2005, "Histone dynamics in living cells revealed by photobleaching", *DNA Repair (Amst)*, vol. 4, no. 8, pp. 939-950.

**Klochendler-Yeivin**, A., Muchardt, C., & Yaniv, M. 2002, "SWI/SNF chromatin remodeling and cancer", *Curr.Opin.Genet.Dev.*, vol. 12, no. 1, pp. 73-79.

**Kobayashi**, J., Antoccia, A., Tauchi, H., Matsuura, S., & Komatsu, K. 2004, "NBS1 and its functional role in the DNA damage response", *DNA Repair (Amst)*, vol. 3, no. 8-9, pp. 855-861.

**Kornberg**, R. D. & Lorch, Y. 1999, "Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome", *Cell*, vol. 98, no. 3, pp. 285-294.

**Kumagai**, A., Lee, J., Yoo, H. Y., & Dunphy, W. G. 2006, "TopBP1 activates the ATR-ATRIP complex", *Cell*, vol. 124, no. 5, pp. 943-955.

**Laurent**, E., Talpaz, M., Kantarjian, H., & Kurzrock, R. 2001, "The BCR gene and philadelphia chromosome-positive leukemogenesis", *Cancer Res.*, vol. 61, no. 6, pp. 2343-2355.

**Lee, J. H.** & Paull, T. T. 2004, "Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex", *Science*, vol. 304, no. 5667, pp. 93-96.

**Lee**, J., Kumagai, A., & Dunphy, W. G. 2007, "The Rad9-Hus1-Rad1 Checkpoint Clamp Regulates Interaction of TopBP1 with ATR", *J.Biol.Chem.*, vol. 282, no. 38, pp. 28036-28044.

**Lee** J, Kumagai A, Dunphy WG. 2003 "Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17." Mol Cell. 2003 Feb;11(2):pp.329-40.

**Li**, H., Lee, T. H., & Avraham, H. 2002, "A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer", *J.Biol.Chem.*, vol. 277, no. 23, pp. 20965-20973.

**Li**, L. & Sacks, D. B. 2007, "Functional interactions between calmodulin and estrogen receptor-alpha", *Cell Signal.*, vol. 19, no. 3, pp. 439-443.

**Lim**, D. S., Kim, S. T., Xu, B., Maser, R. S., Lin, J., Petrini, J. H., & Kastan, M. B. 2000, "ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway", *Nature*, vol. 404, no. 6778, pp. 613-617.

**Lin**, W. C., Lin, F. T., & Nevins, J. R. 2001, "Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation", *Genes Dev.*, vol. 15, no. 14, pp. 1833-1844.

**Lipkin**, S. M., Rozek, L. S., Rennert, G., Yang, W., Chen, P. C., Hacia, J., Hunt, N., Shin, B., Fodor, S., Kokoris, M., Greenson, J. K., Fearon, E., Lynch, H., Collins, F., & Gruber, S. B. 2004, "The MLH1 D132H variant is associated with susceptibility to sporadic colorectal cancer", *Nat.Genet.*, vol. 36, no. 7, pp. 694-699.

**Liu**, K., Lin, F. T., Ruppert, J. M., & Lin, W. C. 2003, "Regulation of E2F1 by BRCT domain-containing protein TopBP1", *Mol.Cell Biol.*, vol. 23, no. 9, pp. 3287-3304.

**Liu** K., Luo Y., Lin F. T>, Lin W.C., 2004, "TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival." Genes Dev. 2004 Mar 15;18(6):pp.673-86.

**Liu**, K., Paik, J. C., Wang, B., Lin, F. T., & Lin, W. C. 2006, "Regulation of TopBP1 oligomerization by Akt/PKB for cell survival", *EMBO J.*, vol. 25, no. 20, pp. 4795-4807.

**Liu**, S., Bekker-Jensen, S., Mailand, N., Lukas, C., Bartek, J., & Lukas, J. 2006, "Claspin operates downstream of TopBP1 to direct ATR signaling towards Chk1 activation", *Mol.Cell Biol.*, vol. 26, no. 16, pp. 6056-6064.

**Lo**, T. L., Yusoff, P., Fong, C. W., Guo, K., McCaw, B. J., Phillips, W. A., Yang, H., Wong, E. S., Leong, H. F., Zeng, Q., Putti, T. C., & Guy, G. R. 2004, "The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer", *Cancer Res.*, vol. 64, no. 17, pp. 6127-6136.

**Lodish** H., 2004. "Molecular Cell Biology" W H Freeman & Co

**Loizou**, J. I., Murr, R., Finkbeiner, M. G., Sawan, C., Wang, Z. Q., & Herceg, Z. 2006, "Epigenetic information in chromatin: the code of entry for DNA repair", *Cell Cycle*, vol. 5, no. 7, pp. 696-701.

**Lowndes**, N. F. & Toh, G. W. 2005, "DNA repair: the importance of phosphorylating histone H2AX", *Curr.Biol.*, vol. 15, no. 3, p. R99-R102.

**Lu**, K. P. & Means, A. R. 1993, "Regulation of the cell cycle by calcium and calmodulin", *Endocr.Rev.*, vol. 14, no. 1, pp. 40-58.

**Ludwig**, T., Fisher, P., Ganesan, S., & Efstratiadis, A. 2001, "Tumorigenesis in mice carrying a truncating Brca1 mutation", *Genes Dev.*, vol. 15, no. 10, pp. 1188-1193.

**Lupardus**, P. J. & Cimprich, K. A. 2006, "Phosphorylation of Xenopus Rad1 and Hus1 defines a readout for ATR activation that is independent of Claspin and the Rad9 carboxy terminus", *Mol.Biol.Cell*, vol. 17, no. 4, pp. 1559-1569.

**Lusser**, A. & Kadonaga, J. T. 2003, "Chromatin remodeling by ATP-dependent molecular machines", *Bioessays*, vol. 25, no. 12, pp. 1192-1200.

**Machida,** Y., Murai, K., Miyake, K., & Iijima, S. 2001, "Expression of chromatin remodeling factors during neural differentiation", *J.Biochem.(Tokyo)*, vol. 129, no. 1, pp. 43-49.

**Madoz-Gurpide**, J., Canamero, M., Sanchez, L., Solano, J., Alfonso, P., & Casal, J. I. 2007, "A proteomic analysis of cell signaling alterations in colorectal cancer", *Mol.Cell Proteomics.*

**Makiniemi**, M., Hillukkala, T., Tuusa, J., Reini, K., Vaara, M., Huang, D., Pospiech, H., Majuri, I., Westerling, T., Makela, T. P., & Syvaoja, J. E. 2001, "BRCT domain-containing protein TopBP1 functions in DNA replication and damage response", *J.Biol.Chem.*, vol. 276, no. 32, pp. 30399-30406.

**Malkin**, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., & . 1990, "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms", *Science*, vol. 250, no. 4985, pp. 1233-1238.

**Maniwa**, Y., Yoshimura, M., Bermudez, V. P., Yuki, T., Okada, K., Kanomata, N., Ohbayashi, C., Hayashi, Y., Hurwitz, J., & Okita, Y. 2005, "Accumulation of hRad9 protein in the nuclei of nonsmall cell lung carcinoma cells", *Cancer*, vol. 103, no. 1, pp. 126-132.

**Manke**, I. A., Lowery, D. M., Nguyen, A., & Yaffe, M. B. 2003, "BRCT repeats as phosphopeptide-binding modules involved in protein targeting", *Science*, vol. 302, no. 5645, pp. 636-639.

**Manson**, M. M. 2005, "Inhibition of survival signalling by dietary polyphenols and indole-3-carbinol", *Eur.J.Cancer*, vol. 41, no. 13, pp. 1842-1853.

**Maser**, R. S., Mirzoeva, O. K., Wells, J., Olivares, H., Williams, B. R., Zinkel, R. A., Farnham, P. J., & Petrini, J. H. 2001, "Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis", *Mol.Cell Biol.*, vol. 21, no. 17, pp. 6006-6016.

**Masumoto**, H., Sugino, A., & Araki, H. 2000, "Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast", *Mol.Cell Biol.*, vol. 20, no. 8, pp. 2809-2817.

**Matsuoka**, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., & Elledge, S. J. 2000, "Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 19, pp. 10389-10394.

**Mayo**, M. W., Denlinger, C. E., Broad, R. M., Yeung, F., Reilly, E. T., Shi, Y., & Jones, D. R. 2003, "Ineffectiveness of histone deacetylase inhibitors to

induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway", *J.Biol.Chem.*, vol. 278, no. 21, pp. 18980-18989.

**McKenna** N. J., O'Malley B. W., 2000 An issue of tissues: divining the split personalities of selective estrogen receptor modulators. 2000 Nat Med., Sep;6(9):960-2.

**McKie**, J. A., Bhagwat, S. S., Brady, H., Doubleday, M., Gayo, L., Hickman, M., Jalluri, R. K., Khammungkhune, S., Kois, A., Mortensen, D., Richard, N., Sapienza, J., Shevlin, G., Stein, B., & Sutherland, M. 2004, "Lead identification of a potent benzopyranone selective estrogen receptor modulator", *Bioorg.Med.Chem.Lett.*, vol. 14, no. 13, pp. 3407-3410.

**Melchior**, F. 2000, "SUMO--nonclassical ubiquitin", *Annu.Rev.Cell Dev.Biol.*, vol. 16, pp. 591-626.

**Melo**, J. A., Cohen, J., & Toczyski, D. P. 2001, "Two checkpoint complexes are independently recruited to sites of DNA damage in vivo", *Genes Dev.*, vol. 15, no. 21, pp. 2809-2821.

**Migliaccio**, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., & Auricchio, F. 1996, "Tyrosine kinase/p21ras/MAP-kinase pathway

activation by estradiol-receptor complex in MCF-7 cells", *EMBO J.*, vol. 15, no. 6, pp. 1292-1300.

**Migliaccio**, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., & Auricchio, F. 1998, "Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor", *EMBO J.*, vol. 17, no. 7, pp. 2008-2018.

**Mirzoeva** OK, Kawaguchi T, Pieper RO 2006 "The Mre11/Rad50/Nbs1 complex interacts with the mismatch repair system and contributes to temozolomide-induced G2 arrest and cytotoxicity." Mol Cancer Ther. 2006 Nov;5(11):2757-66.

**Miyake**, T., Hu, Y. F., Yu, D. S., & Li, R. 2000, "A functional comparison of BRCA1 C-terminal domains in transcription activation and chromatin remodeling", *J.Biol.Chem.*, vol. 275, no. 51, pp. 40169-40173.

**Monteiro**, A. N. & Humphrey, J. S. 1998, "Yeast-based assays for detection and characterization of mutations in BRCA1", *Breast Dis.*, vol. 10, no. 1-2, pp. 61-70.

**Monteiro**, A. N., August, A., & Hanafusa, H. 1996, "Evidence for a transcriptional activation function of BRCA1 C-terminal region", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 24, pp. 13595-13599.

**Monteiro**, A. N., August, A., & Hanafusa, H. 1997, "Common BRCA1 variants and transcriptional activation", *Am.J.Hum.Genet.*, vol. 61, no. 3, pp. 761-762.

**Morishima**, K., Sakamoto, S., Kobayashi, J., Izumi, H., Suda, T., Matsumoto, Y., Tauchi, H., Ide, H., Komatsu, K., & Matsuura, S. 2007, "TopBP1 associates with NBS1 and is involved in homologous recombination repair", *Biochem.Biophys.Res.Commun.*, vol. 362, no. 4, pp. 872-879.

**Morris**, J. S., Nixon, C., Bruck, A., Nasir, L., Morgan, I. M., & Philbey, A. W. 2007, "Immunohistochemical expression of TopBP1 in feline mammary neoplasia in relation to histological grade, Ki67, ERalpha and p53", *Vet.J.*

**Muchardt**, C. & Yaniv, M. 1993, "A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor", *EMBO J.*, vol. 12, no. 11, pp. 4279-4290.

**Muchardt**, C. & Yaniv, M. 1999, "The mammalian SWI/SNF complex and the control of cell growth", *Semin.Cell Dev.Biol.*, vol. 10, no. 2, pp. 189-195.

**Muchardt**, C., Yaniv M., 2001 "When the SWI/SNF complex remodels...the cell cycle." Oncogene 2001 May 28;20(24):pp. 3067-75.

**Muller**, S., Hoege, C., Pyrowolakis, G., & Jentsch, S. 2001, "SUMO, ubiquitin's mysterious cousin", *Nat.Rev.Mol.Cell Biol.*, vol. 2, no. 3, pp. 202-210.

**Murphy**, D. J., Hardy, S., & Engel, D. A. 1999, "Human SWI-SNF component BRG1 represses transcription of the c-fos gene", *Mol.Cell Biol.*, vol. 19, no. 4, pp. 2724-2733.

**Musarrat**, J., Arezina-Wilson, J., & Wani, A. A. 1996, "Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis", *Eur.J.Cancer*, vol. 32A, no. 7, pp. 1209-1214.

**Nagashima** G., Asai J., Suzuki R., Fujimoto T. 2005 "Different distribution of c-myc and MIB-1 positive cells in malignant meningiomas with reference to TGFs, PDGF, and PgR expression" Brain Tum. Path. 2001;18(1):pp.1-5

**Naiki**, T., Kondo, T., Nakada, D., Matsumoto, K., & Sugimoto, K. 2001, "Chl12 (Ctf18) forms a novel replication factor C-related complex and functions redundantly with Rad24 in the DNA replication checkpoint pathway", *Mol.Cell Biol.*, vol. 21, no. 17, pp. 5838-5845.

**Narlikar,** G. J., Fan, H. Y., & Kingston, R. E. 2002, "Cooperation between complexes that regulate chromatin structure and transcription", *Cell*, vol. 108, no. 4, pp. 475-487.

**Oda,** E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., & Tanaka, N. 2000, "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis", *Science*, vol. 288, no. 5468, pp. 1053-1058.

**Oda,** K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., & Taya, Y. 2000, "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53", *Cell*, vol. 102, no. 6, pp. 849-862.

**O'Driscoll** M, Dobyns WB, van Hagen JM, Jeggo PA. 2007 "Cellular and clinical impact of haploinsufficiency for genes involved in ATR signaling." Am J Hum Genet. 2007 Jul;81(1):77-86.

**Ooi,** L., Belyaev, N. D., Miyake, K., Wood, I. C., & Buckley, N. J. 2006, "BRG1 chromatin remodeling activity is required for efficient chromatin binding by repressor element 1-silencing transcription factor (REST) and facilitates

REST-mediated repression", *J.Biol.Chem.*, vol. 281, no. 51, pp. 38974-38980.

**Owen-Hughes, T. & Workman**, J. L. 1996, "Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted trans-displacement of histones", *EMBO J.*, vol. 15, no. 17, pp. 4702-4712.

**Ozen**, M. & Ittmann, M. 2005, "Increased expression and activity of CDC25C phosphatase and an alternatively spliced variant in prostate cancer", *Clin.Cancer Res.*, vol. 11, no. 13, pp. 4701-4706.

**Park**, J. H., Park, E. J., Lee, H. S., Kim, S. J., Hur, S. K., Imbalzano, A. N., & Kwon, J. 2006, "Mammalian SWI/SNF complexes facilitate DNA double-strand break repair by promoting gamma-H2AX induction", *EMBO J.*, vol. 25, no. 17, pp. 3986-3997.

**Paull**, T. T., Cortez, D., Bowers, B., Elledge, S. J., & Gellert, M. 2001, "Direct DNA binding by Brca1", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 11, pp. 6086-6091.

**Paull**, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., & Bonner, W. M. 2000, "A critical role for histone H2AX in recruitment of

repair factors to nuclear foci after DNA damage", *Curr.Biol.*, vol. 10, no. 15, pp. 886-895.

**Pediconi**, N., Ianari, A., Costanzo, A., Belloni, L., Gallo, R., Cimino, L., Porcellini, A., Screpanti, I., Balsano, C., Alesse, E., Gulino, A., & Levrero, M. 2003, "Differential regulation of E2F1 apoptotic target genes in response to DNA damage", *Nat.Cell Biol.*, vol. 5, no. 6, pp. 552-558.

**Peng**, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., & Piwnica-Worms, H. 1997, "Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216", *Science*, vol. 277, no. 5331, pp. 1501-1505.

**Perera** D, Perez-Hidalgo L, Moens PB, Reini K, Lakin N, Syväoja JE, San-Segundo PA, Freire R. 2004 "TopBP1 and ATR colocalization at meiotic chromosomes: role of TopBP1/Cut5 in the meiotic recombination checkpoint." Mol. Cell. Biol 2004 Apr;15(4):1568-79.

**Peterson**, C. L. & Logie, C. 2000, "Recruitment of chromatin remodeling machines", *J.Cell Biochem.*, vol. 78, no. 2, pp. 179-185.

**Peterson**, C. L. & Workman, J. L. 2000, "Promoter targeting and chromatin remodeling by the SWI/SNF complex", *Curr.Opin.Genet.Dev.*, vol. 10, no. 2, pp. 187-192.

**Peterson**, C. L. 2000, "ATP-dependent chromatin remodeling: going mobile", *FEBS Lett.*, vol. 476, no. 1-2, pp. 68-72.

**Phelan**, M. L., Sif, S., Narlikar, G. J., & Kingston, R. E. 1999, "Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits", *Mol.Cell*, vol. 3, no. 2, pp. 247-253.

**Plummer** G., Perreault H. R., Holmes C. F., Posse De Chaves E. I., 2005 "Activation of serine/threonine protein phosphatase-1 is required for ceramide-induced survival of sympathetic neurons" Biochem J. 2005 Feb 1;385(Pt 3):pp 685-93..

**Recht**, J. & Osley, M. A. 1999, "Mutations in both the structured domain and N-terminus of histone H2B bypass the requirement for Swi-Snf in yeast", *EMBO J.*, vol. 18, no. 1, pp. 229-240.

**Reid**, J. L., Bannister, A. J., Zegerman, P., Martinez-Balbas, M. A., & Kouzarides, T. 1998, "E1A directly binds and regulates the P/CAF acetyltransferase", *EMBO J.*, vol. 17, no. 15, pp. 4469-4477.

**Reyes**, J. C., Barra, J., Muchardt, C., Camus, A., Babinet, C., & Yaniv, M. 1998, "Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha)", *EMBO J.*, vol. 17, no. 23, pp. 6979-6991.

**Roberts**, S. G., Ha, I., Maldonado, E., Reinberg, D., & Green, M. R. 1993, "Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation", *Nature*, vol. 363, no. 6431, pp. 741-744.

**Rodriguez**, M., Yu, X., Chen, J., & Songyang, Z. 2003, "Phosphopeptide binding specificities of BRCA1 COOH-terminal (BRCT) domains", *J.Biol.Chem.*, vol. 278, no. 52, pp. 52914-52918.

**Rupinder**, K. R. & Pratap, K. 2006, "Ovulation induction and ovarian cancer: is there a link?", *Med.J.Malaysia*, vol. 61, no. 1, pp. 132-136.

**Saiardi**, A., Resnick, A. C., Snowman, A. M., Wendland, B., & Snyder, S. H. 2005, "Inositol pyrophosphates regulate cell death and telomere length through

phosphoinositide 3-kinase-related protein kinases", *Proc.Natl.Acad.Sci.U.S.A*, vol. 102, no. 6, pp. 1911-1914.

**Saka**, Y. & Yanagida, M. 1993, "Fission yeast cut5+, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene rad4+", *Cell*, vol. 74, no. 2, pp. 383-393.

**Saka**, Y., Esashi, F., Matsusaka, T., Mochida, S., & Yanagida, M. 1997, "Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1", *Genes Dev.*, vol. 11, no. 24, pp. 3387-3400.

**Salghetti**, S. E., Muratani, M., Wijnen, H., Futcher, B., & Tansey, W. P. 2000, "Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 7, pp. 3118-3123.

**Sanders**, S. L., Portoso, M., Mata, J., Bahler, J., Allshire, R. C., & Kouzarides, T. 2004, "Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage", *Cell*, vol. 119, no. 5, pp. 603-614.

**Schmidt**, D. & Muller, S. 2003, "PIAS/SUMO: new partners in transcriptional regulation", *Cell Mol.Life Sci.*, vol. 60, no. 12, pp. 2561-2574.

**Seipel,** K., Georgiev, O., & Schaffner, W. 1992, "Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions", *EMBO J.*, vol. 11, no. 13, pp. 4961-4968.

**Shanahan** F., Seghezzi W., Parry D., Mahoy D., 1999. Cyclin E associates with BAF155 and BRG1, components of the mammalian SWI-SNF complex, and alters the ability of BRG1 to induce growth arrest. Mol Cell. Biol 1999 Feb;19(2):pp.1460-9..

**Shen,** W., Xu, C., Huang, W., Zhang, J., Carlson, J. E., Tu, X., Wu, J., & Shi, Y. 2007, "Solution structure of human Brg1 bromodomain and its specific binding to acetylated histone tails", *Biochemistry*, vol. 46, no. 8, pp. 2100-2110.

**Shiloh,** Y. 1997, "Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart", *Annu.Rev.Genet.*, vol. 31, pp. 635-662.

**Shim,** E. Y., Ma, J. L., Oum, J. H., Yanez, Y., & Lee, S. E. 2005, "The yeast chromatin remodeler RSC complex facilitates end joining repair of DNA double-strand breaks", *Mol.Cell Biol.*, vol. 25, no. 10, pp. 3934-3944.

**Sonoda**, E., Takata, M., Yamashita, Y. M., Morrison, C., & Takeda, S. 2001, "Homologous DNA recombination in vertebrate cells", *Proc.Natl.Acad.Sci.U.S.A*, vol. 98, no. 15, pp. 8388-8394.

**Spiga**, M. G. & D'Urso, G. 2004, "Identification and cloning of two putative subunits of DNA polymerase epsilon in fission yeast", *Nucleic Acids Res.*, vol. 32, no. 16, pp. 4945-4953.

**Stevens**, C., Smith, L., & La Thangue, N. B. 2003, "Chk2 activates E2F-1 in response to DNA damage", *Nat.Cell Biol.*, vol. 5, no. 5, pp. 401-409.

**Stewart**, G. S., Maser, R. S., Stankovic, T., Bressan, D. A., Kaplan, M. I., Jaspers, N. G., Raams, A., Byrd, P. J., Petrini, J. H., & Taylor, A. M. 1999, "The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder", *Cell*, vol. 99, no. 6, pp. 577-587.

**Strobl**, J. S., Wonderlin, W. F., & Flynn, D. C. 1995, "Mitogenic signal transduction in human breast cancer cells", *Gen.Pharmacol.*, vol. 26, no. 8, pp. 1643-1649.

**Stucki**, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., & Jackson, S. P. 2005, "MDC1 directly binds phosphorylated histone H2AX to

regulate cellular responses to DNA double-strand breaks", *Cell*, vol. 123, no. 7, pp. 1213-1226.

**Suzuki**, T., Urano, T., Tsukui, T., Horie-Inoue, K., Moriya, T., Ishida, T., Muramatsu, M., Ouchi, Y., Sasano, H., & Inoue, S. 2005, "Estrogen-responsive finger protein as a new potential biomarker for breast cancer", *Clin.Cancer Res.*, vol. 11, no. 17, pp. 6148-6154.

**Swift**, M., Morrell, D., Massey, R. B., & Chase, C. L. 1991, "Incidence of cancer in 161 families affected by ataxia-telangiectasia", *N.Engl.J.Med.*, vol. 325, no. 26, pp. 1831-1836.

**Takata**, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., & Takeda, S. 1998, "Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells", *EMBO J.*, vol. 17, no. 18, pp. 5497-5508.

**Taricani**, L. & Wang, T. S. 2006, "Rad4TopBP1, a scaffold protein, plays separate roles in DNA damage and replication checkpoints and DNA replication", *Mol.Biol.Cell*, vol. 17, no. 8, pp. 3456-3468.

**Taricani,** L. & Wang, T. S. 2007, "Rad4TopBP1 associates with Srr2, an Spc1 MAPK-regulated protein, in response to environmental stress", *J.Biol.Chem.*, vol. 282, no. 12, pp. 8793-8800.

**Thomas, D. & Tyers,** M. 2000, "Transcriptional regulation: Kamikaze activators", *Curr.Biol.*, vol. 10, no. 9, p. R341-R343.

**Tian**, W., Zhang, Z., & Cohen, D. M. 2000, "MAPK signaling and the kidney", *Am.J.Physiol Renal Physiol*, vol. 279, no. 4, p. F593-F604.

**Tsukuda**, T., Fleming, A. B., Nickoloff, J. A., & Osley, M. A. 2005, "Chromatin remodelling at a DNA double-strand break site in Saccharomyces cerevisiae", *Nature*, vol. 438, no. 7066, pp. 379-383.

**Tyler**, J. K. & Kadonaga, J. T. 1999, "The "dark side" of chromatin remodeling: repressive effects on transcription", *Cell*, vol. 99, no. 5, pp. 443-446.

**Urano**, T., Hosoi, T., Shiraki, M., Toyoshima, H., Ouchi, Y., & Inoue, S. 2000, "Possible involvement of the p57(Kip2) gene in bone metabolism", *Biochem.Biophys.Res.Commun.*, vol. 269, no. 2, pp. 422-426.

**Vance** K. W., Campo M. S., Morgan I. M. 1999. "An enhanced epithelial response of a papillomavirus promoter to transcriptional activators." J. Biol Chem. 1999 Sep 24;274(39):27839-44.

**Van Haaften**, G., Vastenhouw, N. L., Nollen, E. A., Plasterk, R. H., & Tijsterman, M. 2004, "Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 35, pp. 12992-12996.

**Vargas** DA, Takahashi S, Ronai Z. 2003 "Mdm2: A regulator of cell growth and death" Adv Cancer Res. 2003;89:1-34.

**Varon**, R., Vissinga, C., Platzer, M., Cerosaletti, K. M., Chrzanowska, K. H., Saar, K., Beckmann, G., Seemanova, E., Cooper, P. R., Nowak, N. J., Stumm, M., Weemaes, C. M., Gatti, R. A., Wilson, R. K., Digweed, M., Rosenthal, A., Sperling, K., Concannon, P., & Reis, A. 1998, "Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome", *Cell*, vol. 93, no. 3, pp. 467-476.

**Varshavsky**, A. 1997, "The ubiquitin system", *Trends Biochem.Sci.*, vol. 22, no. 10, pp. 383-387.

**Venclovas**, C. & Thelen, M. P. 2000, "Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes", *Nucleic Acids Res.*, vol. 28, no. 13, pp. 2481-2493.

**Verger**, A., Perdomo, J., & Crossley, M. 2003, "Modification with SUMO. A role in transcriptional regulation", *EMBO Rep.*, vol. 4, no. 2, pp. 137-142.

**Von Deimling**, F., Scharf, J. M., Liehr, T., Rothe, M., Kelter, A. R., Albers, P., Dietrich, W. F., Kunkel, L. M., Wernert, N., & Wirth, B. 1999, "Human and mouse RAD17 genes: identification, localization, genomic structure and histological expression pattern in normal testis and seminoma", *Hum.Genet.*, vol. 105, no. 1-2, pp. 17-27.

**Walworth**, N. C. & Bernards, R. 1996, "rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint", *Science*, vol. 271, no. 5247, pp. 353-356.

**Wang**, H. & Elledge, S. J. 2002, "Genetic and physical interactions between DPB11 and DDC1 in the yeast DNA damage response pathway", *Genetics*, vol. 160, no. 4, pp. 1295-1304.

**Wang**, H., Zhai, L., Xu, J., Joo, H. Y., Jackson, S., Erdjument-Bromage, H., Tempst, P., Xiong, Y., & Zhang, Y. 2006, "Histone H3 and H4

ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage", *Mol.Cell*, vol. 22, no. 3, pp. 383-394.

**Wang**, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R., & Crabtree, G. R. 1996, "Diversity and specialization of mammalian SWI/SNF complexes", *Genes Dev.*, vol. 10, no. 17, pp. 2117-2130.

**Wilson**, S. H. 1998, "Mammalian base excision repair and DNA polymerase beta", *Mutat.Res.*, vol. 407, no. 3, pp. 203-215.

**Wong**, A. K., Shanahan, F., Chen, Y., Lian, L., Ha, P., Hendricks, K., Ghaffari, S., Iliev, D., Penn, B., Woodland, A. M., Smith, R., Salada, G., Carillo, A., Laity, K., Gupte, J., Swedlund, B., Tavtigian, S. V., Teng, D. H., & Lees, E. 2000, "BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines", *Cancer Res.*, vol. 60, no. 21, pp. 6171-6177.

**Wu**, J. C. & Fritz, L. C. 1999, "Irreversible caspase inhibitors: tools for studying apoptosis", *Methods*, vol. 17, no. 4, pp. 320-328.

**Wu**, X., Ranganathan, V., Weisman, D. S., Heine, W. F., Ciccone, D. N., O'Neill, T. B., Crick, K. E., Pierce, K. A., Lane, W. S., Rathbun, G., Livingston, D. M., & Weaver, D. T. 2000, "ATM phosphorylation of Nijmegen breakage

syndrome protein is required in a DNA damage response", *Nature*, vol. 405, no. 6785, pp. 477-482.

**Xu**, Z. X., Timanova-Atanasova, A., Zhao, R. X., & Chang, K. S. 2003, "PML colocalizes with and stabilizes the DNA damage response protein TopBP1", *Mol.Cell Biol.*, vol. 23, no. 12, pp. 4247-4256.

**Xue**, Y., Canman, J. C., Lee, C. S., Nie, Z., Yang, D., Moreno, G. T., Young, M. K., Salmon, E. D., & Wang, W. 2000, "The human SWI/SNF-B chromatin-remodeling complex is related to yeast rsc and localizes at kinetochores of mitotic chromosomes", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 24, pp. 13015-13020.

**Yamamoto**, R. R., Axton, J. M., Yamamoto, Y., Saunders, R. D., Glover, D. M., & Henderson, D. S. 2000, "The Drosophila mus101 gene, which links DNA repair, replication and condensation of heterochromatin in mitosis, encodes a protein with seven BRCA1 C-terminus domains", *Genetics*, vol. 156, no. 2, pp. 711-721.

**Yamane**, K. & Tsuruo, T. 1999, "Conserved BRCT regions of TopBP1 and of the tumor suppressor BRCA1 bind strand breaks and termini of DNA", *Oncogene*, vol. 18, no. 37, pp. 5194-5203.

**Yamane**, K., Chen, J., & Kinsella, T. J. 2003, "Both DNA topoisomerase II-binding protein 1 and BRCA1 regulate the G2-M cell cycle checkpoint", *Cancer Res.*, vol. 63, no. 12, pp. 3049-3053.

**Yamane**, K., Kawabata, M., & Tsuruo, T. 1997, "A DNA-topoisomerase-II-binding protein with eight repeating regions similar to DNA-repair enzymes and to a cell-cycle regulator", *Eur.J.Biochem.*, vol. 250, no. 3, pp. 794-799.

**Yamane**, K., Wu, X., & Chen, J. 2002, "A DNA damage-regulated BRCT-containing protein, TopBP1, is required for cell survival", *Mol.Cell Biol.*, vol. 22, no. 2, pp. 555-566.

**Yan**, Y., Spieker, R. S., Kim, M., Stoeger, S. M., & Cowan, K. H. 2005, "BRCA1-mediated G2/M cell cycle arrest requires ERK1/2 kinase activation", *Oncogene*, vol. 24, no. 20, pp. 3285-3296.

**Yang** H., Wei W., Menconi M., Hasselgren P.O. 2007 "Dexamethasone-induced protein degradation in cultured myotubes is p300/HAT dependent." Am. J Phys. Regu. 2007 Jan;292(1):R337-4.

**Yokoi**, M., Masutani, C., Maekawa, T., Sugasawa, K., Ohkuma, Y., & Hanaoka, F. 2000, "The xeroderma pigmentosum group C protein complex XPC-HR23B

plays an important role in the recruitment of transcription factor IIH to damaged DNA", *J.Biol.Chem.*, vol. 275, no. 13, pp. 9870-9875.

**York**, J. D. 2006, "Regulation of nuclear processes by inositol polyphosphates", *Biochim.Biophys.Acta*, vol. 1761, no. 5-6, pp. 552-559.

**Yoshikawa**, K., Honda, K., Inamoto, T., Shinohara, H., Yamauchi, A., Suga, K., Okuyama, T., Shimada, T., Kodama, H., Noguchi, S., Gazdar, A. F., Yamaoka, Y., & Takahashi, R. 1999, "Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases", *Clin.Cancer Res.*, vol. 5, no. 6, pp. 1249-1261.

**Yu**, X., Chini, C. C., He, M., Mer, G., & Chen, J. 2003, "The BRCT domain is a phospho-protein binding domain", *Science*, vol. 302, no. 5645, pp. 639-642.

**Zhan**, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D. A., O'Connor, P. M., & Fornace, A. J., Jr. 1994, "Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis", *Oncogene*, vol. 9, no. 12, pp. 3743-3751.

**Zhang**, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., & El Deiry, W. S. 1998, "BRCA1 physically associates with p53 and

stimulates its transcriptional activity", *Oncogene*, vol. 16, no. 13, pp. 1713-1721.

**Zhao,** H. & Piwnica-Worms, H. 2001, "ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1", *Mol.Cell Biol.*, vol. 21, no. 13, pp. 4129-4139.

**Zhou,** B. B., Chaturvedi, P., Spring, K., Scott, S. P., Johanson, R. A., Mishra, R., Mattern, M. R., Winkler, J. D., & Khanna, K. K. 2000, "Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity", *J.Biol.Chem.*, vol. 275, no. 14, pp. 10342-10348.

**Zhou** XY, Wang X, Wang H, Chen DJ, Li GC, Iliakis G, Wang Y. 2002 "Ku affects the ATM-dependent S phase checkpoint following ionizing radiation." Oncogene. 2002 Sep 12;21(41):6377-81.

**Zofall,** M., Persinger, J., Kassabov, S. R., & Bartholomew, B. 2006, "Chromatin remodeling by ISW2 and SWI/SNF requires DNA translocation inside the nucleosome", *Nat.Struct.Mol.Biol.*, vol. 13, no. 4, pp. 339-346.

**Zou,** L. & Elledge, S. J. 2003, "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes", *Science*, vol. 300, no. 5625, pp. 1542-1548.

**Zou,** L., Liu, D., & Elledge, S. J. 2003, "Replication protein A-mediated recruitment and activation of Rad17 complexes", *Proc.Natl.Acad.Sci.U.S.A.*, vol. 100, no. 24, pp. 13827-13832.