NOVEL MODULATION OF p53 ACTIVITY IN THYROID CANCER

By

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Summary

Thyroid cancer is responsible for more deaths than all other endocrine cancers combined, and its incidence is rising. In contrast to other cancers, thyroid carcinomas rarely display mutations in p53. p53 is a potent tumour suppressor, and serves to inhibit cell transformation. Interestingly, the thyroid gland is also sensitive to the effects of ionising radiation, where WT p53 would normally play a protective role. However, there is evidence to suggest that aberrant expression of cellular oncogenes can result in the functional inactivation of WT p53. PBF is a proto-oncogene found to be overexpressed in thyroid tumours. PBF overexpression causes transformation *in vitro* and tumourigenesis *in vivo.* Work within this thesis describes the functional relationship between PBF and p53 in thyroid cancer. We provide evidence for a direct interaction between PBF and p53 in thyroid cancer cells, and our data demonstrate that oncogenic expression of PBF adversely affects p53 homeostasis by increasing its degradation, with PBF potentially serving as a co-factor in a complex with p53 and HDM2. Surprisingly, PBF had no effect on p53-mediated transcription using focused microarrays. Nonetheless, overexpression of PBF conferred a significant survival advantage following DNA damage, indicating that PBF might potentially facilitate neoplastic growth and tumourigenesis. Besides regulation of transcription, p53 performs a wide range of biological functions. PBF may therefore serve to promote p53 inactivation independently of its role as a transcription factor.

Overall, these data indicate that oncogenic expression of PBF may result in a novel mechanism of p53 inactivation, where PBF binds to p53 and accelerates its degradation in complex with HDM2. These events cause an increase in cell survival following DNA damage, thereby potentially promoting tumourigenesis in thyroid cancers expressing WT p53.

I would like to dedicate this thesis to my family, and my friends.

Thank you for your continual support

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Abbreviations

AD	Activation domain
AKT/PKB	Protein kinase B
APAF-1	Apoptotic protease activating factor 1
APC	Anaphase promoting complex
ARF	ADP Ribosylation Factor
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BAX	BCL-2 associated protein X
BCL-2	B-cell lymphoma 2
BRAF	Serine/threonine-protein kinase
BRCA2	Breast cancer type 2 susceptibility protein
CBP	CREB-binding protein
co-IP	Co-immunoprecipitation
COP-1	Coat protein complex
CT	Computerised Tomography
DBD	DNA binding domain
DNA PKs	DNA protein kinases
DSB	DNA double strand break
E2F3	E2F transcription factor 3
EGR1	Early growth response protein 1
EI24	Etoposide induced protein 2.4 homologue
ERE	Oestrogen response element
ERK	Extracellular signal-related kinase
ERα	Oestrogen receptor α
FGF-2	Fibroblast growth factor 2
GST	Glutathione-S-transferase
HAUSP	Herpes virus associated ubiquitin specific protease 7

HBXAg	Hepatitis B X antigen
HDM2	Human homologue of mouse double minute 2
HMG	High mobility group factors
HPV	Human papilloma virus
MAPK	Mitogen activate protein kinase
MDMX/4	Mdm4 p53 binding protein homolog (mouse)
MEK	Mitogen activate protein kinase kinase
MLL-ELL	Myeloid/lymphiod leukaemia and RNA polymerase II elongation factor
MYC	Myelocytomatosis viral oncogene homologue
NF-1	Neurofibromatosis type I
NLS	Nuclear localisation signal
NTRK1	Neurotrophic tyrosine receptor kinase type 1
PAX8/PPARγ	Paired box 8 gene
PBF	Pituitary tumor transforming binding factor
PI3K	Phosphatidylinositol 3-kinase
PIRH2	p53-induced ubiquitin-protein ligase
PLA	Proximity ligation assay
PRO	Proline rich region
PSI	Plexins, Semaphorins and Integrins
PTEN	Phosphatase and tensin homologUE
PTTG	Pituitary tumor transforming gene
PUMA	p53 upregulated mediator of apoptosis
RAS	Rat Sarcoma
RD	Regulatory domain
RET	Rearranged during transfection (protein kinase)
RET/PTC	Rearranged in transformation/Papillary thyroid carcinomas
SEER	Surveillance epidemiology and end results
SV40	Simian virus 40
T3	Triiodothyronine

T4	Thyroxine
TAD	Transactivation domain
TD	Tetramerisation domain
TNM	Tumour, Node, Metastasis
TOPORS	Topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase
TR	Thyroid hormone receptor
UBE2B/A	Ubiquitin-conjugating enzyme E2
USP10	Ubiquitin specific peptidase 10
WT	Wild-type
YY1	Yin Yang 1

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Chapter 1. **General introduction**

1.1. Pathogenesis of thyroid cancer

1.1.1. Epidemiology and classification

Thyroid cancer is the most frequently diagnosed endocrine malignancy and is responsible for more deaths than all other endocrine cancers combined (Davies *et al*, 2002; Chen *et al*, 2009; Albores-Saavedra *et al*, 2007). Thyroid cancers are fairly uncommon in the general population, accounting for around 1 % of all newly diagnosed malignant disease, which equates to 0.5 % of cancers in males and 1.5 % of cancers in females (Chen *et al*, 2009). The incidence of thyroid cancer is higher in older patients, those with family history, and a history of exposure to ionising radiation (Sharma *et al*, 2010; Johnston *et al*, 2012). However, the worldwide incidence of thyroid cancer has been gradually increasing and has almost tripled between 1975 and 2008 in the UK and other industrialised countries (Burgess & Tucker, 2006; Albores-Saavedra *et al*, 2007; Nikiforov & Nikiforova, 2011). Whilst females are approximately three times more likely to develop thyroid malignancy, the rate of incidence in males has increased at a similar level (Figure 1.1).

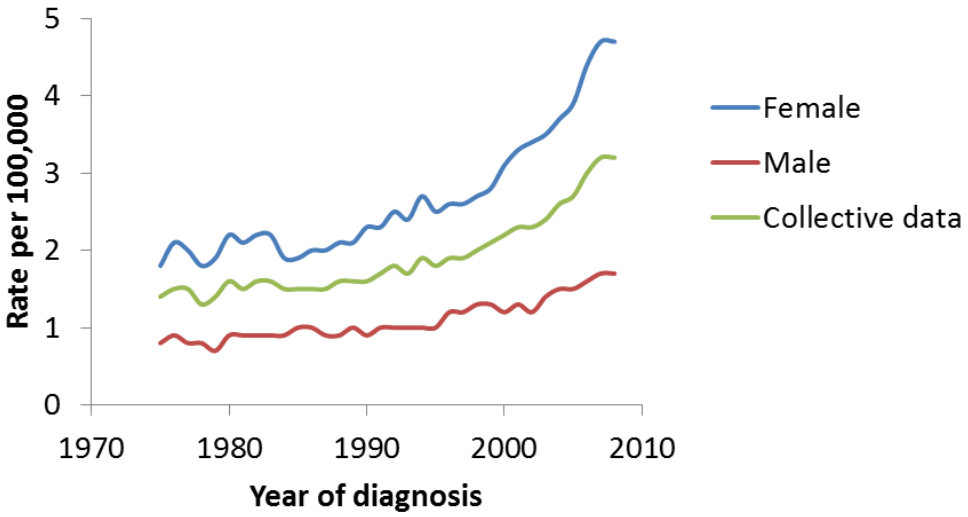


Figure 1.1: Chart representing the rise in incidence of thyroid cancer between 1975 and 2008. The chart data displays European Age-Standardised Incidence Rates per 100,000 Population, by Sex, in Great Britain. Whilst females are 3 times more likely to develop the disease, male incidence rate has risen similarly to females (cancer research UK).

The significant rise in thyroid cancer incidence has been attributed to recent advances in diagnostic techniques, where thyroid ultrasonography provides significant ascertainment bias. Moreover, the steady increase in the use of radiation to the head or neck for diagnosis or treatment (for example through the use of computed tomography (CT) scans), may present a significant risk of thyroid malignancy (Baker & Bhatti, 2006). Whilst differentiated thyroid cancers mostly have good prognosis, the rise in incidence of this disease is higher than for any other human cancer. Furthermore, in comparison to other malignancies, thyroid cancer has the second most rapidly increasing mortality rate (Johnston *et al*, 2012; Figure 1.2).

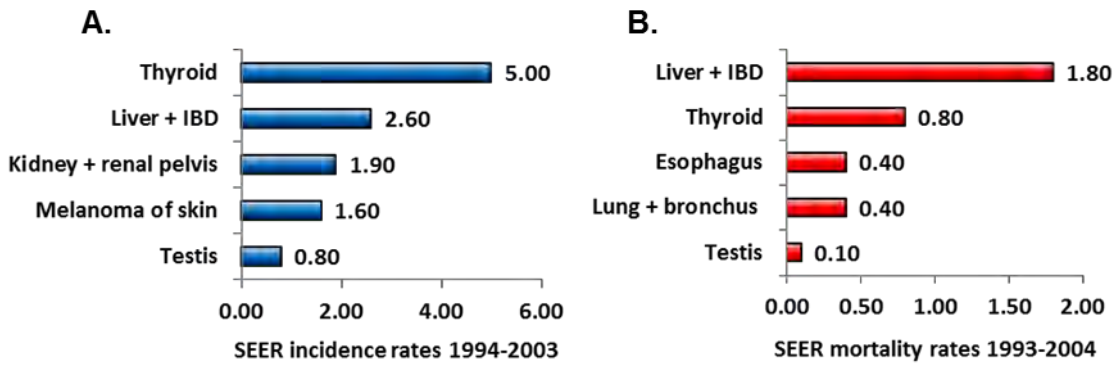


Figure 1.2: Charts representing the SEER incidence and mortality rates for thyroid cancer. ***A:*** *thyroid cancer presents with the greatest rise in incidence compared to other human cancers* ***B:*** *In comparison to other malignancies thyroid cancer also has the second highest increase in mortality rate (Data taken from the National Institute of Health, SEER).*

The thyroid is comprised of two main cell types; with thyroid follicular epithelial cells forming the majority. These cells are responsible for the uptake of iodine at the basolateral membrane and produce thyroglobulin and the thyroid hormones thyroxine (T4) and triiodothyronine (T3). Parafollicular C cells residing in the connective tissue adjacent to the follicles are involved in the production and secretion of calcitonin (Nikiforov & Nikiforova, 2011). The vast majority of thyroid cancers arise from thyroid follicular cells, with only a small minority (3-5 %) originating from parafollicular or C-cells. Thyroid cancers originating from follicular epithelial cells can be subdivided into well-differentiated papillary carcinomas, follicular carcinomas, and undifferentiated anaplastic carcinomas. Anaplastic carcinomas can develop spontaneously, although many arise via stepwise dedifferentiation of both papillary and follicular carcinomas (Nikiforov & Nikiforova, 2011; Nikiforova & Nikiforov, 2009).

1.1.1.1. *Regulation of normal thyroid cell growth*

In order to maintain the functional and structural integrity of the follicular unit, the proliferative renewal of thyroid cells is paramount. Therefore the control of normal thyroid epithelial cell growth and associated proliferation of cells in the surrounding connective tissue and capillaries must be tightly controlled (Kimura *et al*, 2001).

Normal thyroid cell growth is mediated primarily by 2 potentially synergistic mechanisms involving Thyroid stimulating hormone (TSH) and cyclic AMP (cAMP) signalling, and the Insulin-like growth factor 1 (IGF-1) (Brenner-Gati *et al*, 1988).

TSH is produced by the anterior pituitary gland in response to thyroptropin releasing hormone (TRH), which is in turn produced within the hypothalamus. Additionally, the hypothalamus produces and secretes somatostatin which is responsible for inhibiting the production and secretion of TSH. TSH stimulates the thyroid gland to produce and secrete thyroid hormones T3 and T4. Circulating blood levels of T3 and T4 provide an auto regulatory negative feedback loop to further monitor thyroid follicular cell growth and hormone production. For example, when circulating T3/T4 levels are low, the production of TSH is increased; conversely when T3 and T4 levels are high, TSH production is decreased (Lewis *et al*, 2004; Medina *et al*, 1999, 2000). The TSH receptor is expressed on the cell surface of thyroid follicular cells, and following interaction with TSH, is activated and stimulates the production of adenylate cyclase, ultimately serving to promote thyroid cell growth and proliferation via the cAMP pathway. cAMP is an important intracellular second messenger and mediates thyroid cell growth primarily through activation of protein kinase A (PKA) and stimulation mTOR protein kinase, which ultimately serve to activate specific transcription factors

involved in cell cycle progression and DNA synthesis (Deleu *et al*, 1999; Goretzki *et al*, 1987; Kimura *et al*, 2001).

In addition to TSH stimulation of thyroid growth, IGF-1 is able to stimulate thyroid growth via independent mechanisms. However, it is thought that TSH and IGF-1 perform synergistically *in vivo* to mediate successful thyroid cell growth and proliferation (Brenner-Gati *et al*, 1988; Zaballos & Santisteban, 2012). IGF-1 stimulates mitogenic activity in thyroid follicular epithelium via activation of the phosphoinositol-3 kinase (PI3K) pathway. Specifically binding of IGF-1 to the IGF1R activates the receptor's intrinsic tyrosine kinase activities, which results in the phosphorylation of the IRSs (Insulin Receptor Substrates) (Deleu *et al*, 1999; Zaballos & Santisteban, 2012). Tyrosine-phosphorylated IRSs interact with the cytoplasmic protein PI3K through its SH2 (Src Homology -2) domains. PI3K activation then leads to the transduction of the functional effects of IGFs (Zheng *et al*, 2002).

Whilst it is well established that normal thyroid growth and proliferation hinges upon TSH and IGF-1 signalling, the exact molecular mechanisms and specific endpoints regarding mitogenic signals remain poorly characterised. Furthermore, the extent to which TSH and IGF-1 can independently or synergistically drive thyroid cell growth, remains uncertain (Zaballos & Santisteban, 2012)

1.1.2. The molecular biology of thyroid cancer

In a similar manner to other cancer types, thyroid cancer initiation and progression occurs through stepwise accumulation of different genetic and epigenetic events within the cell. Such events include activating and inactivating somatic mutations, alterations in gene expression patterns, dysregulation of microRNAs and aberrant

control of gene methylation (Nikiforov & Nikiforova, 2011). Amongst these alterations, somatic mutations are by far the most common and thoroughly understood. Indeed thyroid cancer represents a neoplastic condition where critical genes are frequently mutated via two distinct molecular mechanisms: point mutations or chromosome rearrangements (Ciampi & Nikiforov, 2007). Point mutations refer to the replacement of a single nucleotide base with another; and may also include insertions or deletions of a single nucleotide base pair within a gene sequence (Namba *et al*, 1990). Chromosomal rearrangements represent abnormalities caused by breakage and fusion of different parts of homologous or non-homologous chromosomes (Fugazzola *et al*, 1995). Interestingly, it has been suggested that these 2 types of somatic mutations associate with different aetiological aspects of thyroid carcinogenesis. It has become increasingly apparent that thyroid tumours arise due to genetic alterations leading to aberrant activation of the MAPK pathway (Xing, 2007; Nikiforov & Nikiforova, 2011 and Figure 1.3). This pathway is of central importance to the regulation of cell growth, differentiation and survival in response to growth factors, hormones, and cytokines all of which interact with receptor tyrosine kinases present on the cell surface (MacCorkle & Tan, 2005). In over 70 % of thyroid papillary carcinomas genetic alterations occur in genes coding for the receptor tyrosine kinases, RET and NTRK1, and also 2 intracellular manipulators of the MAPK pathway: the GTP-binding protein RAS and the serine threonine kinase BRAF. Interestingly these mutations rarely overlap in the same tumour, suggesting that activation of this signalling pathway is a crucial initiating event for thyroid transformation (Romei *et al*, 2012).

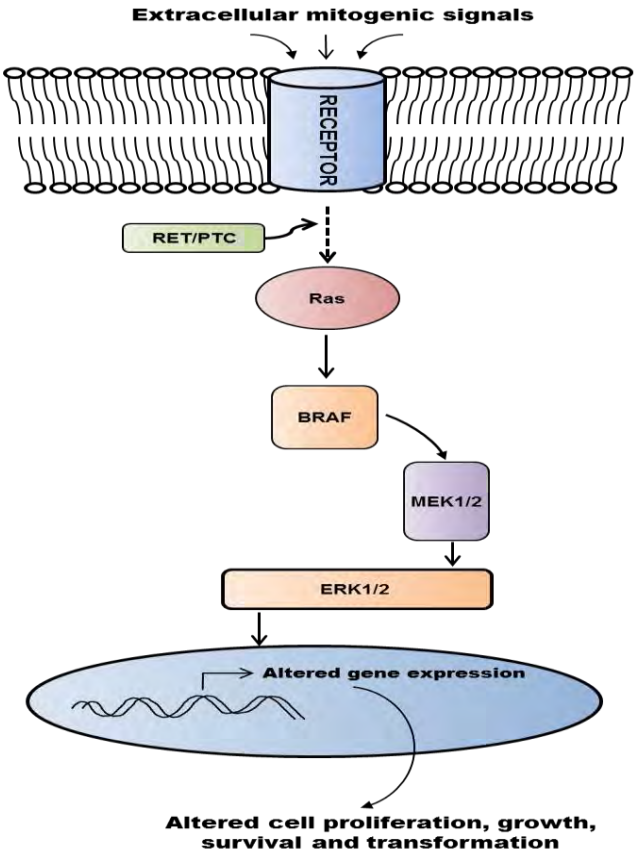


Figure 1.3: Schematic overview of the MAPK signalling pathway. Extracellular mitogenic signals, for example growth factors, activate membrane bound receptors to initiate an intracellular signalling cascade. Upon activation, Ras interacts with members of the Raf protein kinase family (for example the B-type Raf kinase, BRAF). Activated BRAF causes the phosphorylation and activation of MEK1/2, which sequentially phosphorylates and activates ERK1/2; ERK1/2 brings about the phosphorylation and activation of numerous downstream proteins, which ultimately lead to increased expression of genes involved in cell proliferation, growth, survival and tumourigenesis. Thyroid papillary carcinomas may also present with the RET/PTC fusion protein, which brings about aberrant activation of the MAPK pathway upstream of Ras. BRAF is commonly mutated in thyroid papillary carcinomas, leading to aberrant activation of the MAPK pathway (adapted from Xing, 2007).

1.1.3. RET/PTC rearrangements

The RET proto-oncogene is located on chromosome 10q11.2 and encodes a receptor tyrosine kinase. The RET gene encodes three functional domains, including an extracellular ligand binding domain, transmembrane region, and an intracellular tyrosine kinase domain. Receptor tyrosine kinases are activated following ligand:

receptor interaction. RET-TKs are responsive to growth factors belonging to the glial cell-line derived neutrophic factor family (Pachnis *et al*, 1993). Upon ligand binding, RET-TKs undergo dimerization, autophosphorylation of specific tyrosine residues in the intracellular domain, and activation of the MAPK signalling cascade (Ciampi & Nikiforov, 2007 and Figure 1.3).

The Ret proto-oncogene is normally expressed in cells deriving from the neural crest, the kidney and the enteric nervous system (Pachnis *et al*, 1993). In papillary thyroid cancer the RET proto-oncogene is rendered constitutively active by fusion of the RET-TK domain with the 5' terminal sequence of one of a number of different heterologous genes via rearrangements that generate a series of transforming oncogenes collectively described as RET/PTCs (Hamatani *et al*, 2008). RET/PTC rearrangements occur in up to 20 % of thyroid papillary carcinomas, and such rearrangements present with increased metastatic potential. Furthermore, murine transgenic models of RET/PTC rearrangements demonstrate the potential of RET/PTCs to stimulate activation of the MAPK pathway, thereby increasing the risk of thyroid transformation and tumourigenesis (Kim & Zhu, 2009).

1.1.4. RAS mutations

The 3 human RAS genes (HRAS, NRAS and KRAS) encode homologous G-proteins that function via propagation of intracellular signals following activation by membrane bound receptors (Nikiforov & Nikiforova, 2011). Point mutations within important domains of the Ras protein are known to either increase its affinity for GTP (mutations in codons 12/13 of KRAS), or inactivate its autocatalytic GTPase function (mutation of codon 61 of HRAS and NRAS). These mutations give rise to constitutive

RAS function and aberrant stimulation of downstream targets within the MAPK and PI3K pathways, thus promoting tumourigenesis (Figure 1.3).

In contrast to other molecular markers, RAS mutations are not restricted to any particular histological subtype of thyroid cancer. For instance, in thyroid papillary carcinomas, Ras mutations are present in 15-20 % of tumours (Suarez *et al*, 1990; Nikiforov & Nikiforova, 2011). Interestingly, thyroid papillary carcinomas housing RAS mutations frequently present with follicular variant histology. RAS mutations are much more highly associated with conventional follicular thyroid carcinomas, where these mutations are present in 40-50 % of cases (Motoi *et al*, 2000; Nikiforov & Nikiforova, 2011), and to a lesser extent within follicular adenomas, where the prevalence of RAS mutations account for 20-40 % of cases (Manenti *et al*, 1994). Furthermore, RAS mutations are also present in up to 35 % of poorly differentiated and up to 50 % of anaplastic carcinomas, suggesting that RAS mutations may promote tumour aggressiveness and relatively poor prognosis (Garcia-Rostan *et al*, 2003; Basolo *et al*, 2000).

1.1.5. BRAF mutations

Another potent modulator of the MAP kinase pathway is the B-type RAF kinase (BRAF). Mutations in BRAF have been extensively implicated with aberrant regulation of the MAP-K signalling pathway and have a characterised role in human cancers (Davies *et al*, 2002). Interestingly, amongst other human cancers, thyroid cancers demonstrate a high prevalence of BRAF mutations, making this gene of central importance to the initiation and progression of thyroid malignancies (Dhomen & Marais, 2007). Since the discovery of BRAF mutations in human cancers, more than 40 different mutations in BRAF have been identified. Missense point mutations

in the kinase domain of the BRAF gene are located throughout exons 11 and 15, and in over 80 % of cases the T1799A mutation is responsible for cell transformation (Davies *et al*, 2002). The T1799A mutation translates to a V600E amino acid substitution within the BRAF protein product, leading to its constitutive activation. The V600E mutation is thought to render the BRAF kinase constitutively active because the normal valine residue which lies adjacent to the activation phosphorylation site at serine 599, is mutated to a negatively charged glutamate residue, thereby mimicking activation at this site (Dhillon & Kolch, 2004; Mercer & Pritchard, 2003). Therefore the molecular and cellular consequences of the V600E mutation in BRAF result in a constitutively active BRAF kinase by disrupting the interaction of the activation domain with the ATP-binding P-loop, which normally renders BRAF inactive (Wan *et al*, 2004).

BRAF mutations occur in up to 45 % of thyroid papillary carcinomas and in up to 40 % of insular anaplastic carcinomas (Xing, 2007). Such observations imply that BRAF mutations (e.g. $BRAF^{V600E}$) are an early transforming event in thyroid tumourigenesis, and predispose thyroid tumours to increasing dedifferentiation and aggressiveness. Finally, in a mouse model of targeted $BRAF^{V600E}$ overexpression, mice developed significant goiters and an increased prevalence of thyroid papillary carcinomas compared to their wild-type counterparts (Charles *et al*, 2011; Chakravarty *et al*, 2011). Such studies highlight the transforming potential of the V600E mutation in BRAF.

1.1.6. Nuclear receptor mutants

The PAX8/PPARγ rearrangement arises from the fusion between a fragment of the thyroid transcription factor gene 8 (PAX8), and the peroxisome proliferator activator

receptor γ1 (PPARγ) gene, encoding a DNA-binding nuclear receptor that functions to regulate adipocyte differentiation and lipid metabolism (Evans *et al*, 2004; Gregory Powell *et al*, 2004). Whilst the mechanisms of PAX8/PPARγ are particularly poorly understood, the genetic rearrangement occurs in 30-35 % of follicular thyroid carcinomas (Nikiforova *et al*, 2003). Furthermore, it has been documented that mutations in the thyroid hormone receptor (TR) α1 and β1 transcripts play a role in thyroid papillary cancer progression (Puzianowska-Kuznicka *et al*, 2002).

1.1.7. Other oncogenes in thyroid cancer

In addition to *BRAF* and *RAS* mutations, a number of additional oncogenic mutations have been identified in poorly differentiated and anaplastic carcinomas, but not in well-differentiated tumour types, suggesting these mutations are late events in thyroid tumourigenesis. The catenin (cadherin-associated protein) beta 1 (*CTNNB1)* gene encodes a β-catenin involved in cell-adhesion and Wnt signalling, and a point mutation in this gene has been found in up to 60 % of anaplastic carcinomas (Garcia-Rostan *et al*, 1999; García & Santisteban, 2002; Kurihara *et al*, 2004). In addition, studies have demonstrated that poorly differentiated and anaplastic carcinomas harbour mutations of PI3K pathways components including phosphoinositide-3-kinase catalytic alpha polypeptide (*PIK3CA)* itself (10-20 %), phosphatise and tensin homologue (*PTEN)* (5-15%) and v-akt murine thymoma viral oncogene homolog 1 (*AKT1)* (5-10%) (Ricarte-Filho *et al*, 2009; García-Rostán *et al*, 2005; Santarpia *et al*, 2008; Dahia *et al*, 1997).

1.1.8. The prognosis of thyroid cancer

Retrospective studies have enabled the recognition of certain factors that negatively affect the prognosis of thyroid cancer and such risk is assessed in patients with DTC

using a prognostic scoring system. **T**umour size, **N**ode metastases and distant **M**etastases (TNM) is the most frequently used system and is summarised in Table 1-1. While most patients have a good prognosis following surgery and administration of radioiodine, BTA guidelines suggest 10 year disease specific mortality for stage I as 1.7 %, rising to 60.9 % for stage IV disease (British Thyroid Association and Royal College of Physicians 2007; Loh et al. 1997). However, 5-30 % of patients will develop locoregional recurrence (British Thyroid Association and Royal College of Physicians 2007; Jonklaas *et al*, 2006; Mazzaferri, 1993), with subsequent five year survival of 87 %. If distant metastases are present, five year survival falls to 72 % (Jonklaas et al. 2006). Accuracy of the staging and grading systems used in differentiated thyroid cancer has increased (D'Avanzo *et al*, 2004). Factors associated with a worse prognosis are male gender, age greater than 40 years and tumours of the follicular subtype or greater than 1 cm in size (British Thyroid Association and Royal College of Physicians 2007; Mazzaferri, 1993).

Primary tumour
pT1: Intrathyroidal tumour, ≤ 1 cm in greatest dimension
pT2: Intrathyroidal tumour, 1-4 cm in greatest dimension
pT3: Intrathyroidal tumour, > 4 cm in greatest dimension
pT4: Tumour of any size, extending beyond thyroid capsule
pTX: Primary tumour cannot be assessed

Distant metastases
M0: No distant metastases
M1: Distant metastases
MX: Distant metastases cannot be assessed

Regional lymph nodes (cervical or upper mediastinal)
N0: No nodes involved
N1: Regional nodes involved
If possible, subdivided into:
N1a: Ipsilateral cervical nodes
N1b: Bilateral, midline or contralateral cervical nodes or mediastinal nodes
NX: Nodes cannot be assessed

Disease stage	< 45 years	> 45 years	10 year cancer related mortality (%)
STAGE 1	-Any T, any N, M0	-pT1, N0, M0	1.7
STAGE 2	-Any T, any N, M1	-pT1, N0, M0 -pT3, N0, M0	15.8
STAGE 3		-pT4, N0, M0 - Any pT, N1, M0	30.0
STAGE 4		-Any pT, any N, M1	60.9

***All anaplastic carcinomas are stage 4**

*Table 1-1: A table summarising **T**umour size, **N**ode metastases and **D**istance metastases **(TNM)** as a scoring system to predict mortality rates. (Compiled from the British Thyroid Association and Royal College of Physicians 2007 and D'Avanzo* et al*, 2004).*

The gold standard for diagnosing thyroid malignancy remains FNA cytology (FNAC). However, recent advances in molecular analysis of FNA help to provide individualised treatment strategies to patients. Currently, indeterminate cytology indicating an inability to conclusively discern benign from malignant nodules is found in 10-20 % of patients with thyroid nodules and malignancy is diagnosed in only 20 %

of these. Consequently, unnecessary surgery is performed in 60-90 % of these patients (Baloch *et al*, 2002, 2008; Mazzaferri, 1993). Advances in the molecular analysis of the mutational markers described above (see Section 1.1.2), represent opportunities to identify specific cancer subtypes, and to provide highly improved diagnostic accuracy to avoid unnecessary surgical procedures. For example, the collective results of 22 studies of thyroidal FNA samples revealed that in 1,117 nodules deemed positive for the $BRAF^{V600E}$ mutation, 99.3 % were then histologically classified as papillary carcinomas, demonstrating the great accuracy of $BRAF^{V600E}$ mutations as an indicator of cancer (Nikiforov & Nikiforova, 2011; Nikiforova & Nikiforov, 2009; Kim *et al*, 2011).

1.1.9. Ionising radiation and thyroid cancer

The thyroid gland (especially the developing thyroid in children and adolescents), has been reported to be highly sensitive to the carcinogenic effects of ionising radiation (Takeichi *et al*, 1991; Nikiforov *et al*, 1996; Hamatani *et al*, 2008). Data regarding the increased prevalence of thyroid papillary carcinomas of atomic bomb survivors in Hiroshima and Nagasaki, and of populations affected by the Chernobyl nuclear disaster, provide irrefutable evidence that ionising radiation is a significant risk factor for thyroid papillary carcinomas (Kazakov *et al*, 1992; Astakhova *et al*, 1998; Takeichi *et al*, 1991; Imaizumi *et al*, 2006; Hamatani *et al*, 2008). For instance, the Chernobyl nuclear power plant accident resulted in exposure of populations in the Ukraine, Belarus and the Russian Federation to significant volumes of radionuclides (I^{131}, I^{133} and Cs^{137}). The primary source of radiation to the thyroid was incorporated into the food chain through radioiodine contaminated milk and other food products, resulting in cellular damage through β-emissions. Specificity to the thyroid occurs due to the

expression of the sodium iodide symporter (NIS) at the basolateral membrane of thyroid follicular cells, thereby resulting in the accumulation of radioiodides. Children in affected areas received relatively high doses of irradiation due to the small size of the thyroid and the above average consumption of contaminated milk. This exposure in children and adolescents in contaminated areas correlated with a significant increase in thyroid cancer incidence (Kazakov *et al*, 1992; Astakhova *et al*, 1998; Nikiforov *et al*, 1996; Nikiforov & Gnepp, 1994; Fagin, 2005).

Whilst BRAF mutation(s) appear to be the most common genetic change in sporadic papillary carcinomas, the RET/PTC proto-oncogene appears to play a much more prevalent role in radiation induced thyroid cancer. For example, in the most contaminated regions resulting from the Chernobyl nuclear disaster, the resulting prevalence of paediatric thyroid papillary carcinoma was increased by ~100-fold in the highest affected areas, compared to the incident rate before the disaster (Fagin, 2005; Hamatani *et al*, 2008). Genetic analysis of post Chernobyl tumours suggests that RET/PTC rearrangements are highly prevalent; found in 66-87 % of radiation induced papillary tumours compared to a maximum of ~40 % in sporadic paediatric tumours and only 15-12 % in adult sporadic tumours (Klugbauer *et al*, 1995; Fugazzola *et al*, 1995; Ciampi & Nikiforov, 2007). Further to these observations, irradiation of human thyroid carcinoma cell lines and human foetal thyroid cells led to an increased rate of RET/PTC translocations in a dose dependent manner. These findings provide direct *in vitro* evidence that irradiation causes RET/PTC rearrangements in thyroid follicular cells (Ito *et al*, 1993).

More recently, in 2011, another nuclear disaster took place in Fukushima Japan in wake of the Tohoku earthquake and tsunami on 11th March 2011. Fukushima

represents the largest nuclear disaster since Chernobyl of 1986. Whilst this disaster resulted in significant atmospheric efflux of radioiodides, the exposure rates for the surrounding population differ significantly from Chernobyl for the following reasons: The reactors in Chernobyl had no containment vessel, and fires burned for many days resulting in persistent discharge of radioiodides into the atmosphere (Wakeford, 2011a). Furthermore, radioiodides were distributed over larger areas, ingested by grass eating cows, and subsequently ingested as milk by millions of children. These factors resulted in populations around Chernobyl receiving massive doses of irradiation. In contrast, Fukashima appears to have resulted in significantly lower doses of public exposure (Boice, 2012; Wakeford, 2011b; Dauer *et al*, 2011). For example, the Fukushima plant incorporated containment vessels around each reactor, and the prevailing winds primarily deposited radioiodides into the ocean. In addition, successful evacuation of individuals in affected areas and monitoring of local food products and water supply was undertaken (Boice, 2012). Such preventative measures including the distribution of stable iodine pills ensured that individual exposure was minimal. Nonetheless some radioiodides were deposited over populated areas, suggesting that the Fukushima disaster may have a significant effect on thyroid cancer incidence in the future (Yasumura, 2011).

1.2. **p53 is the guardian of the genome**

1.2.1. The discovery of p53

p53 was initially discovered following investigations into the oncogenicity of DNA tumour viruses (Levine & Oren, 2009). DNA tumour viruses archetypally express a small number of viral encoded proteins which stimulate an immune response within the host, leading to the production of virus specific antibodies. Such antibodies were

exploited to investigate and monitor viral proteins encoded by the viral genome. Subsequent investigations revealed that certain proteins (for example the SV40 large T antigen), were important in the transforming potential of virus infected cells. Sera from animals possessing SV40-induced tumours were utilised to immunoprecipitate the SV40 large T antigen, where interestingly, a non-viral protein with a predicted molecular mass of 53 kDa was found to be co-immunoprecipitated (Linzer & Levine, 1979; Lane & Crawford, 1979; Kress *et al*, 1979; Levine & Oren, 2009). Thus it appeared that the SV40 large T antigen was engaging in a specific interaction with an unknown cellular protein of 53 kDa. Future studies revealed that the term p53 is actually a misnomer, with the molecular mass of the p53 protein being only 47.3 kDa. This gross overestimate has since been attributed to the proline rich region within the protein that hinders the migration of the protein through SDS-PAGE (Levine & Oren, 2009).

1.2.2. p53 the tumour suppressor protein

Shortly after its initial discovery, p53 was wrongly characterised as an oncogene. Since SV40 appeared to drive the overexpression and cellular accumulation of p53 in transformed cells, it followed logically that p53 was a positive mediator of cell transformation (Sarnow *et al*, 1982; Linzer *et al*, 1979). Furthermore, it was determined that p53 expression was elevated in tumours, but not in non-transformed cells, further lending weight to the hypothesis that increased p53 expression promoted cell transformation and tumourigenesis (Rotter, 1983; DeLeo *et al*, 1979; Levine & Oren, 2009). This hypothesis logically (but wrongly) identified p53 as an oncogene. The eventual characterisation of p53 as bona fide tumour suppressor was initially hindered following experiments using the first successful clones of the p53

gene from transformed cells. Interestingly, when the sequences of a number of these p53 clones were sequenced it was observed that no 2 clones were identical. Subsequent sequencing of p53 from normal cells revealed that most, if not all of the clones contained inactivating mutations of p53 (Finlay *et al*, 1988; Halevy *et al*, 1991; Eliyahu *et al*, 1988). Moreover, TP53 was shown to be lost or inactivated in a number of malignancies, both human and murine, and is now known to be the most frequently altered gene in human tumours. Finally, it was discovered that the rare disease Li Fraumeni syndrome, which results in a severely increased susceptibility to a number of types of cancers, arises from inactivating germline mutations in p53 (Varley *et al*, 1997). These observations provided irrefutable evidence that p53 is a tumour suppressor protein and not an oncogene.

1.2.3. The molecular structure of p53

Mammalian p53 family members, including p53, p63 and p73 represent an evolutionarily ancient group of transcription factors which may date back as far as the divergence of *animalia* and *fungi* some 2 billion years ago (Nedelcu & Tan, 2007). Sequencing and characterisation of p53 have revealed a number of important domains within the p53 protein that are crucial to its function.

1.2.3.1. *p53 transactivation domain*

Full length wild-type p53 contains 2 tandem, but functionally independent, C-terminal transcription activation domains (TADs). The first, AD1, lies between residues 1-42, and the second, AD2, spans residues 64-92 and encompasses a proline rich domain (PRO) from residues 64-92 (Figure 1.4). Both AD1 and AD2 are able to interact with the basal cellular transcription machinery and also histone acetyl transferases (HATs). Furthermore, both AD1 and AD2 are able to independently drive

transcription when fused to an exogenous DNA binding domain (DBD) (Candau *et al*, 1997). Interestingly, AD1 and AD2 appear to promote distinct subsets of genes to bring about different biological processes; for example, investigations into mutants of p53 containing deleted or non-functional AD1 revealed that this domain was critical for p53-mediated cell cycle arrest in response to genotoxic insult (Zhu *et al*, 1998). However, mutated or deleted AD1 still allows for p53-mediated apoptosis, since the presence of a functional AD2 domain still allows for interaction of p53 with its canonical response elements, and AD1 mutants can still transactivate the potent pro-apoptotic gene BAX (Zhu *et al*, 1998; Johnson *et al*, 2005). These findings suggest that the tandem transcriptional activation domains AD1 and AD2 are responsible for bringing about growth arrest and apoptosis respectively.

Figure 1.4: Schematic representation of the p53 protein, showing important functional domains (not to scale). The p53 protein is 393 amino acids in length, with an N-terminal acidic transcription activation domain (TAD), also known as AD1+ AD2 which activate transcription factors (residues 1-42 + 43-92); a proline rich domain (PRO), is important for p53 apoptotic functions (residues 64-92); a central DNA binding domain (DBD) containing important arginine residues and a zinc atom (residues 102-292); a tetramerisation domain (TD) required for p53 oligermerisation following activation (residues 307-355) - this region also contains a nuclear localisation signal (NLS); and a C-terminal regulation domain (RD – residues 355-393).

1.2.3.2. *p53 DNA binding domain*

The DNA binding domain (DBD) of p53 encompasses residues 102-292 (Figure 1.4). Within these residues lie 4 distinct regions that are highly conserved between species and other p53 family members. These are: conserved region II (residues 117–142), conserved region III (residues 171–181), conserved region IV (residues 234–256), and conserved region V (residues 270–286) (Harms & Chen, 2006). These conserved regions play a critical role in the interaction of tetrameric p53 with p53 consensus sequences (Olivier *et al*, 2002). The recognised p53 response element contains 2 decamers consisting of the following sequence: RRRCWWGYYY, which are separated by a spacer of 0-13 bp, where R=purine, C=cytosine, W=adenine or thymidine, G=guanine, and Y=pyrimidine (el-Deiry *et al*, 1992). Several studies indicate that a monomer binds the pentameric sequence and that a tetramer binds the full consensus site. To bind DNA, the larger part of the DBD forms an antiparallel β-sandwich (two β-sheets packed together). This serves as a scaffold that supports the structures important for the interaction with DNA. These structures include two large loops and a loop-sheet-helix motif (Cho *et al*, 1994). The two large loops are held together by a zinc atom which is coordinated by three cysteine residues and one histidine residue (C176, H179, C238, and C242). C176 and H179 are situated in loop 2 which covers conserved region III. C238 and C242 are positioned in loop 3 which encompasses region IV. Loop 3 also contains S241 and R248, which interact with the DNA phosphate backbone and the minor groove, respectively. The loop-sheet-helix motif spans conserved region V; this motif contains residues that contact the DNA phosphate backbone (R273, A276, R283) as well as the major groove (C277 and R280). Another amino acid residue, K120, is located

within conserved region II and is crucial for the interaction with the major groove and the DNA phosphate backbone (Harms & Chen, 2006; Cho *et al*, 1994; Viadiu, 2008).

The ability of p53 to bind to DNA appears to be of great significance to its function as a tumour suppressor. This statement is supported by the fact that many tumours harbour mutations that alter the affinity of p53 for its consensus sequence. For example, more than 80 % of p53 mutations apparent in human tumours are found in this central DBD (residues 93-292) (Olivier *et al*, 2002). These mutations include contact site alterations, where crucial residues for p53-DNA interactions are mutated, and conformational mutations which alter the shape of the anti-parallel β-sandwich, thereby reducing the proximity of the important DNA binding regions of p53 (Harms & Chen, 2006).

1.2.3.3. *p53 tetramerisation domain*

As previously mentioned, to bind with high affinity to its consensus sequence, and therefore mediate transcription, p53 must do so as a tetramer. The tetramerisation domain (TD) spanning residues 325-356 is responsible for formation of p53 into tetramers (Harms & Chen, 2006 and see Figure 1.4). The secondary structure of the TD is a β-pleated sheet linked to an α-helix, where p53 monomers form dimers through antiparallel β-sheet: antiparallel α-helix interaction (Kamada *et al*, 2011; Mateu & Fersht, 1998). p53 then forms a terameric structure by forming as a dimer of dimers by utilising parallel helix:helix interactions. The tetrameric structure is maintained through formation of a hydrophobic core produced by the helix:helix interaction (Mateu & Fersht, 1998, Figure 1.5). Although the TD is not a mutational hotspot, mutation of this domain has been observed in some Li-Fraumeni syndrome families (Petitjean *et al*, 2007). The L344P mutation lies within the α-helix that is

important for the dimer–dimer interaction and results in monomeric p53 (Scoumanne *et al*, 2005; Zambetti, 2007; see Figure 1.5).

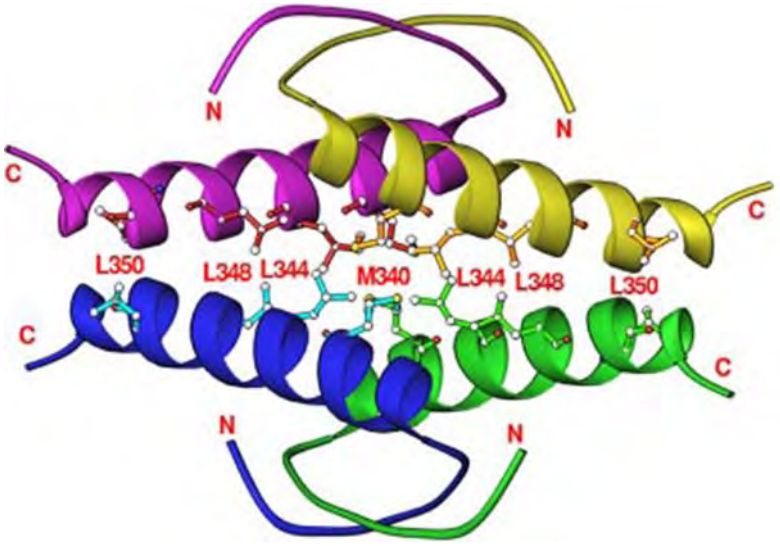


Figure 1.5: Schematic representation of four p53 monomers orientated into tetrameric conformation. p53 initially forms dimers by interaction of antiparallel β-pleated sheets and antiparallel α-helices. Tetramers form as a dimer of dimers by interaction of parallel α-helices to form a stable hydrophobic core. Critical residues such as L344 have been observed to be mutated in Li-Fraumeni syndrome resulting in monomeric p53 –highlighting the importance of p53 tetramer formation in its function as a tumour suppressor (image adapted from Scoumanne et al*, 2005).*

1.2.3.4. *p53 nuclear localisation signal*

To exert its effects as a transcription factor, p53 must shuttle between the cytoplasm and the nucleus. Tetrameric p53 cannot pass freely through the nuclear pore space due to its size, and therefore utilises nuclear import and export signals in order to cross the nuclear membrane. Accordingly, p53 possess a bipartite nuclear localisation signal located between residues 305-322 (Figure 1.4) and also 2 nuclear export signals at the C-terminus and N-terminus of the protein (Scoumanne *et al*, 2005; Harms & Chen, 2006; Viadiu, 2008). It is interesting to note that whilst NLS and NES mutations appear relatively rare, p53 subcellular localisation can be

affected by post translational modifications (Liang & Clarke, 1999). Furthermore, many viral infections, such as cytomegalovirus infection, result in p53 sequestration, thereby inhibiting p53 function (Wang *et al*, 2001).

1.2.3.5. *p53 regulatory domain*

The last 30 amino acids (residues 362-393), make up the regulatory domain of p53 (Figure 1.4). This domain is comprised of a relatively large number of lysine and arginine residues, and therefore is basic in nature (Brady & Attardi, 2010). The basic region of p53 has affinity for DNA binding in a non-sequence-specific manner and also promotes linear diffusion serving as a landing pad to enhance p53 detection of specific response elements. In addition, the p53 regulatory domain is subject to a large number of post-translational modifications including acetylation, methylation, phosphorylation, SUMOylation, neddylation and ubiquitination (Harms & Chen, 2006; Viadiu, 2008; Liu & Kulesz-Martin, 2006). The C-terminus of p53 is unfolded, acting as a flexible linker by acquisition of more stable secondary structure following interaction with other proteins that regulate p53 expression and function (Brady & Attardi, 2010; Bell *et al*, 2002)

1.2.4. The molecular function of p53

p53 is unique amongst the other p53 family members in its potency as a tumour suppressor. Further to co-ordinating effective tumour suppression by mediating crucial cellular responses to cell stress and DNA damage, p53 has an emerging repertoire of roles in diverse range of physiological processes. Such processes include fertility, cell metabolism, mitochondrial function and stem cell maintenance (Hu *et al*, 2007; Kanfi *et al*, 2008; Matoba *et al*, 2006; Gatza *et al*, 2007; Junttila & Evan, 2009). To function effectively as a tumour suppressor, p53 is required to

unerringly distinguish between normal and potentially neoplastic cell growth by promoting the former and preventing the latter (Vogelstein *et al*, 2000). The following paragraphs highlight the molecular mechanisms and cellular biology of p53 tumour suppressor function.

p53 is a sequence specific transcription factor and is widely considered “the guardian of the genome”, owing mostly to its central role in the organisation of cellular responses to genotoxic stress (Wu & Levine, 1997). Under normal conditions, the levels of p53 are tightly controlled within the cell and levels remain low via direct regulation of p53 protein stability (Vogelstein et al 2000). p53 is primarily turned over via ubiquitin mediated proteolysis. Ubiquitination is co-ordinated by a group of enzymes known as ubiquitin ligases. Specifically, E3 ubiquitin ligases are responsible for the direct ubiquitination of target proteins (Ardley & Robinson, 2005). The activity of E3 ligases is augmented by the activity of ubiquitin activating enzymes (E1 ligases) and ubiquitin conjugating enzymes (E2 ligases). For instance, E1 ligases initiate the ubiquitination process by binding specifically to two ubiquitin monomers, making ubiquitin molecules functionally available for transfer to E2 ligases (Nandi *et al*, 2006, Schulman & Harper, 2009). E2 ligases functionally interact with specific E3 ligases; E3 ligases are responsible for the sequential addition of mono-ubiquitin tags, from the E2 ligase to the target protein (Ardley & Robinson, 2005, 3; Nandi *et al*, 2006, Risseeuw *et al*, 2003) Several E3 ligases are known to promote p53 degradation, including HDM2, COP-1, PIRH2, TOPORS and ARF-BP1 (Brooks & Gu, 2006). Of these enzymes, HDM2 is by far the most extensively studied, and the observation that HDM2 null mice die early in development due to p53-dependent massive apoptosis highlights the critical role that this protein plays in p53

homeostasis (Jones *et al*, 1995; Montes de Oca Luna *et al*, 1995). HDM2 contains a number of functional domains including an N-terminal p53 binding domain; NLS and NES regions important for nuclear shuttling (which are also sites of phosphorylation); an acidic region crucial for the ubiquitination and degradation of p53 and HDM2 (also a site of extensive phosphorylation); a zinc finger domain, important for contacting ribosomal proteins; and a RING finger domain critical for mediating the ubiquitination of substrates (Meek & Hupp, 2010 and Figure 1.6). Mechanistically, HDM2 binds directly to, and prevents p53 function by masking specific sites that are essential for interaction with transcriptional co-activators (Brooks & Gu, 2006). Furthermore, HDM2 contains a self and p53 specific ubiquitin ligase that covalently attaches monoubiquitin tags to specific lysine residues within the C-terminus of p53 (Honda *et al*, 1997). This site specific ubiquitination targets the p53 protein for degradation within nuclear and cytoplasmic 26s proteasomes (Sionov & Haupt, 1999; Brooks & Gu, 2006; Meek, 2004). The function of HDM2 is thought to be highly facilitated by MDMX. The MDMX protein shares significant structural homology with HDM2 and appears to have both dependent and independent roles in the modulation of p53 activity (Marine *et al*, 2007; Meek & Hupp, 2010). For instance, MDMX binds directly to the N-terminus of p53, and significantly represses p53 transcriptional activity. However, MDMX lacks intrinsic ubiquitin ligase activity and hence cannot directly influence p53 ubiquitination. MDMX is able to indirectly regulate p53 ubiquitination by activating the ubiquitin ligase function of HDM2 by forming HDM2:MDMX heterodimers by virtue of their mutual RING finger domains (Kawai *et al*, 2007).

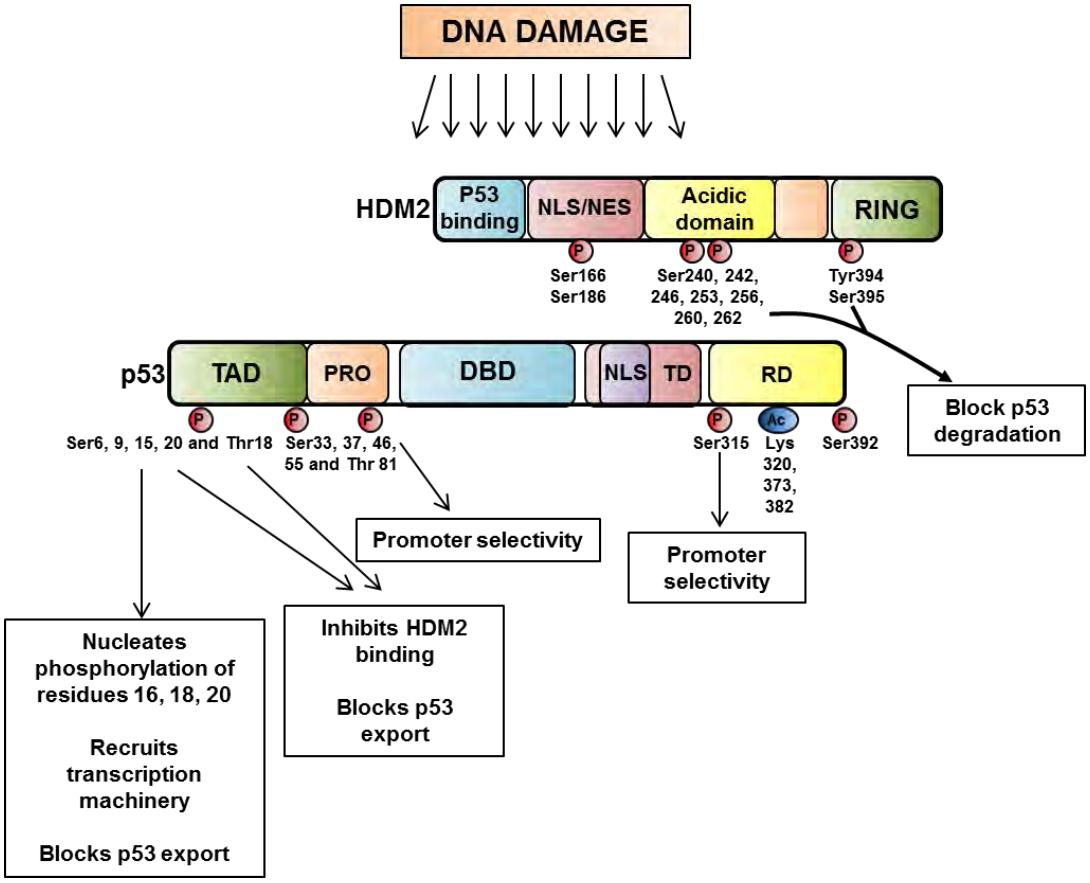


Figure 1.6: Schematic demonstrating DNA damage induced post-translational modification of p53 and HDM2 (not to scale). Important regions of p53 are the transactivation domain (TAD), proline rich region (PRO) and regulatory domain (RD). Important regions of HDM2 include nuclear localisation domains (NLS/NES), the acidic domain and the RING finger domain. Phosphorylation sites are represented by red and blue circles respectively. In each case, target residue numbers are documented and text boxes summarise the biological outcomes of each of the indicated modification sites (adapted from Meek, 2004; Meek & Anderson, 2009).

Central to the HDM2 regulation of p53 levels is the fact that the HDM2 gene itself can be induced by p53 in response to cellular stress. Such induction creates an autoregulatory negative feedback loop, which keeps p53 levels low in the absence of cellular stresses. In addition, this relationship ensures that responses arbitrated by p53 are limited with regard to their extent and duration; for example after a relatively benign cellular insult (Wu & Levine, 1997).

As mentioned previously, the levels of p53 protein are kept at low levels in the absence of cellular stress; nonetheless, the p53 gene is continually transcribed and translated, so that the levels of p53 within the cell are determined by the rate at which it is degraded rather than the rate at which it is made (Vogelstein *et al*, 2000). This allows for a rapid p53 response upon stimulus by relevant stress signals (Meek, 2004). p53 is rapidly stabilised and activated in response to a plethora of genetic insults. Of the diverse stimuli that elicit a p53 response, the molecular mechanism by which p53 is activated following DNA damage are perhaps the most fully understood (Meek, 2004). Stabilisation of p53 is achieved by uncoupling the direct interaction of p53 with its negative regulator HDM2. This event is mediated principally by multisite phosphorylation of both p53 and HDM2, and results in the inhibition of the degradation of p53 (Fang *et al*, 2000). Indeed human p53 has been reported to be post translationally modified in at least 18 different sites (Appella & Anderson, 2000, 2001). For example, in response to DNA damage caused by exposure to ionising radiation, p53 is modified initially through phosphorylation of ser-15 by the protein kinase ATM (Saito *et al*, 2002). This initial phosphorylation event primes the p53 molecule for numerous additional post translational modifications such as phosphorylation of thr-18 by CK1 (reviewed in Appella & Anderson, 2000 and Figure 1.6). In the case of the DNA damage response, such modification of p53 results in the activation of p53 as a transcription factor, which as a tetramer, binds DNA and drives the expression of a multitude of genes responsible for cell-cycle inhibition, apoptosis, genetic stability and inhibition of blood vessel formation (Vogelstein *et al*, 2000 and Figure 1.7). In nearly all mammalian cell types, the direct effect of p53 stimulation is an initial block in the progression of the cell cycle and this effect is

brought about by the p53 driven expression of the potent cyclin dependent kinase inhibitor $p21^{WAF1/CIP}$. p21 inhibits the phosphorylation events mediated by cyclin dependent kinases which would otherwise result in smooth progression though the cell cycle (Waldman *et al*, 1995). Indeed, the classical G1/S phase boundary arrest observed after activation of the p53 response is the result of the inhibition of CDK2/cyclin E by p21 (Waldman *et al*, 1995). This G1/S arrest is critical in preventing the replication of damaged DNA (Figure 1.7).

The example above is just one of an exhaustive list of important events that can positively or negatively regulate cell fate to prevent cell transformation and maintain the integrity of the genome (see Figure 1.7).

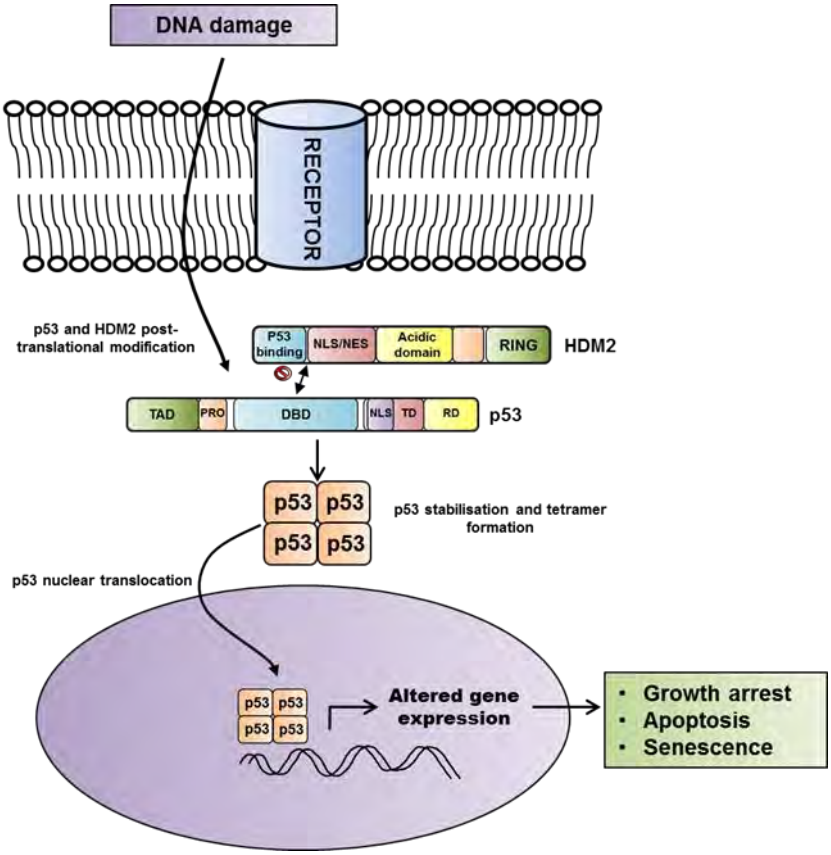


Figure 1.7: Schematic overview of the classical p53 transcriptional response to DNA damage. DNA damage activates specific kinases that post-translationally modify p53 and HDM2, allowing for p53 activation. Activated p53 undergoes further post-translational modifications that further stabilise p53, allowing it to form tetramers. Tetrameric p53 undergoes nuclear translocation, where it binds to p53 response elements and drives the transcription of target genes to bring about cellular responses that ultimately serve to protect against malignant progression (Vogelstein et al*, 2000).*

1.2.5. The importance of p53 in human cancer

Clearly loss or mutation of p53 is strongly associated with an increased susceptibility to cancer and most functions of p53 have been considered in light of how p53 might protect from malignant progression (Vousden & Lane, 2007). As such, p53 has become one of the most studied genes in human disease, primarily because most human cancers have defects in the p53 pathway (Murray-Zmijewski *et al*, 2008). In

fact, gene mutations inactivate p53 in around 50 % of human cancers, although the mutation rate varies dramatically from up to ~70 % of lung cancers, ~60 % of colon cancers, but only 10-12 % of leukaemias (see the IARC TP53 mutation database for further information). Further to this p53 can be functionally inactivated by oncogenic expression of its negative regulators, including HDM2, MDMX, and ARF (Brooks & Gu, 2006; Meek & Anderson, 2009; Hu *et al*, 2006; Sherr, 2006). More recently, expression of novel viral and cellular oncogenes have been observed to be functionally activated in a number of human tumours, further highlighting the importance of wild-type p53 inactivation in human cancers. The following section provides some examples of the molecular mechanisms of p53 functional inactivation caused by viral and cellular oncogenes.

1.2.6. Mechanisms of p53 inactivation by viral oncogenes

The E6 protein from human papilloma virus (HPV) in cervical carcinoma, for example, degrades p53 by ubiquitin mediated proteolysis (Kyo *et al*, 1991; Mishra & Jana, 2008). Another pertinent example includes hepatocarcinoma resulting from hepatitis B infections. 90% of these viruses encode HBXAg; this protein has affinity for the N-terminal domain of p53 and as a result is able to inhibit its transcriptional activity (Truant *et al*, 1995). Finally, the well characterised SV40 viral T antigen associates with a region of the p53 protein responsible for DNA binding and results in the prevention of active p53 from associating with the promoter of target genes (Mietz *et al*, 1992).

1.2.7. p53 inactivation by cellular oncogenes

As mentioned previously, oncogenic expression of the regulators of p53 expression and activity can aberrantly affect the p53 pathway, and HDM2, the master regulator

of p53, is no exception. The HDM2 gene is amplified in a variety of tumours, and HDM2 overexpression without amplification is a common mechanism of p53 inactivation in certain cancers, including breast cancer (Bond *et al*, 2005; Osman *et al*, 1999; Hu *et al*, 2006). Furthermore, MDMX, a protein that shares significant homology to HDM2, has been extensively implicated in the functional inactivation of p53. Unlike HDM2, MDMX does not have intrinsic E3 ligase activity and does not promote p53 degradation. However, MDMX binds to HDM2 through C-terminal RING domain interaction (Shvarts *et al*, 1996, 1997; Tanimura *et al*, 1999; Sharp *et al*, 1999) and stimulates the ability of HDM2 to ubiquitinate and degrade p53. MDMX overexpression has been found in a number of primary tumours or tumour cell lines with wild-type p53 (Riemenschneider *et al*, 1999; Danovi *et al*, 2004; Marine *et al*, 2007), suggesting that MDMX may contribute to p53 inactivation during tumourigenesis.

1.2.8. p53 in thyroid cancer

TP53 mutations are very common in human malignancies, accounting for 50 % of all known cases (Vogelstein *et al*, 2000). However, mutations in TP53 are much rarer in thyroid carcinomas, where they account for only 10 % of cases (Olivier *et al*, 2002; Harvey *et al*, 1995; Malaguarnera *et al*, 2007a). Furthermore, p53 mutations almost exclusively occur in the poorly differentiated and aggressive histotypes, including anaplastic carcinomas (Fagin *et al*, 1993). These observations and the indolent progression of most thyroid tumours have suggested that p53 may play only a minor role in thyroid cancer, where mutations in p53 only serve to promote tumour progression to aggressive or invasive subtypes.

In addition to p53 inactivating mutations, it is becoming increasingly apparent that wild-type p53 is often functionally inactivated by viral or cellular oncogenes (Brooks & Gu, 2006; Meek & Anderson, 2009; Hu *et al*, 2006; Sherr, 2006). It is therefore becoming progressively more important to assess the function of p53 in those cancers that do not present with inactivating mutations, as this may have profound implications on the treatment of such tumours.

1.2.8.1. *p53 inactivation in thyroid cancer*

In cancer types that are not normally associated with mutations in p53 or underlying viral infection, there is evidence for the role of cellular proteins in the functional inactivation of p53. For example the High-mobility group factors are nonhistone proteins, which participate in a variety of cellular processes including gene transcription, integration of retroviruses into chromatin and the promotion of metastatic progression (Reeves, 2001; Sgarra *et al*, 2004). HMGA1a and HMGA1b are together referred to as HMGA1 because they arise from the alternative splicing of a common gene (Johnson *et al*, 1988). HMG factors participate in specific protein:protein and protein:DNA interactions and initiate activity of the promoter/enhancer regions of genes (Reeves, 2001). Interestingly, HMG1A is commonly over expressed in thyroid cancers (Malaguarnera *et al*, 2007a). Subsequently the role of HGM1A in the p53 response was investigated, and the results are summarised below.

In several thyroid cancer cells of different histotypes, silencing of the HMGA1 gene resulted in an increase in p53 transcriptional activity, measured by analysis of p21 promoter activity (Frasca *et al*, 2006). In a similar vein, such studies also revealed that HMGA1 is able to inhibit p53 activity that would normally result in successful

G1/S phase arrest and apoptosis after treatment with DNA damaging agents. Moreover, coimmunoprecipitation experiments demonstrated direct interaction of HMGA1 and p53. The results of this study indicate a novel role of HMGA1 in regulation of the oncosuppressor function of p53 and aberrant control of this gene may play a central role not only in thyroid cancers but many other tumour types as well (Frasca *et al*, 2006).

The above study, amongst others, provides evidence that over expression of other novel cellular proteins can result in the inhibition of p53 activity or p53 responses. Such proteins may be responsible for cell transformation and therefore play a central role in tumourigenesis.

1.3. **Pituitary tumor transforming gene binding factor (PBF)**

Pituitary tumor transforming gene binding factor (PBF or PTTG1IP) is the poorly characterised interacting partner of the pituitary tumor transforming gene (PTTG) (Pei & Melmed, 1997; Chien & Pei, 2000). PTTG stimulates the expression of fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) (McCabe *et al*, 2002). Furthermore, PTTG is the multifunctional human securin, with an established role in the control of mitosis. Here, PTTG functions to prevent premature sister chromatid separation by inhibition of separase activity (Zou *et al*, 1999 and Figure 1.8). In addition, PTTG has also been linked with DNA repair mechanisms and foetal development amongst other roles (Kim *et al*, 2005; Boelaert *et al*, 2003). PTTG has also been implicated in numerous neoplastic conditions, and PTTG overexpression has been reported in tumours of the thyroid, pituitary, colon, ovary and breast (Stratford *et al*, 2005; Puri *et al*, 2001; Heaney *et al*, 2000; Shibata *et al*, 2002).

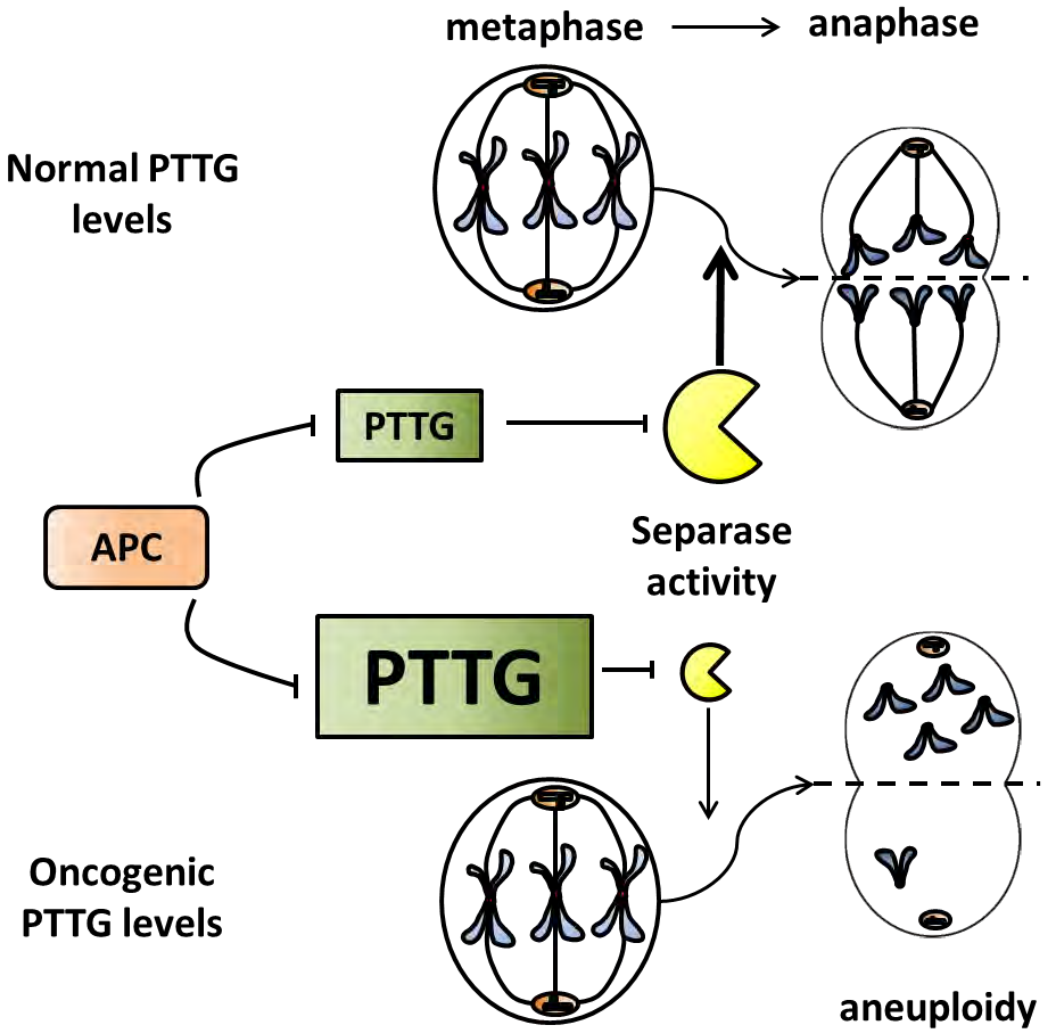


Figure 1.8: Schematic overview of the function of PTTG as human securin and its relevance to human cancers. Overexpression of PTTG leads to aneuploidy: under normal conditions PTTG serves to inhibit the activity of separase and therefore prevents premature sister chromatid separation. At the metaphase-anaphase boundary PTTG is degraded by the anaphase promoting complex (APC) allowing separase to co-ordinate sister chromatid separation (and hence normal metaphase to anaphase transition). However, oncogenic levels of PTTG result in aneuploidy through the inhibition of mitosis progression by the increased repression of separase activity. PBF is thought to facilitate the nuclear entry of PTTG. (Figure adapted from Lewy et al*, 2012).*

The mechanism by which PTTG regulates FGF-2 was determined through discovery and isolation of PBF. Using the yeast 2-hybrid system to identify interacting partners

of PTTG, PBF was isolated and characterised. PBF is located on chromosome 21q21.3 and encodes a protein 180 amino acids in length; with a predicted molecular mass of 22kDa (Chien & Pei, 2000). It is interesting to note that PBF is found in the distal portion of chromosome 21 to which the majority of phenotypic features of Down's syndrome map (Lyle *et al*, 2009).

Whilst PBF appears to lack sequence homology with other human proteins, placing constraints on prediction of physiological role, this suggests unique functionality. Furthermore, PBF is ubiquitously expressed and highly conserved, implying significant evolutionary importance (Yaspo *et al*, 1998; Smith *et al*, 2011). The PBF protein consists of four functional domains including an N-terminal signal peptide, PSI domain, transmembrane region and a C-terminal domain containing a bipartite nuclear localisation signal (NLS) and sorting signal (Yaspo *et al*, 1998; Chien & Pei, 2000 and Figure 1.9). The bipartite nuclear localisation signal, located towards the C-terminus of PBF, appears important for the translocation of PTTG into the nucleus. In fact, ablation of the PBF NLS prevents PTTG regulation of FGF-2, suggesting that PBF may indirectly mediate the promotion of angiogenesis through subcellular distribution of PTTG (Chien & Pei, 2000).

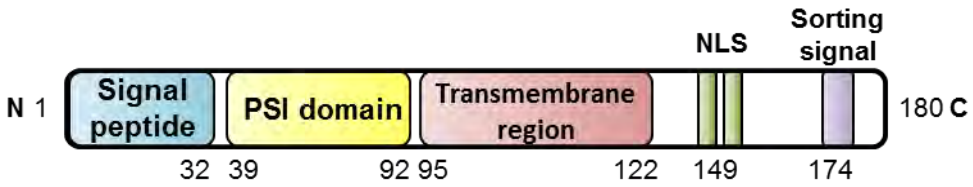
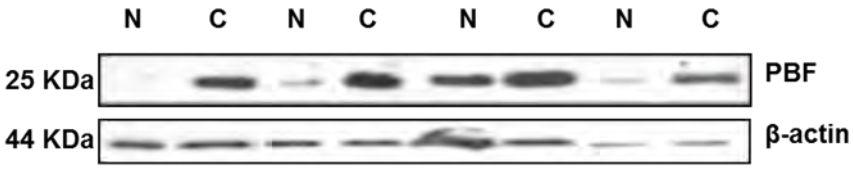
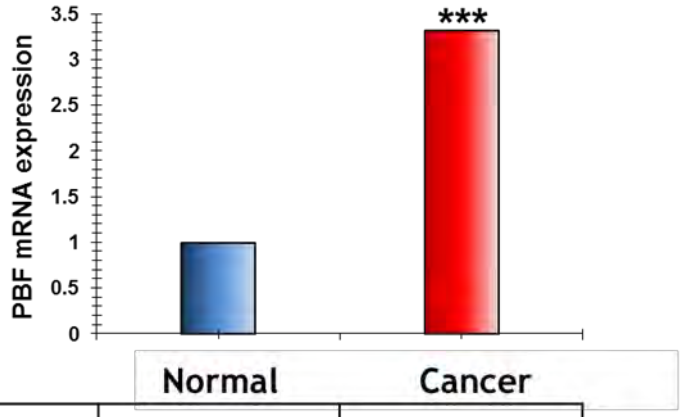


Figure 1.9: Schematic representation of the putative functional domains of the PBF protein. PBF contains an N-terminal signal peptide, a PSI domain (common to plexins, semaphorins and integrins), a bipartite nuclear localisation signal (NLS) and a C-terminal sorting signal.

1.3.1. PBF in thyroid cancer

PBF overexpression has been characterised in thyroid and pituitary tumours, as well as those of the colon and breast, suggesting an oncogenic role for PBF in tumourigenesis (Chien & Pei, 2000; Stratford *et al*, 2005; Watkins *et al*, 2010a) (Figure 1.10). Additionally, PBF overexpression in thyroid tumours was found to be independently associated with early tumour recurrence (Stratford *et al*, 2005). Whilst PBF protein expression was significantly elevated in thyroid carcinomas, no mutations were found in the coding region of PBF when it was sequenced in 24 thyroid tumours, suggesting that PBF, whilst overexpressed, does not appear to be mutated in thyroid cancer. PBF levels, however, significantly correlate with PTTG expression, where overexpression of PTTG in human primary thyrocytes and HCT116 cells significantly increased PBF protein expression (Stratford *et al*, 2005).





Number	11	27
Mean ΔCt ± SEM	12.51 ± 0.43	10.78 ± 0.21

Figure 1.10: Western blotting and Real-time PCR data demonstrating significant elevation of PBF protein and mRNA expression in thyroid papillary carcinomas compared to normal tissues. Overexpression of proteins in tumours may point to an oncogenic role in cell transformation and/or tumourigenesis (data taken from Stratford et al*, 2005. *** P<0.001).*

More recently a study designed to assess the precise contribution of PBF to cell transformation and/or tumourigenesis was performed. NIH3T3 cell lines stably over expressing PBF, as well as wild type and mutated PTTG were constructed, and colony transformation assays conducted. PBF and PTTG overexpression led to significant colony formation, although over-expression of mutant forms of PTTG which were unable to stimulate PBF mRNA expression did not. The results of this study revealed that PBF overexpression could transform NIH3T3 cells *in vitro.* Furthermore, subcutaneous injection of NIH3T3 cells over expressing PBF elicited large tumours in athymic nude mice (Stratford et al 2005). Due to the dynamic

interaction of PBF and PTTG, the precise role of PBF in tumourigenesis remained unclear from this study. However, given that PTTG was not induced in a stable cell line of PBF overexpression it is reasonable to suggest that PBF may be a transforming gene in its own right (Stratford *et al*, 2005).

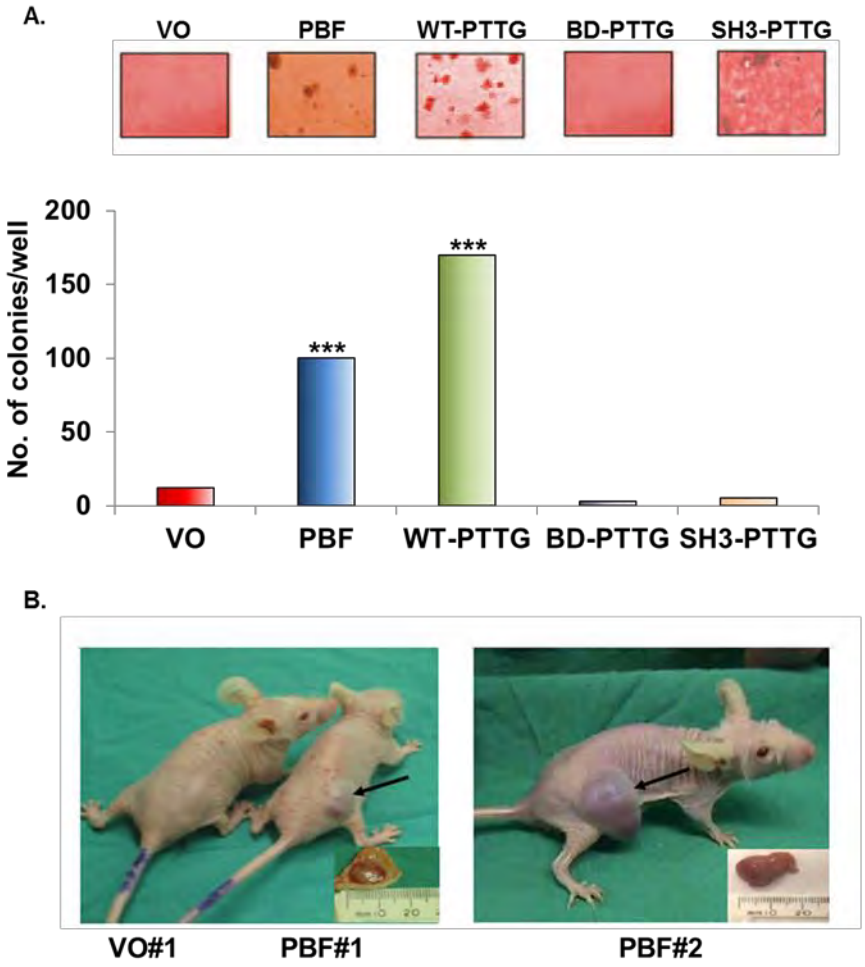


Figure 1.11: PBF is transforming in vitro and tumourigenic in vivo. ***A:*** *Images of cell colony formation highlighting in vitro cell transformation of NIH3T3 cells stably transfected with PBF, WT PTTG, PTTG with mutated binding domain (BD-PTTG) and PTTG with mutated SH3 domain (SH3-PTTG) compared to VO-transfected cells. The graph represents the mean number of colonies/well. Both PBF and PTTG were able to significantly influence colony formation, but mutants of PTTG were not.* ***B:*** *Tumour growth in nude mice injected with NIH3T3 cells overexpressing PBF compared with VO (data taken from Stratford* et al*, 2005. ***p<0001).*

1.3.1.1. *PBF and the sodium iodide symporter*

The sodium iodide symporter (NIS), under normal conditions, functions to mediate the uptake of iodide from the bloodstream and across the basolateral membrane of thyroid follicular cells, where it is utilised for thyroid hormone biosynthesis (Spitzweg *et al*, 2001). The expression of NIS, and therefore the ability to incorporate iodide, is a unique feature of thyroid follicular cells; and as such radioiodine has been central in in the treatment of thyroid tumours for the last 60 years (Shen *et al*, 2001). Whilst treatment of thyroid cancer is relatively successful, leading to particularly good prognosis, significant loco-regional recurrence is observed in up to 30 % of cases (Jonklaas *et al*, 2006; Mazzaferri, 1993). Interestingly, many thyroid tumours that express functional NIS do so at significantly diminished levels. This is due to reduced NIS expression and compromised targeting to, or retention at, the plasma membrane thereby leading to its functional inactivation. Indeed, even with the co-stimulation of the TSH receptor to increase iodide uptake, up to 20 % of differentiated thyroid cancers do not amass enough radioiodine for eradication. Thyroid cancers and their metastases which present as radioiodine refractory are associated with particularly poor prognosis; therefore, understanding the mechanisms whereby NIS regulation is impaired in thyroid tumours is an important consideration in the treatment of thyroid cancer (Kogai *et al*, 2006; Smith *et al*, 2011).

PTTG overexpression was observed to decrease iodide uptake and reduce NIS expression in human primary thyrocytes. Furthermore, high PTTG expression was strongly associated with decreased radioiodine uptake during patient follow-up (Sáez *et al*, 2006; Heaney *et al*, 2001; Smith *et al*, 2011). These data highlighted a potential role for PTTG in NIS regulation and function and led to the investigation of the effects

of PBF on this pathway. Firstly, in a series of thyroid cancers harbouring oncogenic levels of PBF and PTTG, NIS expression was significantly reduced compared to normal thyroid (Boelaert *et al*, 2007). Further study revealed that both PTTG and PBF overexpression caused significant reduction in iodide uptake, with the greatest effects observed following synergistic overexpression of both PTTG and PBF. Further interrogation of this model revealed that PTTG mediated repression of NIS was severely abrogated by administration of FGF-2 antibody, suggesting that the mechanism of PTTG repression by NIS was, at least in part, dependent upon FGF-2 secretion. Conversely, FGF-2 antibody treatment had no effect on PBF repression of iodide uptake, suggesting an independent mechanistic role for PBF in the repression of iodide uptake (Boelaert *et al*, 2007). Recently the novel mechanism of NIS regulation by PBF was elucidated. Subcellular localisation studies revealed that PBF overexpression caused the redistribution of NIS from the plasma membrane into intracellular vesicles, where it was found to colocalise with the tetraspanin CD63. Moreover, cell-surface biotinylation assays demonstrated that PBF was able to significantly able to reduce the membrane expression of NIS compared to VO-transfected controls. Critically GST-pulldown assays and co-immunoprecipitation assays confirmed a direct interaction between NIS and PBF *in vitro.* The functional consequence of this was examined using WT PBF and 3 deletion mutants. Whilst PBF significantly repressed iodide uptake, the 3 PBF mutants that showed significantly different subcellular expression, and did not localise to cytoplasmic vesicles, were unable to significantly affect NIS activity. These findings highlight a potentially important mechanism of NIS regulation by PBF under normal conditions, and highlight the possible role of PBF overexpression in resistance of a subset of

thyroid tumours that resist radioiodine ablation (Boelaert *et al*, 2007; Smith *et al*, 2009). Furthermore, these findings suggest that PBF may have a number of roles to play in normal cellular physiology and in tumourigenesis.

1.3.2. PBF outside the thyroid

When the promoter region of PBF was examined, PBF was found to contain putative oestrogen response elements (EREs). To test whether PBF expression was regulated by oestrogen, ERα positive MCF-7 cells were treated with synthetic oestrogen analogues. Treatment with such analogues resulted in significant elevation of PBF protein and mRNA expression. Furthermore oligonucleotide pulldown assays demonstrated binding of ERα to the PBF promoter. PBF was also observed to be overexpressed in breast tumours, where PBF protein expression positively correlated with ERα status. Interestingly overexpression of PBF significantly increased the invasive capacity of MCF-7 cells compared to controls, when measured using specific Matrigel invasion assays (Watkins *et al*, 2010). These data highlight a putative role for PBF in tumour progression outside the thyroid, and suggest that PBF may play a diverse role in cellular physiology, encompassing a number of different biological functions.

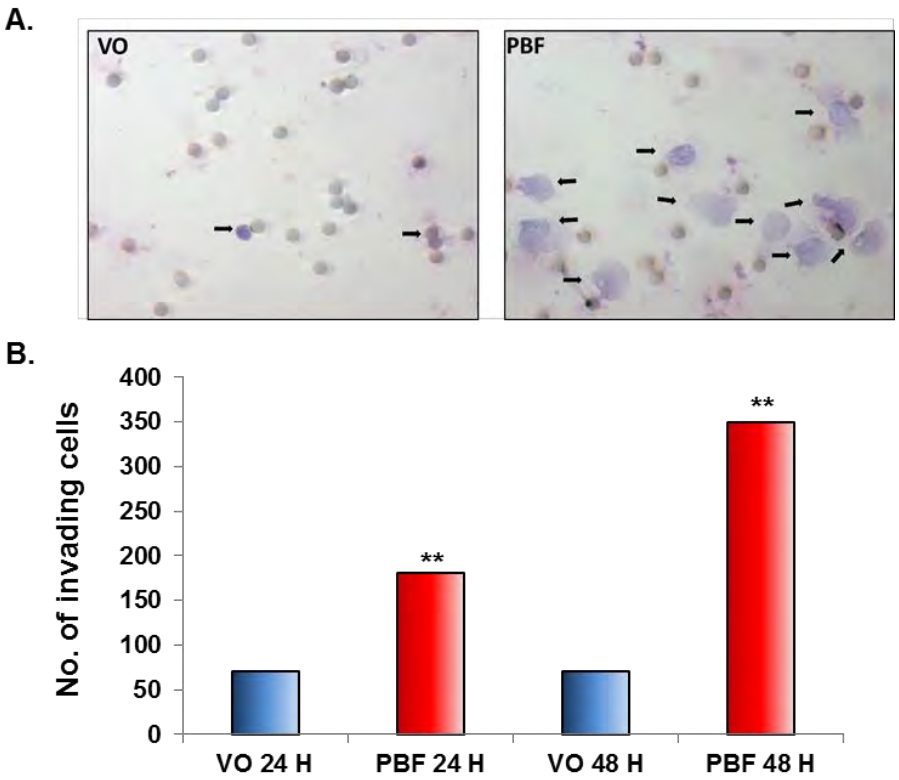


Figure 1.12: PBF overexpression induces breast cell invasion: ***A:*** *Representative images of invading cells (arrowed) from Matrigel invasion assays* ***B:*** *The mean number of invading cells were calculated at 24 and 48 hours and PBF overexpression was observed to significantly increase the invasive capacity of MCF-7 at both time-points (data taken from Watkins* et al*, 2010. **p<0.01).*

1.3.3. Preliminary data

Recent preliminary work from within our group has aimed at characterising the role and function of PBF in tumourigenesis more fully. For instance, Glutathione-S-transferase experiments were performed to assess whether PBF and p53 interact *in vivo.* Specifically, PBF or p53 protein was GST-tagged by insertion of PBF or p53 sequences into a GST-tagged bacterial expression vector and PBF or p53-GST fusion proteins were generated. Additionally, [35^{s}]-methionine PBF or p53 was generated via direct *in vitro* translation. For the detection of the potential interaction

of PBF with p53, Bacterial lysates containing the p53-GST fusion protein were incubated with beads conjugated with glutathione to allow for the immobilisation of p53 to the assay beads. Following immobilisation of p53 onto the assay beads, *in vitro* translated [35^S]-methionine PBF was incubated with the immobilised p53, and after sequential wash steps, proteins were eluted from the assay beads using detergent and subject to SDS-PAGE. [35^S]-labelled proteins were then detected using X-ray film exposure. Glutathione-S-Transferase (GST) assays showed direct binding of PBF to p53, and through additional mutational analysis, 2 specific regions of p53 necessary for PBF interaction were discovered. These regions lie between amino acid residues 1-100 and 318-393. Conversely by disrupting PBF sequences it was also discovered that the N-terminal region of PBF was essential for p53 interaction, with binding repressed by removal of 2 N-terminal regions lying between amino acids 28-93 and 93-149.

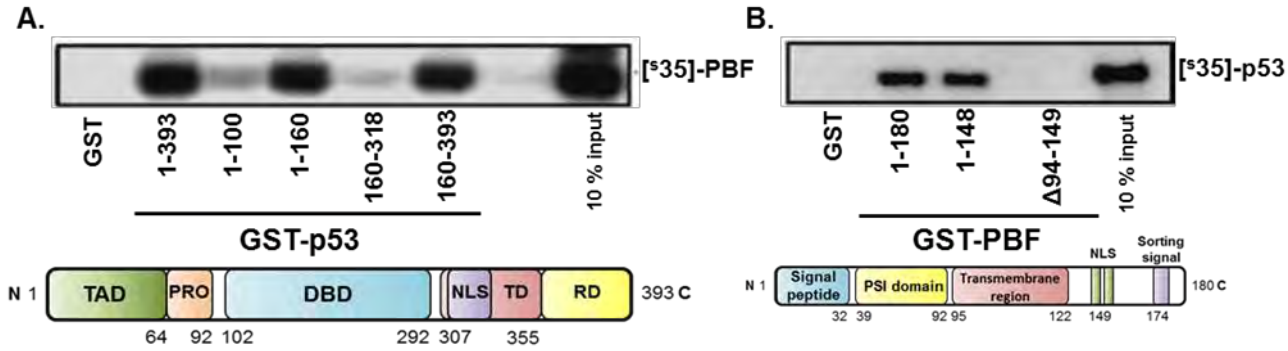


Figure 1.13: PBF binds p53. ***A:*** *Binding of [^{35}S]-PBF to GSTp53 (1-393) and GST-p53 deletion mutants versus a GST-only control.* ***B:*** *Binding of [^{35}S]-p53 to GST-tagged PBF (1-180) and GST-PBF deletion mutants versus a GST-only control (McCabe laboratory unpublished data).*

Other preliminary work within our group involved observing the effects of PBF on p53-mediated gene regulation through HDM2 promoter assays in H1299 (p53-null) cells. Transfection of p53 elicited a 30.7+/-2.6-fold stimulation of HDM2 promoter activity, whereas transfection of PBF failed to modulate promoter activity. On the other hand, co-transfection with PBF significantly repressed p53 transcriptional activity to 16.3+/-1.1-fold (Figure 1.14). Interestingly, alongside this study it was observed that PBF protein expression increased after exposure to gamma irradiation, reaching maximal levels after 24 hours in p53 positive HCT116 cells (McCabe laboratory unpublished data).

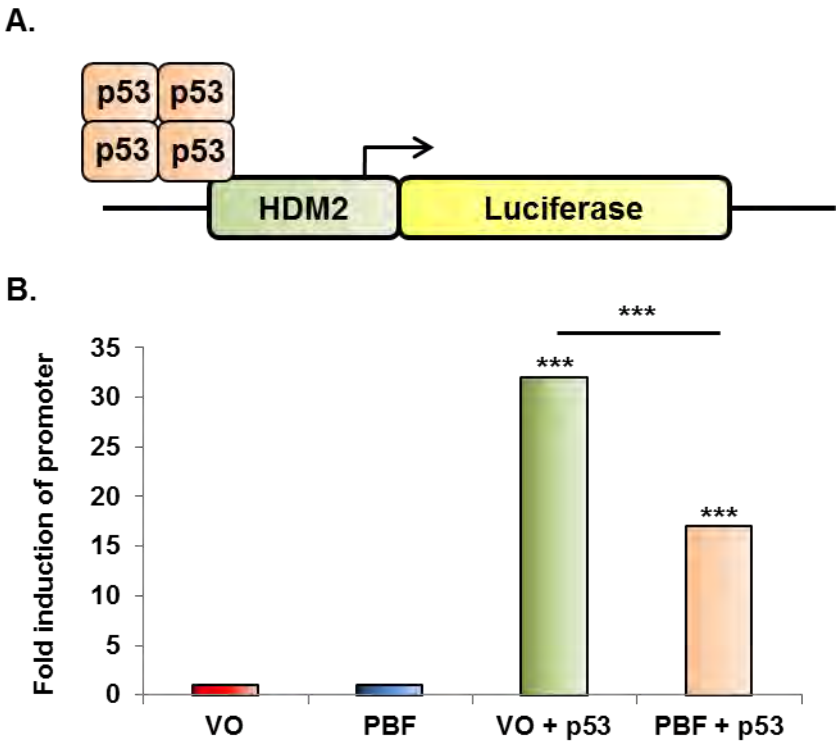


Figure 1.14: HDM2 luciferase reporter assays in p53-null H1299 cells. ***A:*** *Schematic representation of p53 binding to the HDM2 promoter and driving the transcription of the luciferase reporter gene.* ***B:*** *The effects of PBF on HDM2 promoter activity in p53-null H1299 cells. PBF was unable to influence HDM2 promoter activity in the absence of p53. Exogenous expression of p53 significantly increased HDM2 promoter activity 30.7+/-2.6-fold. However, co-expression of PBF with p53*

*significantly reduced p53-mediated HDM2 promoter activity to 16.3+/-1.1-fold (***p<0.001, McCabe laboratory unpublished data).*

The preliminary observations described above are indicative of a role for PBF in the DNA damage response through modulation of the p53 pathway. Given that PBF is overexpressed in several tumours, PBF may have an important role in tumourigenesis by binding specifically to p53 and inhibiting downstream responses involved in the maintenance of cellular and genetic stability.

The wealth of preliminary data supporting a role for PBF in tumourigenesis and more specifically within the context of p53-inhibition has prompted further study. The overall aim of my research project was therefore to characterise novel PBF modulation of p53 function in thyroid cancer.

1.4. **Hypothesis**

Thyroid cancer is often caused by point mutations or chromosomal rearrangements. For example, the BRAF V600E mutation and RET/PTC rearrangements lead to aberrant activation of the MAP kinase pathway, thereby leading to aberrant cell growth, survival and proliferation, thus promoting neoplastic growth and cell transformation (Fagin, 2005; Xing, 2007; Dhomen & Marais, 2007; Ciampi & Nikiforov, 2007). However, in contrast to the majority of human cancers, thyroid carcinomas rarely display mutations in p53; and those cancers that do display p53 mutations are almost exclusively of the poorly differentiated and aggressive histotypes (Vogelstein *et al*, 2000; Olivier *et al*, 2002; Malaguarnera *et al*, 2007a). p53 is a potent tumour suppressor, and when functional serves to inhibit neoplastic growth and cell transformation by inhibiting cell growth, promoting cell cycle arrest,

initiating apoptosis and driving cells into senescence, amongst other antitumourigenic responses (Meek, 2004; Vousden & Lane, 2007). Interestingly, through the study of the Chernobyl nuclear disaster and others, the thyroid gland has been observed to be highly sensitive to the carcinogenic effects of ionising radiation via accumulation of radioiodides, to which functional p53 would normally play an important protective role (Nikiforov *et al*, 1996; Nikiforov & Gnepp, 1994; Klugbauer *et al*, 1995).

In addition to inactivating mutations, it is clear that p53 is also extensively inactivated by viral oncogenes and oncogenic expression of the negative regulators of p53 function. Moreover, novel cellular oncogenes are emerging, that when aberrantly expressed significantly contribute to cell transformation and tumour progression via direct functional inactivation of p53 (Danovi *et al*, 2004; Mietz *et al*, 1992; Havre *et al*, 1995). These findings suggest that p53 inactivation may be present in many tumours that express wild-type p53. The functional inactivation of p53 in thyroid cancer has not been comprehensively investigated, and remains controversial. Nonetheless, novel oncogenes are emerging, including HMG factors that when overexpressed bind directly to p53 and inhibit its transcriptional function (Malaguarnera *et al*, 2007a; Frasca *et al*, 2006).

PBF is a relatively poorly characterised proto-oncogene found to be overexpressed in thyroid and other tumours (Stratford *et al*, 2005; Watkins *et al*, 2010 and our unpublished data). Oncogenic expression of PBF, as found in thyroid tumours causes transformation of cells *in vitro* and subcutaneous injection of cells stably overexpressing PBF into athymic nude mice leads to tumourigenesis *in vivo* (Stratford *et al*, 2005). Recently preliminary data indicate that PBF may interact directly with p53 and repress its transcriptional activity.

Given that p53 mutations are rare in thyroid cancer, yet the thyroid gland is particularly sensitive to the carcinogenic effects of ionising radiation, we hypothesised that p53 functional inactivation is common in thyroid cancers lacking inactivating p53 mutations. Moreover, we hypothesised that PBF overexpression (as observed in thyroid cancers) leads to the functional inactivation of p53 by directly interacting with and abrogating its tumour suppressor function in response to cell stress such as DNA Double strand breaks (DSBs). We further hypothesised that such events ultimately serve to promote cell transformation and malignant progression.

1.5. **Aims**

The aims of this work fall into three categories: firstly, we sought to investigate the potential interaction of PBF and p53 in a more physiologically relevant setting, using co-immunoprecipitation assays. We also aimed to determine the subcellular localisation of PBF and p53 in thyroid cancer cell lines. Secondly, we aimed to assess the functional consequences of the PBF:p53 interaction on p53 biology and function by assessing the effects of PBF on p53 expression and p53-mediated gene transcription in the presence and absence of ionising radiation. Thirdly, we aimed to characterise the gross biological effects of PBF overexpression on cellular physiology in the presence and absence of ionising radiation.

Chapter 2. **Materials and methods**

Unless otherwise determined, all chemicals in this thesis were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.1. **Cell lines**

Thyroid papillary carcinoma TPC-1 cells were kindly provided by Dr Rebecca Schweppe (Division of Endocrinology, Metabolism, & Diabetes, University of Colorado Denver, Aurora, Colorado), and thyroid papillary carcinoma K1 cells were obtained from the Health Protection Agency Culture Collections, UK. Both TPC-1 and K1 papillary carcinoma cells express wild-type p53 (Messina *et al*, 2012; Wyllie *et al*, 1999). All thyroid carcinoma cell lines were routinely cultured in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Invitrogen; EU approved, South American origin), penicillin (105 U/l) and streptomycin (100 mg/l) [Invitrogen]. Cells were passaged twice weekly. Human colorectal cancer HCT116 cells were obtained from the Health Protection Agency Culture Collections, UK. HCT116 cells also express wild-type p53 (Kaeser *et al*, 2004). These cell were routinely cultured in McCoy's 5A (Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Invitrogen; EU approved, South American origin), penicillin (105 U/l) and streptomycin (100 mg/l) [Invitrogen]. Cells were passaged twice weekly.

2.2. **Murine primary thyrocyte culture**

Murine primary thyrocyte culture was performed using an approach previously described by Jeker *et al,* 1999 (Jeker *et al*, 1999). Briefly, wild-type murine thyroids were aseptically dissected prior to mechanical disruption in PBS and subsequent digestion in 0.2 % collagenase for 45 minutes at 37 ºC. Collagenase was inactivated

by addition of culture medium and the cells were centrifuged for 10 minutes at 700 x *g* to obtain a pellet containing single thyroid follicles. After discarding the supernatant, the pellet was resuspended in 1 ml of culture medium and pipetted up and down until no fragments were macroscopically visible. Cells from each thyroid (2 wells for each WT thyroid) were seeded into 12-well plates in medium described by Ambesi-Impiombato *et al*. (Ambesi-Impiombato *et al*, 1980), supplemented with thyrotrophin (300 mU/l), insulin (10 mg/ml), transferrin (5 mg/ml) [Sigma], hydrocortisone (3.5 ng/ml) [Sigma], somatostatin (10 ng/ml) [Sigma], glycyl-L-histidyl-L-lysine acetate (2 ng/ml) [Sigma], penicillin (105 U/l), streptomycin (100 mg/l) and 5 % fetal calf serum. After 72 hours, serum was omitted and experiments were performed between days 7-14 of the culture.

2.3. **Human primary thyrocyte culture**

Collection of thyroid specimens was with approval of the Local Research Ethics committee and following informed written consent from patients. All tissues were surplus to pathological requirement. Human thyroid follicular cells were prepared from surgical specimens, and consisted mainly of multinodular goitres and normal thyroid tissue from the contralateral lobe, as previously described (Eggo *et al*, 1996; Ramsden *et al*, 2002). Thyroid tissue was digested by 0.2 % type II collagenase (Worthington Biochemicals). Follicles were plated in 12-well plates in medium described previously by Ambesi-Impiombato *et al*.(Ambesi-Impiombato *et al*, 1980), supplemented with thyrotrophin (300 mU/l) [Sigma], insulin (100 µg/l) [Sigma], penicillin (105 U/l), streptomycin (100 mg/l) and 5 % fetal calf serum. Serum was omitted after 72 hours of culture, and experiments were performed between days 7-11 of the culture. Generally, these cultures survive and maintain function for more

than 4 weeks and fibroblast as well as endothelial cell contamination is negligible under these conditions (Eggo *et al*, 1996).

2.4. **RNA extraction and reverse transcription**

Total RNA was extracted from cells, utilising a single step acid guanidinium phenol-chloroform extraction method [Tri-reagent (500 µl per well of a 6-well plate), Ambion, Austin, TX, USA] (Chomczynski & Sacchi, 1987). All samples were subjected to one freeze-thaw cycle at -80 °C. Subsequently, 100 µl chloroform (99+ %) was added to each reaction, and samples shaken vigorously for 15 seconds. After standing for 15 minutes at room temperature, samples were centrifuged at 12,000 g for 15 minutes at 4°C. This separates the mixture into 3 phases. The uppermost colourless aqueous phase containing RNA was transferred into a fresh tube and 250 µl isopropanol (99+ %) was added. After mixing, the samples were left to stand at room temperature for 10 minutes before centrifugation at 12,000 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet washed in 500 µl 75 % ethanol then centrifuged at 7500 g for 5 minutes at 4 °C. The ethanol was discarded and the pellet left to air dry before being dissolved in 30 µl of nuclease free water.

500 nanograms of RNA (determined by spectroscopy at λ260 nm using a NanoDrop spectrometer (NanoDrop Products, Wilmington, DE, USA)) was reverse transcribed (RT) with avian myeloblastosis virus (AMV) reverse transcriptase in a total reaction volume of 10 µl, with 5 pmol random hexamer primers, 1 µl 10 AMV reverse transcriptase buffer, 1 µl deoxynucleotide triphosphate (dNTP) mix (200 µM each), 10 units ribonuclease inhibitor (RNasin™) and 7.5 units AMV reverse transcriptase (All reagents from Promega, Madison, WI, USA).

2.5. **Qualitative polymerase chain reaction (QT- RT-PCR)**

Relative fold changes in expression of mRNAs encoding various different proteins were calculated in relation to control samples (that were arbitrarily assigned a value of 1). QT-PCR experiments were performed using an ABI 7500 Sequence Detection System, employing TaqMan™ chemistry for highly accurate measurement of mRNA levels (Wang & Brown, 1999).

TaqMan™ probes consist of a fluorophore FAM (6-carboxy-flourescien) or VIC (full chemical name patent protected) covalently attached to the 5' end, and a quencher, TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end. The quencher molecule suppresses the fluorescence emitted by the fluorophore when excited by the cycler's light source via fluorescence resonance energy transfer (FRET). During FRET the high energy fluorophore acts as a donor by transferring energy to the low energy quencher. As the Taq polymerase extends the primer, the 5' to 3' exonuclease activity of the polymerase degrades the probe. Degradation of the probe releases the fluorophore from it and breaking the close proximity to the quencher, thereby increasing its fluorescent signal by relieving the quencher effect. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and therefore the amount of DNA template present in the PCR.

Quantitative primers and probes were designed using the Primer Express™ software (PE Biosystems, USA). Primer design warranted a unique sequence for each gene, and ensured that the generated PCR product would span at least one exon-exon boundary (therefore avoiding genomic DNA amplification). Alternatively, pre-optimised specific gene expression assays for QT-PCR were purchased externally (Applied Biosystems, Warrington, UK).

PCR was carried out in 25 µl volumes on 96 well plates, in a reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK) [3 mM Mg(OAc)2, 200 µM dNTPs, 1.25 units Ampli-Taq Gold polymerase, 1.25 units AmpErase UNG], 175 nM TaqMan probe and 900 nM primers with 1 µl of the RT reaction. All target gene probes were labelled with FAM, and the housekeeping genes, 18S and DS-CAM, with VIC. All reactions were multiplexed where possible. For specific gene-expression assays that were not validated for multiplexing, reactions were singleplexed. The 18S primers and probe are provided as a pre-optimised control system (Applied Biosystems, Warrington, UK), eNAbling data to be expressed in relation to an internal reference, to allow for differences in RT efficiency. Reactions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes; then 44 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

As per the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt = Ct of the target gene minus Ct of the housekeeping gene 18S). Measurements were carried out in duplicate for each sample. The fold change of mRNA in an experimental group compared to the control group is calculated using the equation fold change = $2^{-(\Delta Ct \text{ of experimental group} - \Delta CT \text{ of control group})}$ or $2^{-\Delta\Delta Ct}$.

2.6. **Western blot analysis**

Proteins were prepared from thyroid tissues in lysis buffer (100 mmol/L sodium chloride, 0.1 % Triton X-100, and 50 mmol/L Tris, pH 8.3) containing enzyme inhibitors (1 mmol/L phenylmethylsulphonylfluoride, 0·3 µmol/L aprotinin, and 0·4 mmol/L leupeptin). Protein concentration was determined using the BCA Protein

Assay kit (Thermo-Fisher Scientific, Rockford, IL, USA) with bovine serum albumin standards. 10-60 µg of protein was denatured in 4 x laemlli buffer (Bio-Rad, Hertfordshire, UK) for 5 minutes at 95 ºC.

Western blot analyses were performed as we have described previously (Boelaert et al. 2003a; Kim et al. 2005; Smith et al. 2009). Soluble proteins (10-60 µg) were separated by electrophoresis in 12.5 % sodium dodedecyl sulphate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5 % non-fat milk in tris-buffered saline with 0.1 % Tween, followed by incubation with appropriate primary antibodies for 16 hours at 4 °C. After washing in tris-buffered saline plus 0·1 % Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. After further washes, antigen-antibody complexes were visualised by the ECL plus and ECl advance chemiluminescence detection systems (GE Healthcare, UK) on x-ray film (Kodak, UK). Actin expression was determined in all Western blot analyses (monoclonal anti-β-Actin Clone AC-15 (Sigma-Aldrich), used at 1:10 000) to assess potential differences in protein loading. Scanning densitometry studies were performed to quantify differences in protein expression (Image J Software).

2.7. **Immunofluorescence**

COS-7, HCT116, TPC-1 and K1 cells growing on coverslips in 35 mm dishes were fixed at room temperature for 20 minutes in fixing solution (for 40 mls, 20 ml 0.2 M phosphate buffer, 0.2 M Na_2HPO_4 pH7.4, 0.8 g PFA (2 %), 0.8g glucose (2 %) 8 ml 0.1% Sodium Azide (0.02%) and 12mls water). Cells were then washed in PBS and permeabilised in 100 % methanol for 10 minutes at -20 ^{0}C, blocked for 30 minutes in 10% new-born calf serum in PBS and subsequently incubated in relevant primary

antibodies (in 1 % Bovine serum albumin-PBS). Cells were then washed and incubated in secondary antibodies (Alexa-fluor-594 Goat anti-rabbit and Alexa-fluor-488 goat anti-mouse) at a concentration of 1:250 (also included was hoeschst stain at 1:1000 concentration) in 1% BSA and 1% NCS in PBS. Finally washes were then performed with PBS and coverslips were mounted onto microscope slides using fluorescence mounting medium (Invitrogen). Coverslips were sealed using clear nail varnish and cell were visualised on a confocal fluorescence microscope.

2.8. **Statistical analysis**

Statistical analysis was only performed on data sets with a minimum of 3 independent biological replicates Data were analysed using Sigma Stat (SPSS Science Software UK Ltd). The Kolmogorov-Smirnov test determined whether the data followed normal distribution. If the data set to be tested followed normal distribution, it was analysed using a two sample Student's t-test. However, if the data set was non-parametric, the Mann-Whitney Rank Sum test was used for comparison between two groups of data. Furthermore samples with multiple variances were assessed via analysis of variance (ANOVA).Significance was taken as $p< 0.05$. For quantitative real time PCR, all statistics were performed on the ΔCt values to avoid potential bias through transformation of the data with the equation $2^{-\Delta\Delta Ct}$.

Chapter 3. **The p53 response to ionising radiation in thyroid papillary carcinoma cells**

3.1. **Introduction**

Induction of DNA damage in TPC-1 and K1 thyroid papillary carcinoma cells and resulting p53 activity has not been well documented. Of the various cellular stresses that initiate a p53 response, the molecular mechanisms by which p53 is activated following DNA double strand breaks (DSBs) are perhaps the most comprehensively understood (Meek, 2004; Brooks & Gu, 2006). Activation of p53 by DNA damage occurs at 2 levels: stabilisation of the p53 protein leading to its accumulation, and activation of biochemical functions encompassed within the p53 protein (Meek, 2004). DSBs are considered the most cytotoxic form of DNA lesion; and they can be introduced by a number of exogenous agents including γ-irradiation, topoisomerase poisons, radiomimetic drugs (e.g bleomycin and neocarcinostatin), and by various cellular processes such as V(D)J recombination and stalled replication forks (Mahaney *et al*, 2009). As described earlier, p53 is stabilised in response to DNA damage through inhibition of its degradation by HDM2, mediated by multi-site phosphorylation of both p53 and HDM2. The initial and fundamental post-translational modification of p53 in response to DNA damage is phosphorylation of ser-15 by the ATM protein kinase. This initial event propagates a host of secondary modifications, resulting in further p53 stability and biochemical activation (Ashcroft & Vousden, 1999). Activated p53 acts as a node for the transduction of stress signals (i.e. DSBs). Predominantly p53 functions as a transcription factor to bring about cellular responses with the primary biological end-points of growth arrest or apoptosis. p53 is likely to directly regulate a plethora of different gene activity, although a sub-set of genes have been directly implicated with p53 regulation. Such genes include CDKN1A and HDM2 (involved in growth arrest and p53 regulation

respectively), and BAX and PUMA (both key mediators of apoptosis). As mentioned previously CDKN1A encodes the protein p21, which is a potent cyclin-dependent kinase inhibitor, transcribed by p53 in response to DNA damage to initiate a G1/S phase growth arrest (Waldman *et al*, 1995). HDM2, the human homologue of the mouse double minute 2 (HDM2), encodes a protein of the same name which contains a self and p53-specific ubiquitin ligase, binding directly to p53 and targeting it for degradation (Brooks & Gu, 2006). BAX (or BCL-2 associated X protein) shares significant amino acid homology to the anti-apoptotic BCl-2 protein. BCL-2 can form homodimers or heterodimers, where homodimeric BCL-2 results in anti-apoptotic conditions; however, when the frequency of BCL-2-BAX heterodimers increases, programmed cell death is accelerated and the anti-apoptotic effects of BCL-2 are countered (Oltval *et al*, 1993; Basu & Haldar, 1998; Wolter *et al*, 1997). Furthermore, the expression of *BAX* is upregulated by the tumour suppressor protein p53, and BAX has been shown to be involved in p53-mediated apoptosis (Basu & Haldar, 1998). The p53 upregulated mediator of apoptosis (PUMA), also known as the BCL-2 binding component 3 (BBC3), is another pro-apoptotic member of the BCL-2 protein family (Jeffers *et al*, 2003; Nakano & Vousden, 2001). As the name implies, PUMA is directly induced by p53 at the level of transcription (Jeffers *et al*, 2003).

In order to determine the effects of PBF on p53 function, it is necessary to exogenously induce DNA damage to activate the p53 pathway and bring about reliable and repeatable p53 responses against which the effects of PBF can be measured. It has been well documented that the thyroid is particularly sensitive to DNA damage, for example exposure of the developing thyroid to ionising radiation due to human disasters (Hiroshima and Chernobyl) and exposure to ionising

radiation as a result of medical care, lead to an increased incidence of thyroid papillary carcinomas (Thompson *et al*, 1994; Nikiforov *et al*, 1996; Sadetzki *et al*, 2006). With this in mind, treatment of human papillary carcinoma cell lines with ionising γ-irradiation may be a valid and robust mechanism for inducing DNA damage and propagating a detectable p53 response.

One of the first cellular responses to the introduction of DSBs is phosphorylation of H2AX at ser-139 to generate γ-H2AX, one of three types of histone H2A molecules in eukaryotic cells (Rogakou *et al*, 1998; Kuo & Yang, 2008), an event mediated by the PI3 kinase-like family of protein kinases, including DNA PKs, ATM and ATR. Interestingly, formation of DSBs can initiate this response, but other lesions, including SSBs do not (Paull *et al*, 2000). After treatment with DNA damaging agents, γ-H2AX forms foci that localise to subnuclear volumes containing damaged DNA. γ-H2AX foci formation precedes the formation of the Rad51-MRE11-Nbs1 complex, but appears to serve as an important "landing pad" for the recruitment and retention of these central components of DNA-repair. The importance of recruitment of γ-H2AX to sites of DNA breaks is highlighted following treatment of cells with Wortmannin to inhibit the phosphorylation of H2AX. In the absence of γ-H2AX, localisation of the central DNA repair machinery (RAD51-MRE11-Nbs1) is significantly impaired, suggesting a causal link between γ-H2AX accumulation and subsequent colocalisation of DNA repair machinery (Paull *et al*, 2000; Kuo & Yang, 2008; Kinner *et al*, 2008).

PBF has been reported to be overexpressed but not mutated in tumours of the thyroid, breast pituitary and colon (Stratford *et al*, 2005; Watkins *et al*, 2010 and our unpublished data). Furthermore at oncogenic levels, PBF has been characterised as transforming *in vitro* and tumourigenic *in vivo* (Stratford *et al*, 2005). Despite these

findings PBF remains a relatively uncharacterised protein associated with multiple cellular processes, such as nuclear entry of PTTG, FGF-2 upregulation and NIS mRNA expression (Stratford *et al*, 2005; Watkins *et al*, 2010b). Although the role of PBF in oncogenesis has not been well documented, recent preliminary data from our laboroatory suggests potential interaction between PBF and p53 *in vitro* through the use of GST-Pulldown assays, potentially highlighting a role for PBF in p53 activity. Therefore, observing the effects of DNA damage on PBF expression and determining whether PBF localises to sites of DNA breaks may provide useful insights into any potential role of PBF in the DNA damage response, which may in turn influence p53 functionality.

The aims of this study were therefore to explore the effects of ionising radiation on the induction of p53 and associated down-stream responses in thyroid papillary carcinoma cell lines, and establish an optimal dose response and time point at which p53 activity appears greatest (without inducing excessive cytotoxicity), from which PBF function can be measured. Furthermore, the effects of ionising radiation on PBF expression and localisation following ionising radiation are unknown; therefore we aimed to observe how PBF protein expression changes following treatment with ionising radiation, and to determine whether PBF localises to sites of DNA damage by observing its relationship with the important DNA repair protein γ-H2AX.

3.2. **Materials and methods**

3.2.1. Cell lines and plasmids

Human thyroid papillary carcinoma TPC-1 and K1 cells, and human colorectal carcinoma HCT116 cell lines, were routinely cultured as described in Section 2.1 . For experiments involving ionising radiation treatment, TPC-1 and K1 cells were seeded into 6-well dishes at a density of $1X10^5$/well, and HCT116 cells at $2.5x10^5$/well, and incubated at 37 ^oC for 24 hours before treatment. A Caesium-137 γ-emitter was used to treat cells with specific doses of γ-radiation. At our time of research the emitter delivered 38.5 mGy of γ-irradiation per second of exposure. Cells were treated with the required dose and returned to incubation until harvesting at the relevant time point. For experiments requiring transient overexpression of PBF, TPC-1 and K1 cells were seeded into 6-well dishes at a density of $1X10^5$/well and incubated at 37 ^oC for 24 hrs. After incubation human thyroid papillary carcinoma cells were transfected using FUGENE6™ (Roche Diagnostics Corporation, Indianapolis, USA), as per the manufacturer's instructions. However an optimised ratio of 3 µl FUGENE6: 1 µg plasmid DNA was used (6 µl FUGENE6: 2 µg DNA in a 6-well dish). Cells were transfected with the mammalian expression vector pCDNA3™ (Invotrogen™) with full length wild-type PBF cDNA with a haemaglutinin (HA) tag insert. Cells were then incubated for 24 hours to allow for exogenous PBF protein overexpression before being treated or harvested.

3.2.2. Murine primary thyrocyte culture

Murine primary thyrocytes were harvested and cultured using methods described in Section 2.2. After a minimum of 7 days in culture, thyrocytes were irradiated using a

Caesium-137 γ-emitter and returned to incubation before harvesting at the relevant time-point.

3.2.3. SDS-PAGE/ Western blotting

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see Section 2.6). Blocked membranes were subsequently incubated with primary antibodies. The antibodies used were, our rabbit anti-human PBF-8 polyclonal (1:1000). Our polyclonal antibody to PBF was generated using a peptide corresponding to amino acid sequence 44–57 (TNKTCEECLKNVXC). The antibody was purified using HPLC, and its subsequent specificity was validated through peptide blocking studies (Stratford *et al*, 2005), rabbit anti-human phosphor-histone (ser 139) H2AX at 1:1000 (NEWENGLAND Biolabs, MA, USA), mouse anti-human p53 (D0-1) monoclonal at 1:5000 (Santa Cruz, CA, USA), monoclonal anti-β-actin clone AC-15, (used at 1:10,000, Sigma-Aldrich, Poole, UK). Scanning densitometric analysis of Western blotting assessment of PBF and p53 expression was normalised to β-actin.

3.2.4. RNA extraction and Quantitative Real-time PCR

Total RNA was extracted from TPC-1, K1, HCT116 cells and murine primary thyrocytes as described in Section 2.4. Reverse transcription and QT-PCR techniques were as described above (see Sections 2.4 and 2.5). Gene-specific expression assays for CDKN1A, HDM2, PUMA and BAX were purchased from Applied Biosystems (Warrington, UK). Primers and probes for PBF mRNA expression were designed according to the guide-lines mentioned in Section 2.5 and the exact sequences are presented in Table 3-1.

Sequence name:	Sequence:
PBF/PTTG1IP forward primer	CTCTTCTCAGTTTGTGAAACGCTAA
PBF/PTTG1IP reverse primer	CTGCCCTGGGAGAATGACA
PBF/PTTG1IP probe	AAGCCGCCTGACGGCACCCAGC

Table 3-1: Oligonucleotide sequences of PCR primers and TaqMan™ probes used. Each probe possessed a 5' FAM reporter molecule and a 3' TAMRA quencher molecule. All TaqMan primers run at 59°C and yield amplicons of 70-150bp.

3.2.5. Immunofluorescence

COS-7, HCT116, TPC-1 and K1 cells were seeded onto coverslips (in 6-well dishes), irradiated using a Caesium-137 γ-emitter and prepared for fluorescence immunocytochemistry using the methods described in Section 2.7. PBF was detected using our rabbit anti-human PBF antibody (1:100) (Read *et al*, 2011), and γ-H2AX detected using a mouse anti-human γ-H2AX phosphor ser-139 specific antibody (Pierce, UK). Cells were then visualised on a fluorescence confocal microscope.

3.2.6. Statistical analysis

Data were analysed as described in Section 2.8.

3.3. **Results**

3.3.1. Treatment with γ-irradiation: establishing an optimal dose.

To assess the p53 response to DNA damage in TPC-1 and K1 papillary carcinoma cell lines, cells were treated with a range (0-40 Gy) of doses of γ-irradiation and total cellular protein was harvested 8 hours post treatment (Figure 3.1). A significant

increase in p53 protein expression was observed in TPC-1 cells at all doses of γ-irradiation, with maximal induction of p53 protein expression observed between doses of 5 and 15 Gy (Figure 3.1A). At the highest dose of 40 Gy, p53 protein expression was elevated compared to untreated controls, but to levels below that of the lowest treatment dose. This dose correlated with significant levels of cytotoxicity that were not present at lower doses of γ-irradiation (data not shown). Similar results were obtained following treatment of K1 cells with ionising radiation (Figure 3.1B).

To determine whether these effects were thyroid specific, experiments were repeated using the human colorectal cancer (HCT116) cell line over a dose range of 0-40 Gy. In these cells, again, a large and significant increase in p53 protein expression was observed throughout all doses of γ-irradiation. However, unlike thyroid papillary carcinoma cells, HCT116 cells displayed maximal p53 protein expression at the highest dose of 40 Gy (Figure 3.1C).

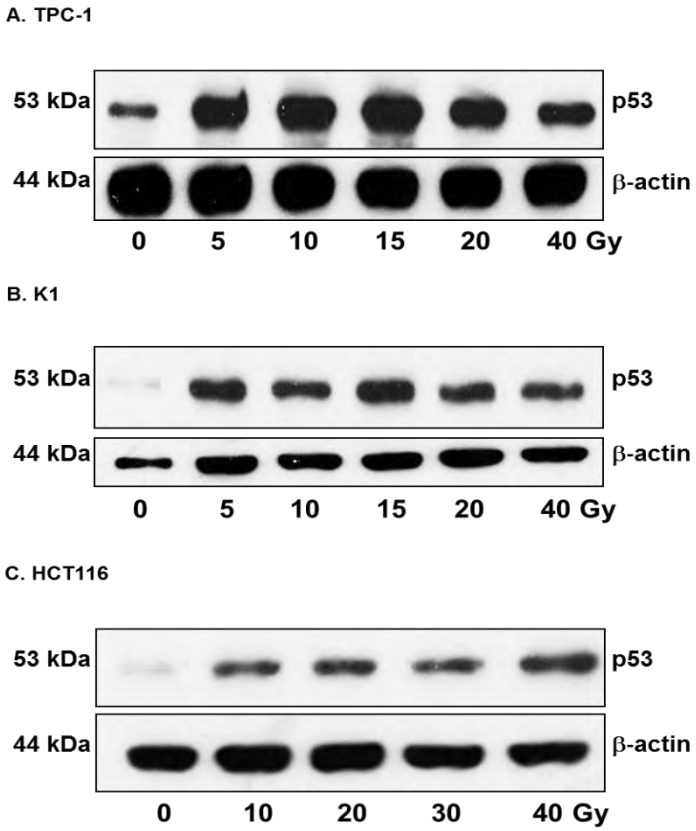
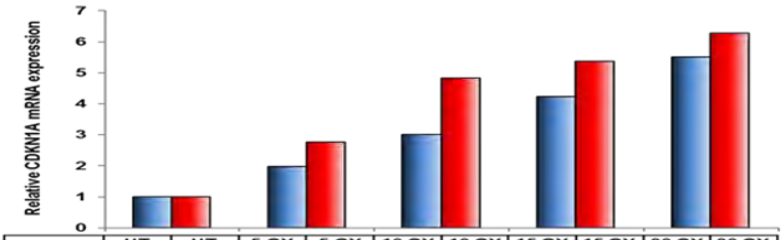


Figure 3.1. Induction of p53 protein expression 8 hours after treatment with a range of doses of γ-irradiation in TPC-1, K1 and HCT116 cell lines. ***A-C****: Western blot analysis of p53 protein expression between doses of 0-40 Gy γ-irradiation in TPC-1 and K1 and HCT116 cells respectively.*

In order to determine whether the increased p53 expression observed through treatment of different doses of γ-irradiation correlated with increased p53 activity, the above dose response experiments were repeated, and cellular RNA harvested 8 and 24 hours after treatment. QT-PCR was then performed to assess the induction of CDKN1A mRNA expression in these cell lines. CDKN1A is a gene transcribed directly by p53 in response to DNA damage, giving rise to the protein p21, which functions as a potent cyclin-dependent kinase inhibitor, and results in G1-S-phase growth arrest (Vousden & Lu, 2002).

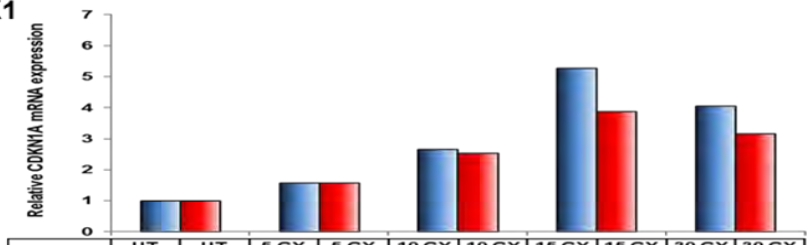
CDKN1A mRNA expression in TPC-1 cells was increased in a dose dependent manner both 8 and 24 hours after treatment, with maximal responses occurring after 24 hours (Figure 3.2A). CDKN1A mRNA expression was highest at 30 Gy where expression was increased by 5.5-fold (after 8 hours) and 6.3-fold (after 24 hours) compared to time matched untreated controls. At the 15 Gy dose CDKN1A mRNA expression was increased by 4.24-fold and 5.36-fold at the 8 and 24 hour time point respectively (Figure 3.2A). K1 cells displayed a somewhat different pattern of CDKN1A mRNA expression in response to γ-irradiation, where CDKN1A mRNA expression initially increased in a dose dependent manner. However, in this cell line, the strongest induction of CDKN1A mRNA expression was observed at the 15 Gy dose (5.3-fold at 8 hours Figure 3.2B). At the highest dose of 30 Gy CDKN1A was induced by only 4.05-fold at 8 hours. Furthermore CDKN1A expression was most apparent 8 hours after treatment with γ-irradiation (compared to TPC-1 cells which showed maximum responses at 24 hours). For example, cells treated with a 15 Gy dose of γ-irradiation were induced by 5.3-fold compared to untreated controls after 8 hours, in contrast to a 3.8-fold response after 24 hours (see Figure 3.2B). Additionally, the broad effects of γ-irradiation on the induction of CDKN1A mRNA expression in non-thyroidal HCT116 cells were determined; once more, in these cells transcription of CDKN1A mRNA was dose dependent. At the highest dose of 40 Gy, HCT116 cells elicited the largest response of 7.01-fold after 24 hours (Figure 3.2C).

A. TPC-1



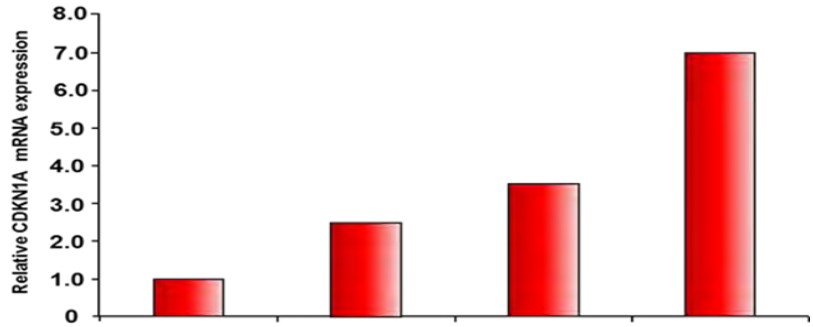
SAMPLE	UT 8 H	UT 24 H	5 GY 8H	5 GY 24 H	10 GY 8H	10 GY 24 H	15 GY 8H	15 GY 24 H	30 GY 8 H	30 GY 24 H
ΔCt ± SE	10.86 +/ 0.12	11.018 +/- 0.024	9.88 +/- 0.17	10.23 +/- 0.015	9.27 +/- 0.03	8.74 +/- 0.0081	8.78 +/- 0.49	8.59 +/- 0.21	8.40 +/- 0.012	8.36 +/- 0.11

B. K1



SAMPLE	UT 8 H	UT 24 H	5 GY 8H	5 GY 24 H	10 GY 8H	10 GY 24 H	15 GY 8H	15 GY 24 H	30 GY 8 H	30 GY 24 H
ΔCt ± SE	12.22 +/- 0.022	13.43 +/- 0.36	11.57 +/- 0.042	12.78 +/- 0.62	10.82 +/- 0.35	12.10 +/- 0.9	9.82 +/- 0.013	11.44 +/- 0.19	10.20 +/- 0.177	11.78 +/- 0.54

C. HCT116



Sample	UT	5 GY	10 GY	40 GY
ΔCt ± SE	13.66 +/- 0.15	12.35 +/- 0.12	11.59 +/- 0.13	10.86 +/- 0.83

D.

K1	UT 8 H	5 GY 8 H	10 GY 8 H	15 GY 8 H	30 GY 8 H
Ct 18s	9.3	9.02	9.4	9.07	9.08
Ct p21	21.5	20.3	20.2	19.8	19.7

HCT	UT 24 H	5 GY 24 H	10 GY 24 H	40 GY 24 H
Ct 18s	12.2	11.9	12.4	12.2
CT p21	25.8	24.4	24	23.05

Figure 3.2: Induction of CDKN1A mRNA expression over a range of doses of γ-irradiation 8 and 24 hours after treatment. ***A+B:*** *Induction of CDKN1A mRNA expression in TPC-1 and K1 cells over a dose range of 0-30 Gy.* ***C:*** *Induction of CDKN1A mRNA expression over a dose range of 0-40 Gy in HCT116 cells (24 hour time-point only).* ***D:*** *Exemplar of raw Ct data in K1 and HCT116 cells demonstrating that ionising radiation treatment increases relative CDKN1A mRNA expression as a function of decreasing CDKN1A Ct threshold values as opposed to increasing 18s control Ct threshold values.*

This series of preliminary experiments demonstrated that both thyroid papillary and human colorectal carcinoma cell lines are responsive to γ-irradiation where p53 is stabilised in a dose dependent manner. γ-irradiation resulted in the greatest responses in the thyroid carcinoma cell lines at 15 Gy (where higher doses resulted in visible cytotoxicity) and in the colorectal cancer cell line at 40 Gy. When p53 activity was assessed (by observing the expression of a p53 inducible gene) in response to γ-irradiation, p53 activity was increased in a dose dependent manner in the 3 cell lines. In TPC-1 cells the greatest levels of p53 protein expression were observed at 15 Gy; in contrast the greatest level of p53-mediated gene induction was observed at 30 Gy. However, due to the observed cytotoxicity of the larger 30 Gy dose in these cells, and the significant induction of CDKN1A mRNA expression at the 15 Gy dose, a dose of 15 Gy was chosen for subsequent experiments in this thesis. K1 cells displayed the most significant induction of p53 protein expression and p53-mediated gene expression after 15 Gy of γ-irradiation, and so the 15 Gy dose was deemed optimal for these cells. Finally, in HCT116 cells, p53 protein expression and p53-mediated gene activation occurred at the highest treatment dose of 40 Gy and so in this radio-resistant cell line, a 40 Gy dose was used to induce DNA damage during experiments within this thesis.

3.3.2. Treatment with γ-irradiation: establishing an optimal time-point

With the aim of finding the time-point at which our chosen dose-response was most apparent, TPC and K1 cells were treated with 15 Gy of γ-irradiation and harvested over a 24 hour time-course at 2, 8 and 24 hours. In a similar manner to experiments described in Section 3.3.1 protein was extracted from cells to determine the levels of p53 protein expression and RNA isolated in order to monitor p53-mediated gene

transcription. TPC-1 cells treated with 15 Gy of γ-irradiation displayed increased p53 protein expression from 2 hours post irradiation; levels increased at 4 hours and were greatest after 8 hours (Figure 3.3A). After 24 hours, irradiation induced p53 protein expression was apparent, but at levels considerably below that of the 8 hour time point (Figure 3.3A). Irradiation induced p53 protein expression was observed from 4 hours in K1 cells and remained similarly elevated to at 8 hours; again, after 24 hours, p53 protein expression was only marginally increased compared to negative controls (Figure 3.3B). Finally, HCT116 cells were treated at the chosen higher dose of 40 Gy and harvested over the same time-course. p53 protein expression was induced after 8 hours. p53 protein expression remained elevated to similar levels even 24 hours after irradiation (Figure 3.3C).

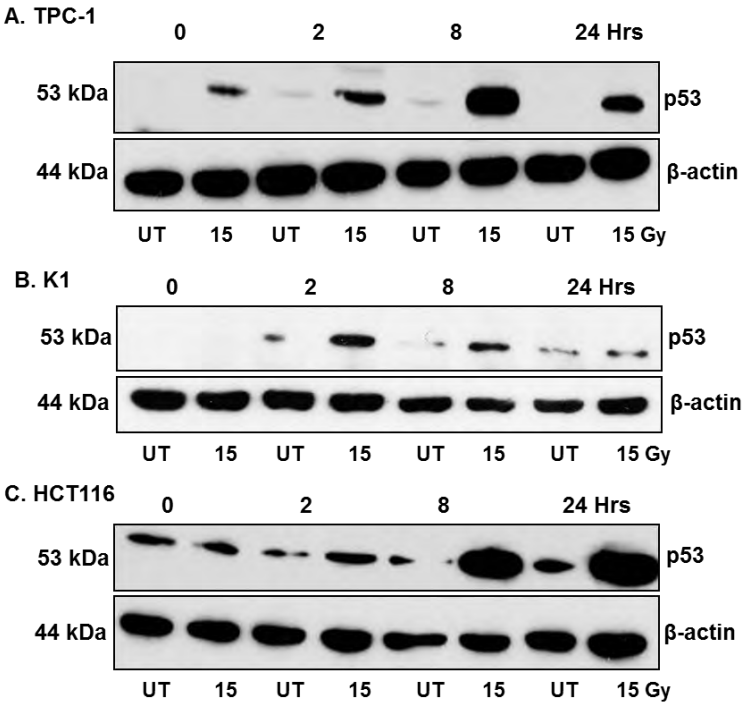
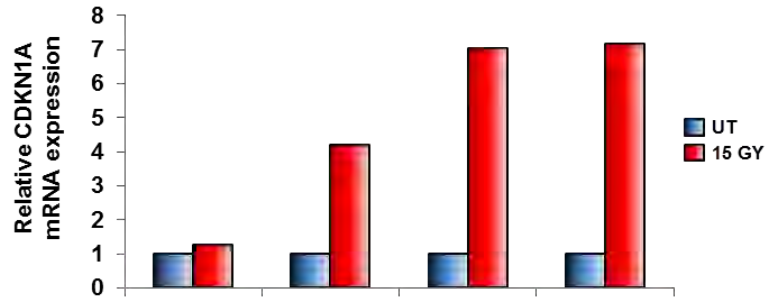


Figure 3.3: Time-course demonstrating induction of p53 protein expression in TPC-1, K1 and HCT116 cell lines following treatment with γ-irradiation 0, 2, 8 and 24 hours after treatment. ***A+B****: p53 expression levels after treatment with 15 Gy of γ-irradiation in K1 and TPC-1 cells. C. Time-course displaying p53 expression levels after treatment with 40 Gy of γ-irradiation in HCT116 cells.*

TPC-1 and K1 cells were treated with 15 Gy of γ-irradiation and harvested across a 24 hour time course as previously described. QT-PCR was then performed to assess the relative induction of CDKN1A expression, and hence p53 activity (Figure 3.4). TPC-1 cells demonstrated a robust response to irradiation from 2 hours (4.2-fold) compared to time-matched untreated controls. This response increased to 7-fold after 8 hours and remained elevated at similar levels after 24 hours (7.2-fold) (Figure 3.4A). K1 cells showed induction of CDKN1A mRNA expression from 2 hours (3.4-fold) which was slightly lower than that of TPC-1 cells at this time-point. After 8 hours the greatest induction of CDKN1A mRNA expression was observed (4.1-fold) in contrast to the 7.18-fold induction detected in TPC-1 cells at this time-point. After 24

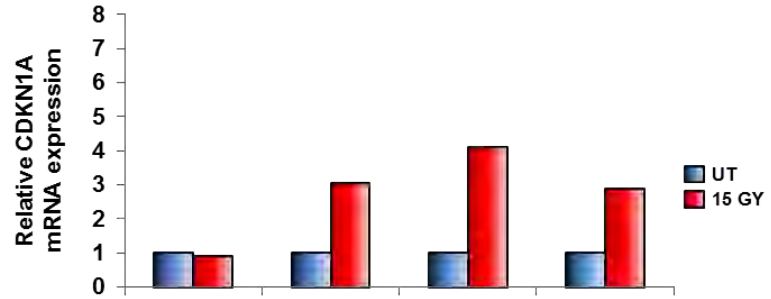
hours CDKN1A expression was reduced to 2.9-fold suggesting a shorter length of p53 activity in these cells treated with a 15 Gy dose of γ-irradiation (Figure 3.4B). CDKN1A mRNA expression was also observed in HCT116 cells 8 and 24 hours after the higher treatment dose of 40 Gy. After 8 hours CDKN1A mRNA expression was elevated to 6-fold compared to untreated controls, and mRNA expression continued to increase at the 24 hour time point to 7.1-fold that of untreated controls (Figure 3.4C).

A. TPC-1



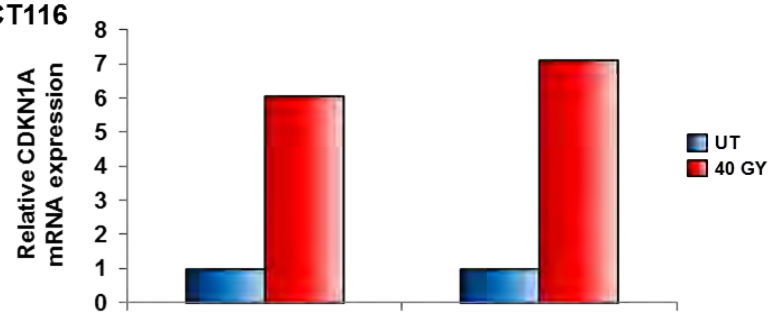
Sample	UT 0 H	15 GY 0 H	UT 2 H	15 GY 2 H	UT 8 H	15 GY 8 H	UT 24 H	15 GY 24 H
ΔCt ± SE	11.40 +/- 0.097	11.071 +/- 0.10	11.52 +/- 0.256	9.44 +/- 0.085	10.82 +/- 0.13	8.01 +/- 0.11	11.85 +/- 0.19	9.56 +/- 0.46

B. K1



Sample	UT 0 H	15 GY 0 H	UT 2 H	15 GY 2 H	UT 8 H	15 GY 8 H	UT 24 H	15 GY 24 H
ΔCt ± SE	10.93 +/- 0.21	11.056 +/- 0.61	10.83 +/- 0.013	9.23 +/- 0.37	10.99 +/- 0.18	8.95 +/- 0.41	10.24 +/- 0.21	8.71 +/- 0.07

C. HCT116



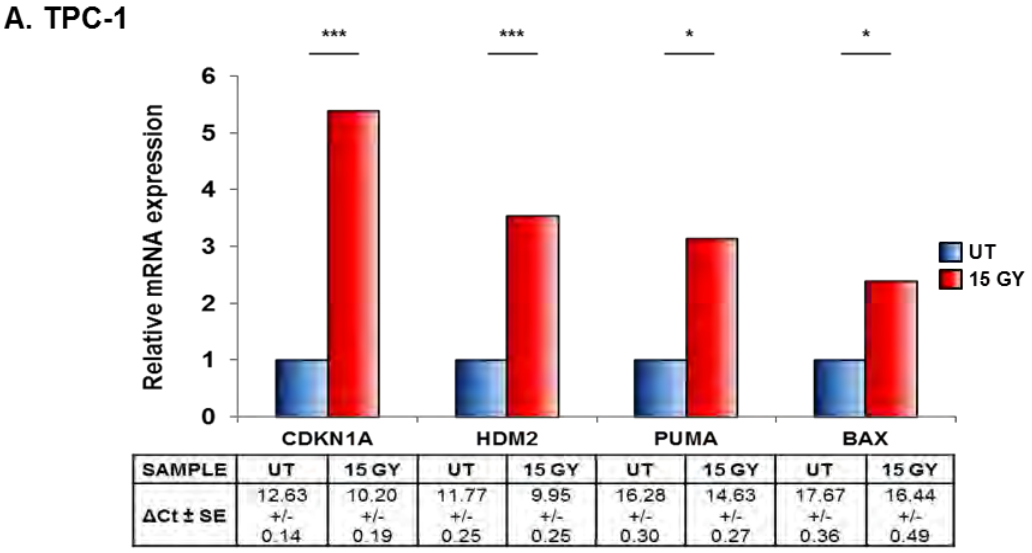
SAMPLE	UT 8 H	40 GY 8 H	UT 24 H	40 GY 24 H
ΔCt ± SE	11.99 +/- 0.26	9.40 +/- 0.45	13.004 +/- 0.037	10.17 +/- 0.60

Figure 3.4: Time-course demonstrating the relative induction of CDKN1A mRNA expression in response to treatment with γ-irradiation in TPC-1, K1 and HCT116 cells respectively. ***A+B:*** *Fold induction of CDKN1A mRNA expression in TPC-1 and K1 cells after treatment with 15 Gy γ-irradiation over a time-course of 0, 2, 8 and 24 hours, compared to time-matched untreated controls.* ***C:*** *Fold induction of CDKN1A mRNA expression in HCT116 cells tretated with 40 Gy γ-irradiation 8 and 24 hours after treatment.*

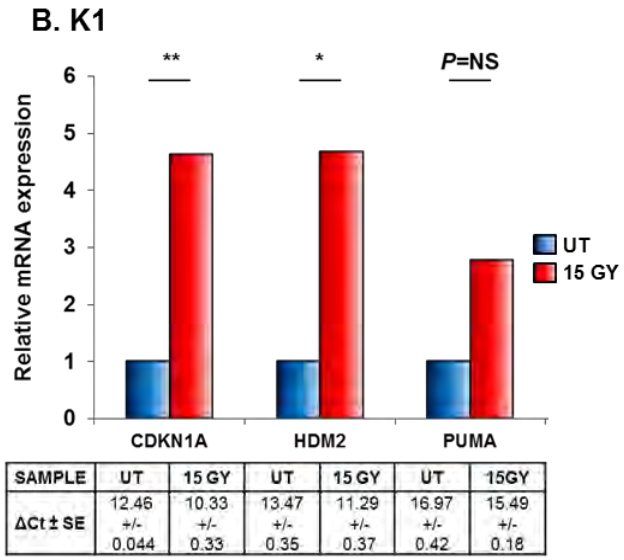
An optimal dose to treat the 2 thyroid papillary carcinoma cells (15 Gy) and the HCT116 colorectal cell line (40 Gy) were established to induce a robust p53 response. In addition to this, time-courses established optimal time points following treatments to observe maximal p53 responses in each cell line. In TPC-1 cell lines, maximal p53 protein expression was observed 8 hours after a 15 Gy dose of γ-irradiation, coupled with the greatest (~7-fold) induction of p53-mediated gene expression at both 8 and 24 hours. K1 cells showed the most significant induction of p53 protein expression 4 and 8 hours after treatment with γ-irradiation and maximal p53-mediated gene expression at 8 hours. HCT116 cells, at the higher dose of 40 Gy, displayed the highest levels of p53 protein expression at 8 and 24 hours, and p53-mediated gene expression was substantially elevated at both 8 and 24 hours. From these data treatment of the K1 and TPC-1 cell lines with a 15 Gy dose of γ-irradiation and harvesting cellular protein and RNA after 8 hours resulted in significant induction of both p53 protein expression and p53-mediated gene expression which could then be used to measure the effects of PBF on the induction of this pathway. In HCT116 cells treatment with the higher dose of 40 Gy provided significant induction of p53 protein expression and activity. Therefore assessing p53 expression and activity at this dose and time-points (both 8 and 24 hours) resulted in optimal conditions for analysis of the effects of PBF on the induction of the p53 response to cellular stress.

Further to these preliminary experiments, the validity of the chosen doses and time-points was assessed. QT-PCR was performed to determine the induction of a panel of p53-mediated genes, including CDKN1A. In TPC-1 cells the induction of CDKN1A, HDM2, BAX and PUMA mRNA expression were analysed 8 hours after treatment

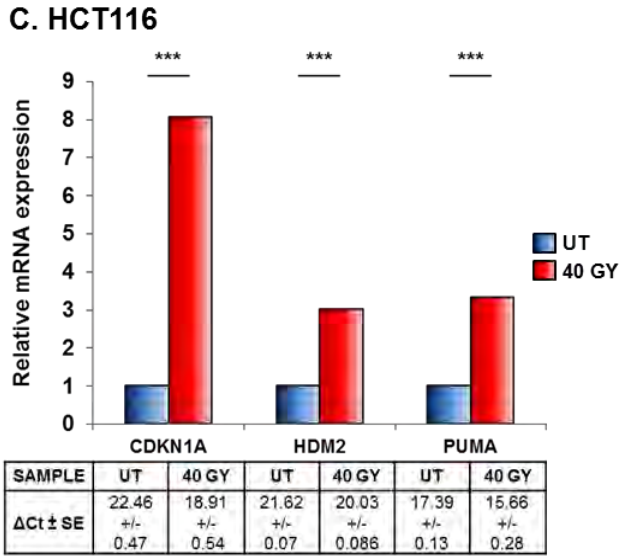
with 15 Gy of γ-irradiation. As expected TPC-1 cells treated with 15 Gy γ-irradiation produced significant repeatable induction of CDKN1A mRNA expression (5.4-fold, n=4, p<0.001; Figure 3.5A). HDM2 mRNA expression was also significantly up-regulated under these conditions (3.5-fold, n=4, p<0.001). The pro-apoptotic genes BAX and PUMA were also activated (3.1-fold, n=3, p<0.05 and 2.4-fold, n=3, p<0.05 respectively) compared to untreated controls. CDKN1A, HDM2 and PUMA mRNA expression were analysed in K1 cells in a similar manner to TPC-1 cells (Figure 3.5B). CDKN1A expression was highly elevated in response to DNA damage (4.6-fold, n=3, p<0.01) and HDM2 mRNA expression significantly induced to similar levels (4.7-fold, n=3, p<0.05) compared to untreated controls. However, unlike TPC-1 cells, PUMA mRNA expression in K1 cells was non-significantly up-regulated in response to DNA damage (2.8-fold, n=3, p=NS). HCT116 cells treated with the higher dose of 40 Gy displayed a striking increase in CDKN1A mRNA expression after 8 hours (8-fold, n=4, p<0.001; Figure 3.5C). HDM2 and PUMA were also significantly induced under these conditions but at lower levels (3-fold, n=3, p<0.001 and 3.3-fold, n=3, p<0.001 respectively).



SAMPLE	UT	15 GY	UT	15 GY	UT	15 GY	UT	15 GY
ΔCt ± SE	12.63 +/- 0.14	10.20 +/- 0.19	11.77 +/- 0.25	9.95 +/- 0.25	16.28 +/- 0.30	14.63 +/- 0.27	17.67 +/- 0.36	16.44 +/- 0.49



SAMPLE	UT	15 GY	UT	15 GY	UT	15GY
ΔCt ± SE	12.46 +/- 0.044	10.33 +/- 0.33	13.47 +/- 0.35	11.29 +/- 0.37	16.97 +/- 0.42	15.49 +/- 0.18

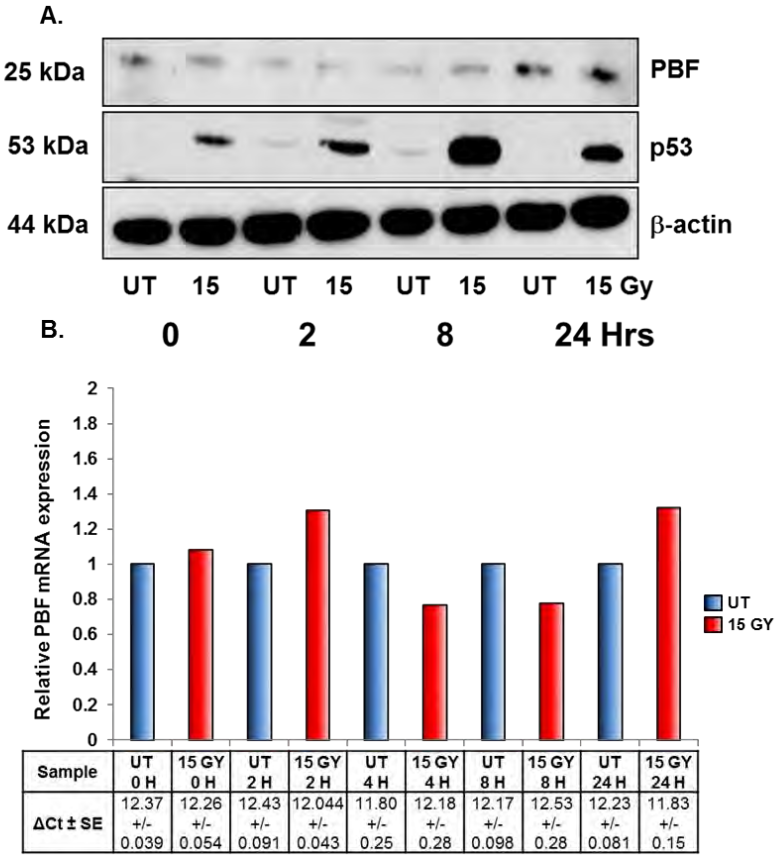


SAMPLE	UT	40 GY	UT	40 GY	UT	40 GY
ΔCt ± SE	22.46 +/- 0.47	18.91 +/- 0.54	21.62 +/- 0.07	20.03 +/- 0.086	17.39 +/- 0.13	15.66 +/- 0.28

Figure 3.5: Induction of genes mediated by p53 in response to DNA damage in TPC-1, K1 and HCT116 cells. ***A:*** *Significant induction of CDKN1A (n=4, p<0.001), HDM2 (n=4, p<0.001), PUMA (n=3, p<0.05) and BAX (n=3, p<0.05) mRNA expression 8 hours after treatment with 15 Gy γ-irradiation in TPC-1s.* ***B:*** *Induction of CDKN1A (n=3, p<0.01), HDM2 (n=3, p<0.05) and PUMA (n=3, p=NS) mRNA expression 8 hours after treatment with 15 Gy γ-irradiation in K1s.* ***C:*** *Significant induction of CDKN1A (n=3, p<0.001), HDM2 (n=3, p<0.001) and PUMA (n=3, p<0.001) mRNA expression after treatment with 40 Gy γ-irradiation in HCT116 cells (p<0.05 =*, p<0.01 =**, p<0.001 =***).*

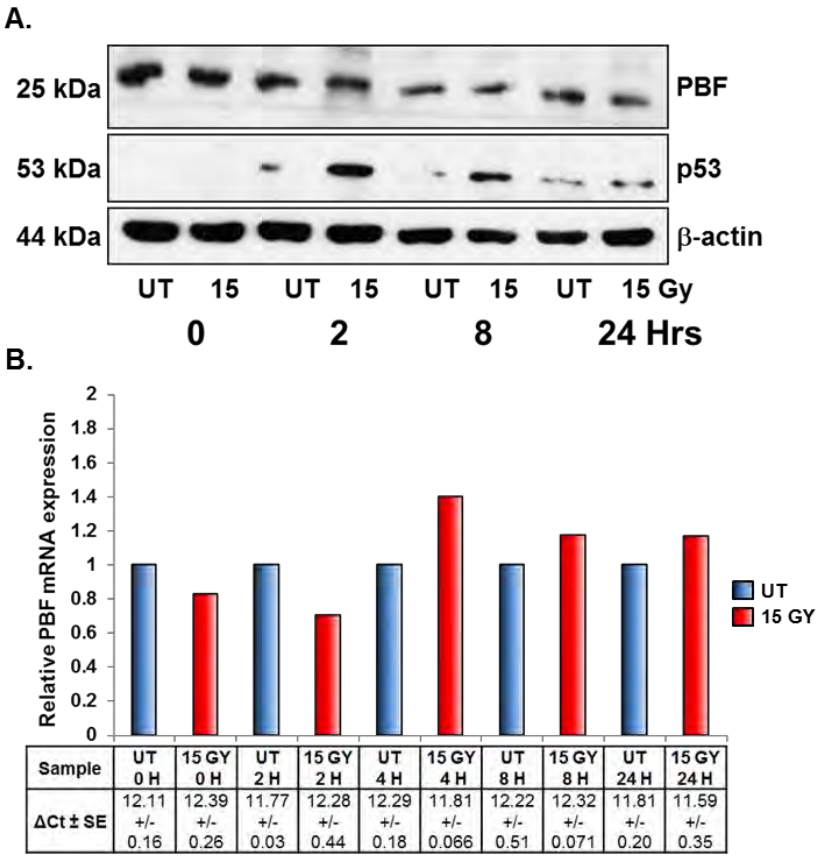
3.3.3. The effects of DNA damage on PBF expression

The effects of DNA damage on PBF expression have not been documented. This thesis concerns the assessment of oncogenic PBF (as reported in tumours of the breast, thyroid, pituitary and colon) and its potential effects on p53 biology. We therefore investigated the effects of DNA damage on PBF mRNA and protein expression. Firstly, TPC-1 and K1 cells were treated with γ-irradiation and PBF protein expression was analysed over a 24-hour time course (Figure 3.6). Western blotting did not reveal any significant difference in PBF protein expression over the 24 hour time-course in either TPC-1 (Figure 3.6A; *n*=5) or K1 cells (Figure 3.7A; *n*=5), with levels of PBF protein remaining constant in the same way as the time-matched negative controls. In-keeping with these observations PBF mRNA expression remained unchanged over 24 hours (*n*=3) (Figure 3.6B + Figure 3.7B) in both cell lines. These experiments were repeated in HCT116 cells with varying results. Over a 24 hour time-course, PBF protein expression appeared to be elevated 8 hours after treatment with respect to negative controls, and remained elevated at 24 hours (Figure 3.8A). However, this result was not reliably repeatable. Furthermore when the effects of increasing doses of γ-irradiation on PBF protein expression in HCT116 cells were observed, PBF protein expression appeared to increase in a dose dependent manner 24 hours post irradiation treatment, from doses as small as 10 Gy, and PBF expression was observed to increase in a similar manner to that of p53 (Figure 3.8B). PBF mRNA expression remained unchanged in response to increasing doses of γ-irradiation, suggesting that the effects of DNA damage on PBF protein expression are not due to upregulation of PBF at the level of gene transcription (Figure 3.8C).



Sample	UT 0 H	15 GY 0 H	UT 2 H	15 GY 2 H	UT 4 H	15 GY 4 H	UT 8 H	15 GY 8 H	UT 24 H	15 GY 24 H
ΔCt ± SE	12.37 +/- 0.039	12.26 +/- 0.054	12.43 +/- 0.091	12.044 +/- 0.043	11.80 +/- 0.25	12.18 +/- 0.28	12.17 +/- 0.098	12.53 +/- 0.28	12.23 +/- 0.081	11.83 +/- 0.15

Figure 3.6: The effects of DNA damage on PBF mRNA and protein expression in TPC-1 cells. ***A:*** *PBF protein expression 0, 2, 8 and 24 hours after treatment with 15 Gy of γ-irradiation. DNA damage did not induce any change in PBF protein expression at any time point (n=5).* ***B:*** *PBF mRNA expression 0, 2, 4, 8, and 24 hours after treatment with 15 Gy of γ-irradiation. DNA damage similarly failed to induce any change in PBF mRNA expression at any time-point (n=3, p=NS).*



Sample	UT 0 H	15 GY 0 H	UT 2 H	15 GY 2 H	UT 4 H	15 GY 4 H	UT 8 H	15 GY 8 H	UT 24 H	15 GY 24 H
ΔCt ± SE	12.11 +/- 0.16	12.39 +/- 0.26	11.77 +/- 0.03	12.28 +/- 0.44	12.29 +/- 0.18	11.81 +/- 0.066	12.22 +/- 0.51	12.32 +/- 0.071	11.81 +/- 0.20	11.59 +/- 0.35

Figure 3.7: The effects of DNA damage on PBF mRNA and protein expression in K1 cells. ***A:*** *PBF protein expression 0, 2, 8 and 24 hours after treatment with 15 Gy of γ-irradiation. (n=3).* ***B:*** *PBF mRNA expression 0, 2, 4, 8, and 24 hours after treatment with 15 Gy of γ-irradiation. (n=3, p=NS).*

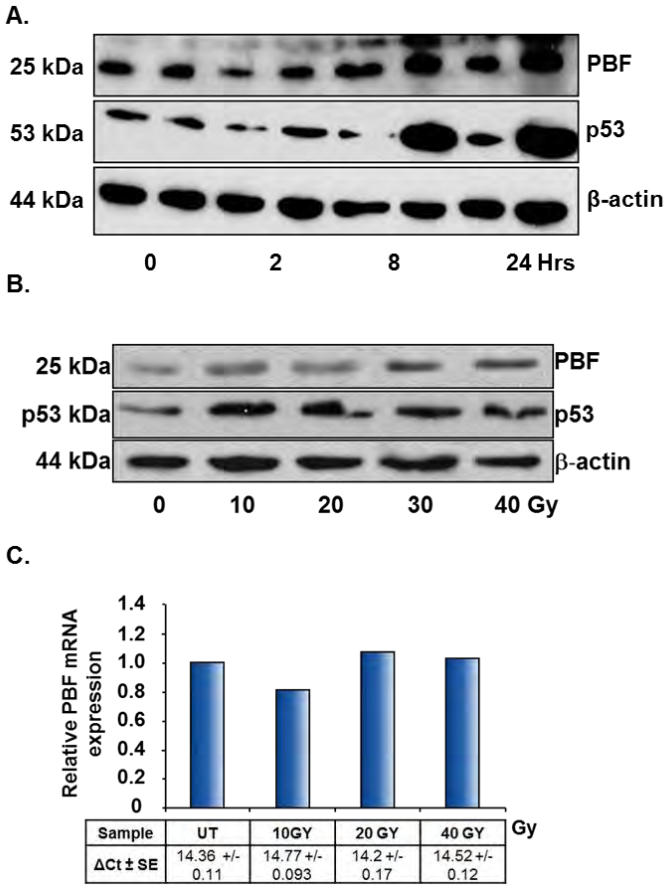


Figure 3.8: The effects of DNA damage on PBF mRNA and protein expression in HCT116 cells. ***A:*** *PBF protein expression 0, 2, 8 and 24 hours after treatment with 15 Gy of γ-irradiation. DNA damage resulted in a non-significant increase in PBF protein expression 8 and 24 hours after treatment (n=3).* ***B:*** *PBF protein expression appeared to increase in a dose dependent manner 24 hours after treatment with γ-irradiation, in a similar manner to the observed increase in p53 stability.* ***C:*** *PBF mRNA expression 0, 2, 4, 8, and 24 hours after treatment with 15 Gy of γ-irradiation. DNA damage did not induce any change in PBF mRNA expression at any time-point (n=3, p=NS).*

PBF overexpression has been reported in tumours of the thyroid, breast and colon (Stratford *et al*, 2005; Watkins *et al*, 2010b; Kim *et al*, 2005), and PBF is therefore likely to be overexpressed in both the thyroid papillary carcinoma cell lines and the colorectal cancer cell line. To test whether the presence of PBF over-expression in transformed cell lines was masking a potential PBF response to DNA damage,

murine primary thyrocytes were grown in culture and subsequently irradiated with 15 Gy of γ-irradiation, and protein harvested after 24 hours. Western blotting revealed a significant increase in PBF protein expression 24 hours after treatment with γ-irradiation (Figure 3.9A+B 2.17-fold, n=3, p<0.05). Critically, when PBF mRNA expression levels were analysed in response to ionising radiation, no significant difference was detected compared to time-matched untreated controls, either at 8 or 24 hours (Figure 3.9C, n=3, p=NS), implying that PBF protein expression is increased in response to γ-irradiation due to an effect on protein stability rather than increased transcription and translation.

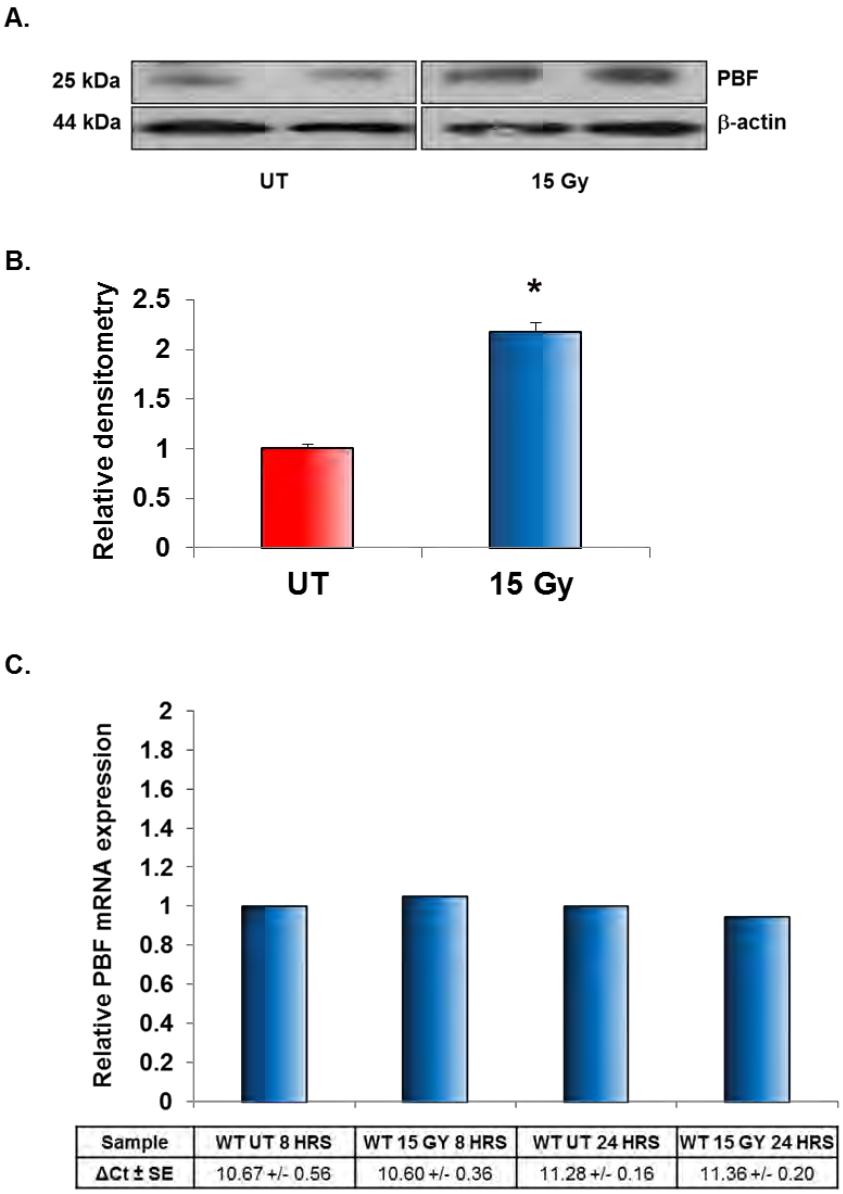


Figure 3.9: The effects of DNA damage on PBF protein and mRNA expression in untransformed murine primary thyrocytes. ***A:*** *Western blot demonstrating increased PBF protein expression 24 hours after treatment with 15 Gy γ-irradiation* ***B:*** *Scanning densitometry revealed a significant increase in expression after treatment with γ-irradiation compared to untreated controls (2.18-fold, n=3, p<0.05).* ***C:*** *DNA damage had no effect on PBF mRNA expression (n=3, p=NS).*

3.3.4. 3 A role for PBF in the DNA damage response?

To investigate a potential role for PBF in the DNA damage response and repair pathways upstream of p53 activity, the critical DNA damage protein H2AX was assessed to determine whether PBF localises to sites of DSBs. Firstly the optimal doses and time-points chosen for maximal p53 activity were challenged in light of a γ-H2AX response. In dose responses similar to those described earlier in this chapter, TPC-1, K1 and HCT116 cells were irradiated with a range of doses of γ-irradiation, and total protein was harvested 2 hours post treatment (Figure 3.10). All three cell lines displayed a dose dependent increase in γ-H2AX induction following treatment with γ-irradiation (Figure 3.10A-C). γ-H2AX was readily detectable at the established dose of 15 Gy in TPC-1 cells and K1 cells and at the higher dose of 40 Gy in HCT116 cells. When time courses were performed to assess the most suitable time-point for detection of γ-H2AX, all 3 cell lines displayed a robust γ-H2AX response to DNA damage with the most detectable levels recorded 2 hours after treatment (Figure 3.10D-F). The phosphorylation of H2AX is an early event in the DNA damage response and levels were detected at the 0 hour time-point (time taken to harvest cells following irradiation ~15 mins). Levels increased up to 2 hours following treatment, but were reduced significantly at the 8 and 24 hour time-points in all cell lines, most notably in K1 cells, where γ-H2AX levels were undetectable from 8 hours along this time-course (Figure 3.10 D-F). Using the optimal doses and time-points established, fluorescence immunocytochemistry experiments were performed to assess the localisation of PBF in respect to γ-H2AX foci present at sites of DSBs (Figure 3.11). Such studies were initially performed in green-monkey kidney COS-7 cells, a large cell type especially amenable to immunofluorescent studies. These

cells, treated with 15 Gy of γ-irradiation displayed numerous sub-nuclear foci of γ-H2AX 2 hours following treatment. PBF expression was predominantly nuclear, with significant levels present within the cytosol and intracellular vesicles. Interestingly nuclear PBF expression appeared to form distinct foci scattered through the nucleus. Analysis of the resultant merged image of these 2 proteins revealed a degree of colocalisation, whereby PBF appeared to colocalise to some γ-H2AX foci (Figure 3.11). These experiments were repeated in TPC-1, K1 and HCT116 cells with similar results, where γ-H2AX foci were numerous and readily detectable, and the numerous PBF sub-nuclear foci appeared to colocalise with many of them.

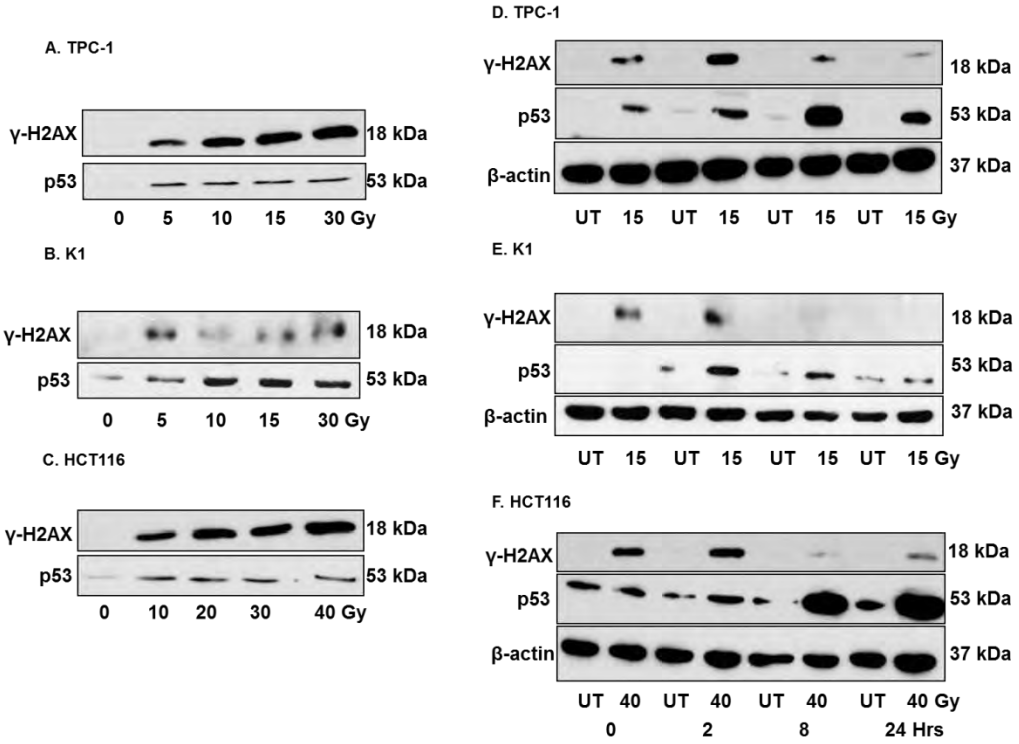


Figure 3.10: Induction of DNA damage to elicit γ-H2AX induction in TPC-1, K1 and HCT116 cells. ***A-C:*** *Induction of H2AX expression 2 hours after treatment with a range of doses of γ-irradiation in TPC-1, K1 and HCT116 cells respectively.* ***D-E:*** *Time-course demonstrating maximal γ-H2AX expression in TPC-1 and K1 cells over a 24 hour time-course following treatment with 15 Gy γ-irradiation.* ***F:*** *Time-course demonstrating maximal γ-H2AX expression in HCT116 cells over a 24 hour time-course following treatment with 40 Gy γ-irradiation.*

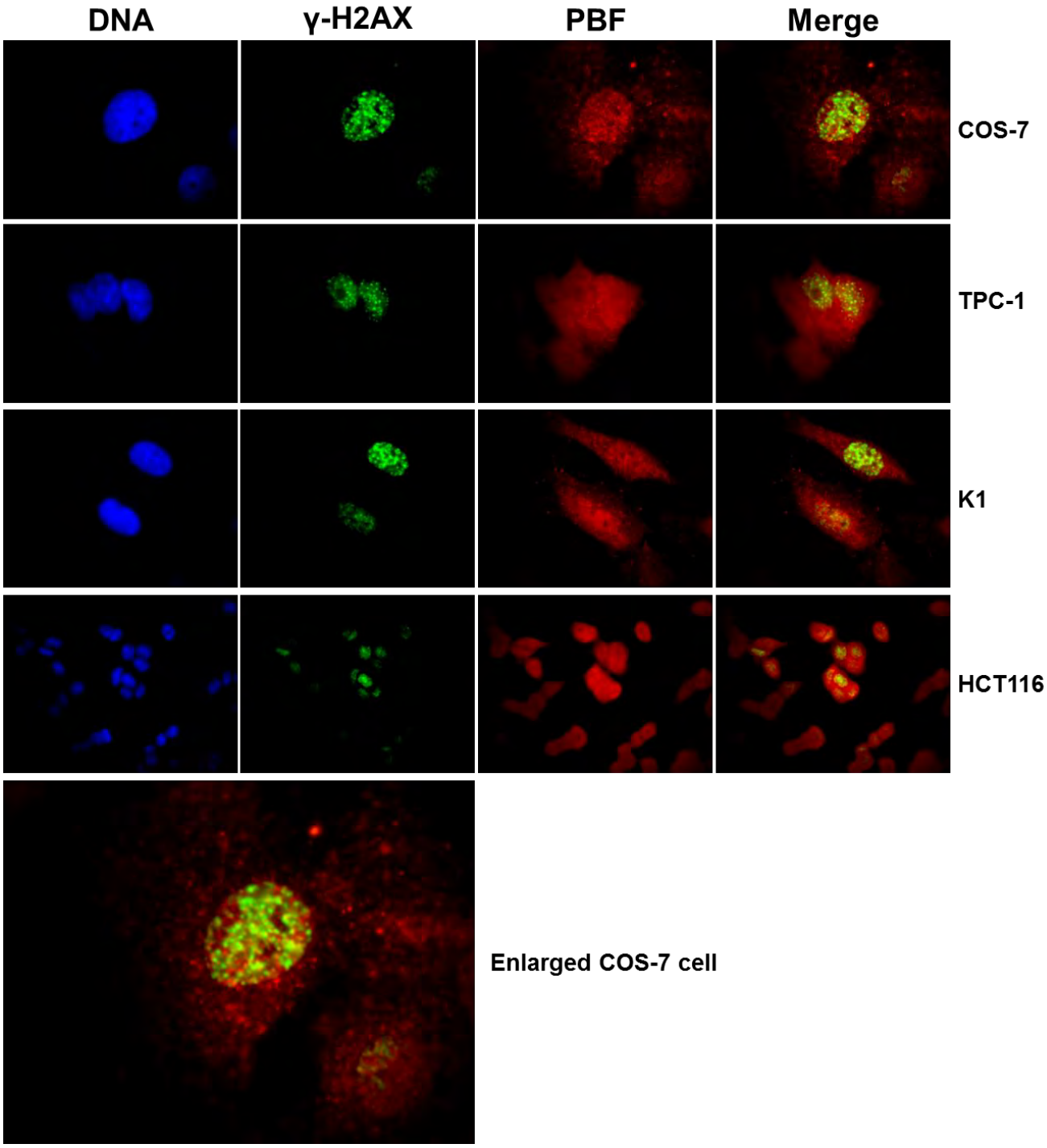


Figure 3.11: Localisation of γ-H2AX and PBF 2 hours after treatment with I.R. in COS-7, TPC-1, K1 and HCT116 cells. γ-H2AX (green) forms numerous discrete sub-nuclear foci following DNA damage, and detection is exclusively nuclear in all cell types. PBF expression (red) is located within the nucleus, cytosol and intracellular vesicles in all cell types. The merge of the γ-H2AX and PBF imaging demonstrated a degree of colocalisation (yellow) of PBF to γ-H2AX sub-nuclear foci.

Finally, in light of the apparent colocalisation of PBF with sites of DNA damage, specifically to γ-H2AX foci, the effects of PBF overexpression on γ-H2AX expression were investigated (Figure 3.12). Thyroid papillary carcinoma cells transiently overexpressing exogenous PBF were treated with 15 Gy γ-irradiation and total protein was harvested 2 and 8 hours after treatment. TPC-1 and K1 thyroid papillary carcinoma cell lines exhibited a robust p53 response following treatment with I.R. after 2 and 8 hours, compared to untreated controls (Figure 3.12A+B). γ-H2AX expression was also increased in response to I.R. at both time-points However, the induction of γ-H2AX was not affected by PBF overexpression at any time-point compared to VO-transfected (VO) controls (TPC-1 *n*=2, K1 *n*=2, Figure 3.12A+B). Despite the apparent colocalisation of PBF with γ-H2AX foci, these preliminary data suggest that PBF has no effect on the γ-H2AX expression in response DNA damage. This initial finding was also repeated in HCT116 cells with similar results (*n*=1, data not shown).

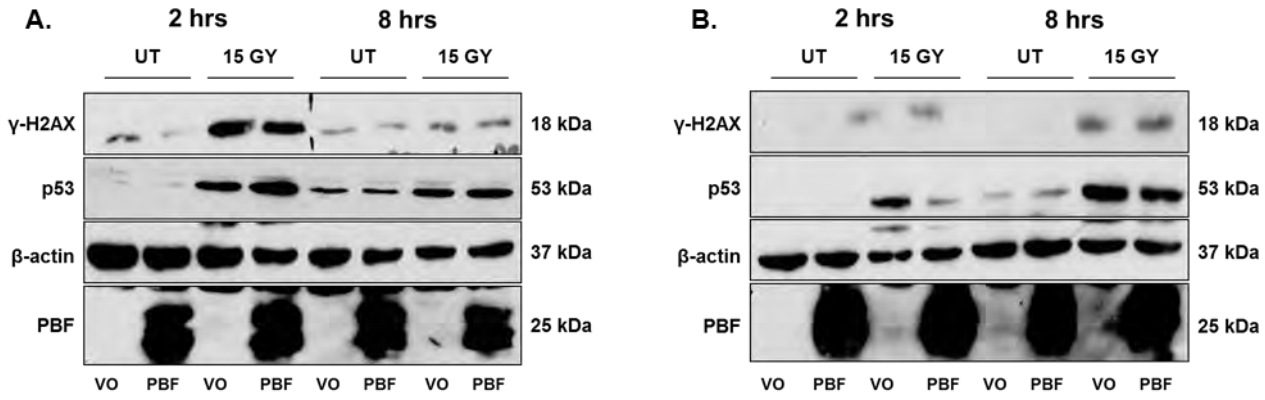


*Figure 3.12: Western blot analysis to determine the effects of PBF overexpression on the induction of γ-H2AX in response to DNA damage in **A:** TPC-1 cells (n=2) and **B:** K1 cells (n=2).(UT= untreated).*

3.4. **Discussion**

The effects of DNA damage, resultant p53 responses and downstream effects in TPC-1 and K1 thyroid papillary carcinoma cells have not been well documented. Of the diverse and increasing number of cellular stresses that initiate a classical p53 response, the molecular events following the induction of DNA DSBs are the most fully understood (Appella & Anderson, 2001; Saito *et al*, 2002; Vousden & Lu, 2002). Exposure of the developing thyroid to ionising radiation through human disasters such as Hiroshima and Chernobyl, and through the administration of γ-irradiation via medical treatment, have revealed that the thyroid gland is highly sensitive to the carcinogenic effects of ionising radiation (Thompson *et al*, 1994; Nikiforov *et al*, 1996; Sadetzki *et al*, 2006). We therefore investigated the effects of DNA damage in the form of ionising γ-irradiation on TPC-1 and K1 cells (expressing wild-type p53) to attempt to establish optimal dose responses and time-points for the measurements of the effects of PBF on this pathway. Furthermore, the effects of DNA DSBs on the expression, and subcellular localisation of PBF remain uncharacterised. Therefore we assessed the effects of DNA damage on PBF expression and subcellular localisation in these cells. Finally, to determine whether responses detected were thyroid specific, we endeavoured to repeat our investigations in the HCT116 colorectal carcinoma cell line.

3.4.1. Treatment with γ-irradiation: establishing an optimal dose.

TPC-1 and K1 papillary carcinoma cells displayed robust p53 protein accumulation and phosphorylation of ser-15 (data not shown), indicative of p53 activation in response to DNA DSBs induced by γ-irradiation. Furthermore, both cell lines displayed a dose dependent increase in p53 accumulation following treatment. The

reduced p53 accumulation observed at higher doses of γ-irradiation correlated with gross cytotoxicity indicative of necrosis caused by exposure to high and lethal doses of γ-irradiation (Raghavan *et al*, 2012). In HCT116 cells p53 accumulation also occurred in a dose-dependent manner, however, accumulation of p53 continued to occur even at the highest dose delivered (40 Gy) with no observable cytotoxicity. This suggests that TPC-1 and K1 thyroid papillary carcinoma cells, whilst displaying robust p53 accumulation following genotoxic stress, are more sensitive to higher doses of irradiation than the radio-resistant HCT116 colorectal cancer cells. p53 activity measured as induction of CDKN1A mRNA expression was also increased in a dose-dependent manner in both TPC-1 and K1 cells. K1 cells displayed increasing CDKN1A mRNA expression in response to DNA damage up to a dose of 15 Gy after which, in-keeping with the cytotoxicity observed at the higher doses (above 20 Gy), CDKN1A mRNA expression levels began to fall. HCT116 cells displayed a dose dependent increase in CDKN1A mRNA expression with maximal induction at the highest treatment dose of 40 Gy, in direct correlation with the observed increase in p53 protein expression. Taken together these data indicate that the human thyroid papillary carcinoma cell lines, TPC-1 and K1s are responsive to a range of doses of γ-irradiation, resulting in a vigorous increase in p53 protein expression and activation of p53 as a transcription factor. The dose at which p53 accumulation and activation is most apparent in the thyroid papillary carcinoma cells is 15 Gy (taking into account the global cytotoxicity observed at higher doses) and treatment of cells with 15 Gy of γ-irradiation provides robust p53 activity from which the effects of PBF can be measured.

3.4.2. Treatment with γ-irradiation: establishing an optimal time-point

In addition to observing the p53 response to DNA damage over a range of doses of γ-irradiation it was also necessary to determine the time-points at which maximal p53 activity occurred. TPC-1, K1 and HCT116 cells all displayed significant induction of CDKN1A mRNA expression over a 24 hour time-course, with maximal responses observed at 8 and 24 hours in each cell-line.

Different cell types often display different biological reactions to any given stress (Gudkov & Komarova, 2003), and the decision between apoptosis and growth arrest appears to be governed by p53 activity (Vousden & Lu, 2002). However, this process is by no means random, for example haematopoietic stem cells only respond to γ-irradiation via induction of apoptosis (Vousden & Lu, 2002; Meek, 2004); and fibroblasts only appear to respond even to very high doses of γ-irradiation through growth-arrest; apoptosis is never initiated (Gudkov & Komarova, 2003). However, many cell types may undergo either growth arrest or apoptosis, and the p53 'decision' to drive one or both of these pathways is influenced by the levels of genotoxic stress sustained. TPC-1 cells are an example of a cell-type that can undergo either growth arrest or apoptosis depending on the level of genotoxic insult sustained (Abou-El-Ardat *et al*, 2008). We found that γ-irradiation induced p53-mediated gene expression of the growth arrest gene CDKN1A most significantly, with the pro-apoptic genes BAX and PUMA significantly induced but at lower levels. These data correlate with the findings of Abou-El-Ardat *et al*, 2008, who observed growth-arrest as the major response to both high and low-doses of ionising radiation, whilst observing a degree of apoptosis in TPC-1 cells. In a similar fashion to TPC-1 cells, K1 cells also displayed significant upregulation of CDKN1A mRNA expression,

suggesting that in this cell line growth arrest pathways are highly stimulated in response to 15 Gy of γ-irradiation. However, in contrast to the TPC-1 cell line PUMA mRNA expression was non-significantly upregulated (2.4-fold) following treatment, indicating that in these cells, induction of apoptosis may be less frequent. This result comes in apparent support of the finding of Challeton *et al*, who observed a weak effect of the dose rate of ionising radiation on the survival of K1 thyroid papillary carcinoma cells (Challeton *et al*, 1997). Finally, in HCT116 cells CDKN1A mRNA expression was significantly elevated 40 Gy of γ-irradiation, suggesting that this dose is inducing a strong p53-mediated growth arrest response in this cell line. In addition, PUMA mRNA expression was also significantly upregulated, but to a lesser degree, demonstrating that at this dose, p53-mediated apoptotic pathways are also activated. That p53-mediated gene transcription of CDKN1A expression was significantly elevated in all cell lines may suggest that treatment of TPC-1 and K1 cells with 15 Gy and HCT116 cells with 40 Gy of γ-irradiation predominantly results in growth arrest responses. However, the potency of gene induction does not always reflect the severity of the biological response. Therefore to determine the biological outcome (apoptosis or growth arrest) of these doses of ionising radiation, further experiments are required. For instance analysis of classical BrdU staining via FACS would determine the extent of growth arrest exhibited by TPC-1, K1 and HCT116 cells following treatment with ionising radiation, and caspase 3/7 cleavage assays would determine the levels of apoptosis in these cells. In conclusion, whilst these data do not demonstrate a reliable mechanism for determining the overall cellular response to ionising radiation in TPC-1, K1 and HCT116 cells, they demonstrate that treatment of

TPC-1 and K1 cells with 15 Gy and treatment of HCT116 cells with 40 Gy significantly activate the p53 response to DNA damage.

3.4.3. The effects of DNA damage on PBF expression

The effects of ionising radiation on PBF expression are unknown. To assess whether PBF is responsive to DNA damage we treated TPC-1 and K1 cells with 15 Gy γ-irradiation and harvested total cellular protein and RNA over a 24 hour time-course (we also repeated these experiments using HCT116 cells at the higher dose of 40 Gy). In TPC-1 and K1 cells no detectable difference in PBF protein or mRNA expression was observed across a 24 hour time-course following treatment with γ-irradiation. These cells were also subjected to a range of doses to γ-irradiation to observe whether the PBF response was dependent on dose (i.e. low or high dose response), and again, showed no change in PBF protein expression. These data suggest that PBF expression is not effected by γ-irradiation, and that in these cell lines PBF is not responsive to γ-irradiation treatment. HCT116 cells treated with 40 Gy of γ-irradiation displayed increased PBF protein expression from 8 and 24 hours after treatment, but this result could not be repeated to statistical significance. Furthermore when dose responses were performed PBF protein expression appeared to increase in a dose-dependent manner, but again, these observations were not reliably repeatable in this cell line. PBF mRNA expression was not induced in response to γ-irradiation. As mentioned earlier, PBF protein is overexpressed in tumours of the thyroid, breast pituitary and colon, and so investigation into the effects of γ-irradiation in thyroid papillary carcinoma cells and human colorectal cells may represent a futile system of observation whereby physiological PBF responses are saturated. We therefore assessed the effects of PBF expression in response to γ-

irradiation in primary murine thyrocytes. In this system, PBF protein expression was significantly elevated 24 hours after treatment with 15 Gy of γ-irradiation. Interestingly, PBF mRNA expression was unaltered in response to 15 Gy of γ-irradiation at both 8 and 24 hours post irradiation. The lack of induction of PBF mRNA expression suggests that PBF protein induction in these cells occurred as a result of the induction of PBF protein stability rather than transcription and translational events. That increased PBF protein expression correlates with ionising radiation treatment in murine primary thyrocytes may suggest that PBF plays a role in the normal cellular response to genotoxic insult in the form of DNA DSBs, with oncogenic expression of PBF resulting in aberrant cellular responses to DNA damage, resulting in cell transformation or malignant progression.

3.4.4. A role for PBF in the DNA damage response

In order to determine the relationship between PBF, sites of DNA damage and DNA damage and repair proteins upstream of p53, we assessed the relationship between PBF and the DNA repair protein γ-H2AX. Phosphorylation of the H2AX protein at serine-139 (yielding γ-H2AX), mediated by ATM, is an early event following the induction of DNA lesions (Kinner *et al*, 2008). γ-H2AX was induced in a dose dependent manner in TPC-1 and HCT116 cells, 2 hours after treatment with ionising radiation. γ-H2AX was more difficult to detect in K1 cells, but was readily induced by DNA damage. Time-courses were also performed to ascertain the optimal time-point to study the relationship between PBF and γ-H2AX. In all cell lines the greatest accumulation of γ-H2AX occurred 2 hours after treatment, with levels readily detectable at time 0 (time taken to remove cells from the irradiator and harvest cellular protein ~15 mins). Using this information, we assessed whether PBF

localised to sites of DNA damage using immunofluorescence cytochemistry. Treatment of COS-7, TPC-1, K1 and HCT116 cells with ionising radiation induced a number of distinct subnuclear foci of γ-H2AX to sites of DNA lesions; PBF expression was located throughout the cytoplasm, intracellular vesicles and the nucleus, with some but not ubiquitous colocalisation with γ-H2AX foci. These data lie in support of the multi-functional role of PBF, for example in shuttling of PTTG into the nucleus and in the regulation of NIS function amongst others, where distinct isoforms of PBF (cleavage products or differential post-translational modifications) have distinct biological roles (Smith *et al*, 2009, 2012 and our unpublished data). However, if PBF plays an important role within the DNA damage response, these comments fail to explain why PBF does not colocalise to all sites of γ-H2AX foci preventing the derivation of firm conclusions concerning the relationship of PBF within the DNA damage response.

Whilst this thesis concerns the relationship of oncogenic PBF levels on p53 function, we decided to investigate these findings further to attempt to ascertain whether PBF plays a role in the DNA damage response. To do this TPC-1, K1 and HCT116 cells were transfected with our PCDNA3-PBF plasmid to induce transient overexpression of PBF and the effects of PBF overexpression on the accumulation of γ-H2AX in response to ionising radiation were assessed. Preliminary data (TPC-1, *n=2;* K1, *n=2;* HCT116 *n=1*), demonstrated that PBF overexpression had no effect on the induction of γ-H2AX following treatment with γ-irradiation. These findings indicate that there is no functional relationship between PBF and γ-H2AX accumulation at sites of DNA damage.

3.4.5. Concluding statements

This Chapter provides evidence that TPC-1, K1 and HCT116 cells display a robust p53 response to ionising radiation. For instance, p53 was highly stabilised at the protein level and p53-mediated gene expression was significantly elevated following treatment of TPC-1 and K1 cells with 15 Gy of γ-irradiation and HCT116 cells with 40 Gy of γ-irradiation.

Induction of DNA damage within TPC-1, K1 and HCT116 cells did not correlate with any significant differences in PBF protein or mRNA expression, however, in normal murine thyrocytes, PBF protein expression was significantly elevated in response to γ-irradiation. These data suggest that PBF may be activated following genotoxic insult in normal (non-transformed cells), and may indicate a normal cellular role for PBF in the cellular response to DNA damage.

Finally, despite observing a degree of colocalisation of PBF and γ-H2AX nuclear foci, PBF overexpression had no effect on the induction of γ-H2AX; suggesting that whilst a subset of PBF nuclear foci are localise to sites of DNA breaks, PBF does not influence the activation of this protein in response to DNA damage. Further study into the DNA damage and repair pathways are required to fully elucidate the role of PBF in the DNA damage response upstream of p53 activity.

Chapter 4. **The relationship between PBF and p53**

4.1. **Introduction**

As mentioned previously, p53 is inactivated by mutation in over 50 % of human cancers, but is also observed to be functionally inactivated by viral or cellular oncogenes. Indeed, numerous avenues of study implicate the functional inactivation of p53 with initiation and progression of tumourigenesis (Vogelstein *et al*, 2000; Vousden, 2000). Many viral oncogenes manipulate p53 function to allow for replication, with the outcome for the infected cell being increased susceptibility to transformation. For example, the viral onco-protein (HPV16) E6 counteracts cellular responses to unscheduled proliferation by binding directly to p53 and targeting it for proteasomal degradation (Havre *et al*, 1995; Massimi *et al*, 2007).

Recently, cellular oncogenes have been characterised to inhibit p53 function in certain tumours. For instance, chromosomal rearrangements in acute myeloid and lymphoblastic leukaemias result in the generation of a chimeric protein MLL-ELL. Recently MLL-ELL has been shown to inhibit p53 transcriptional activity, and the 2 proteins have been observed to colocalise within the nucleus. Furthermore, a direct interaction of the p53 transactivation domain and the ELL protein has been observed. The binding of p53 and MLL-ELL results in the disruption of numerous important p53 interactions in response to cellular stress, including the association of p53 with its key transcriptional co-activator p300/CBP (Wiederschain *et al*, 2003). More importantly in thyroid papillary carcinomas, oncogenic expression of the high mobility group A factors (HMGA1a, HMGA1b, and HMGA2) in thyroid epithelial cells results in malignant transformation (Frasca *et al*, 2006). The mechanism by which HMGA1 promotes transformation and tumourigenesis has been characterised, with the functional inactivation of p53 playing a key role. HMGA1 binds directly to p53 in the

region of the C-terminal oligomerisation domain, and oncogenic expression of HMGA1 represses the DNA binding capacity of p53 and causes the inhibition of p53 apoptotic function (Frasca *et al*, 2006; Pierantoni *et al*, 2007; Malaguarnera *et al*, 2007a).

In some cases, the functional inactivation of p53 is brought about by its physical removal (sequestration) from sites of activity by another protein. For example this mechanism may result in p53 accumulation in the cytoplasm thereby abrogating its tumour suppressive action. Originally, this was discovered in mammalian models of viral oncogenesis. The adenovirus E1B 55 kDa protein (in conjunction with E1A and E1B 19 kDa proteins) contributes to cellular transformation (Yew & Berk, 1992; Yew *et al*, 1994; Hartl *et al*, 2008). This protein interacts directly with the transactivation domain (TAD) of p53 perturbing its tumour suppressive role. Whilst the E1B 55 kDa protein appears to disrupt p53 on many levels, it has been observed to cause the sequestration of p53 into cytoplasmic aggresomes (Zhao & Liao, 2003; Hartl *et al*, 2008). This is a brief description of a small number of the total proteins that have either evolved to bypass the protective effect of p53, as in the case of viral oncogenes, or functionally inactivate p53 responses following oncogenic expression (as in the case of cellular oncogenes). Interestingly, of the p53 disruptive oncogenes described, all bind directly to p53 to elicit their oncogenic properties.

Numerous cellular roles for the proto-oncogene PBF have been described. For instance PBF binds directly to PTTG and facilitates its transportation to the nucleus via its C-terminal bi-partite nuclear localisation signal. This property allows PTTG to function as a Securin, by inhibiting separase activity to prevent premature sister-chromatid separation (Chien & Pei, 2000). PBF also plays a role in the regulation of

NIS function whereby PBF binds directly to NIS causing its redistribution from the plasma membrane to intracellular vesicles (Smith *et al*, 2009). Additionally, the Membrane transporter MCT-8 (a key regulator of TH secretion from the thyroid gland) has been observed to undergo significant subcellular relocalisation Following interaction with PBF (Smith *et al*, 2012a). PBF has therefore been characterised with a number of diverse roles, suggesting that PBF is able to functionally interact with a number of cellular proteins.

We hypothesised that aberrant expression of PBF promotes tumourigenesis by functional inhibition of p53 activity, and that, in keeping with the cellular oncogenes described, an interaction between the 2 proteins may be required. Given that PBF is known to interact functionally with other proteins we therefore assessed the potential interaction between PBF and p53. We have previously described an interaction between PBF and p53 from HCT116 cells *in vitro* with the use of GST pull down assays (see Section 1.3.3). However, in light of the fact that GST pull down assays represent an artificial system, where proteins may bind in a physiologically irrelevant manner, the observed interaction may not occur under normal cellular conditions. To validate or refute these preliminary GST data in a setting that is more physiological, we aimed to assess this interaction using the technique of co-immunoprecipitation in TPC-1, K1 and HCT116 cells, both in the presence and absence of ionising radiation. In addition to these studies, assessment of the subcellular relationship between PBF and p53 was investigated, both in the presence and absence of DNA damage. We also aimed to characterise the effects of PBF overexpression on the subcellular localisation of p53, especially in light of the previously described roles for PBF in the

subcellular distribution of other important proteins (Chien & Pei, 2000; Boelaert *et al*, 2007; Smith *et al*, 2009, 2012a).

The overall aims of this chapter were therefore to determine whether PBF and p53 interact within the intracellular environment and assess the subcellular localisation of PBF and p53 in the presence and absence of DNA damage.

4.2. **Materials and methods**

4.2.1. Cell lines and plasmids

TPC-1, K1 and HCT116 cells were routinely subcultured using the methods described in Section 2.1. For experiments requiring transient PBF overexpression, and /or γ-irradiation exposure, cells were treated using the procedures described in Section 3.3

4.2.2. Co-immunoprecipitation assays

TPC-1, K1 and HCT116 cells growing in T25 culture flasks were harvested in 500 ul RIPA buffer with 60 µl/ml protease inhibitor (100 mM sodium chloride, 0.1 % Triton X-100 and 50 mM Tris (pH8.3)). Samples were then sonicated for 30 seconds on the medium setting of the Diagenode Bioruptor and centrifuged for 20 minutes at 13,000 rpm (4 ^{0}C). Protein concentration was then measured using BCA estimation (PIERCE) and 30 µg of protein lysate were retained before addition of primary antibody (DO-1 at 1:100 concentration) overnight at 4 ^{0}C, with end over end mixing. Samples were then incubated in 50 µl of protein-G sepharose/RIPA slurry for 2 hours at 4 ^{0}C with end over end mixing. Beads were then pulsed and the supernatant discarded and unbound protein was washed using 4X500 µl RIPA buffer. Subsequently protein was eluted with 1:20 β-mercaptoethanol and 1 % SDS in

Laemlli buffer and incubation for 30 minutes at 37 ^{0}C. Equal volumes of this solution were added to the cell lysates and samples were loaded onto a 12 % acrylamide gel for subsequent analysis via Western blotting.

4.2.3. SDS-PAGE/Western blotting

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see Section 2.6). Blocked membranes were subsequently incubated with primary antibodies. Antibodies used were; our rabbit anti-human PBF-8 polyclonal (1:1000) (Stratford *et al,* 2005), rabbit anti-human HA (Covance) mouse anti-human p53 (D0-1) monoclonal at 1:5000 (Santa Cruz, CA, USA), and monoclonal anti-β-actin clone AC-15 (used at 1:10,000, Sigma-Aldrich, Poole, UK). To test for the purity and equal loading of nuclear and cytoplasmic protein subfractions mouse anti-human lamin and mouse anti-human α-tubulin specific antibodies were used respectively (Santa Cruz, CA, USA). Scanning densitometric analysis of Western blotting assessment of PBF and p53 expression was normalised to β-actin.

4.2.4. Immunofluorescence

COS-7, HCT116, TPC-1 and K1 cells were seeded onto coverslips, irradiated using a Caesium-137 γ-emitter and prepared for fluorescence immunocytochemistry using the methods described in Section 2.7. PBF was detected using our rabbit anti-human PBF-8 antibody (1:100) and p53 detected using the mouse anti-human p53 (DO-1) monoclonal at 1:250 (Santa Cruz, CA, USA). Cells were then visualised on a fluorescence confocal microscope.

4.2.5. Subcellular fractionation experiments

For subcellular fractionation experiments, cells were seeded into 6-well dishes and nuclear and cytosolic protein subfractions were prepared using a commercial nuclear extraction kit (Active Motif Europe, Rixenhart, Belgium) as per manufacturer's instructions. Protein concentration before loading was measured by the Bradford assay with BSA as standard.

4.2.6. Statistical analysis

Data were analysed as described in Section 2.8.

4.3. **Results**

4.3.1. PBF and p53 interact in vitro

To determine whether PBF and p53 interact *in vitro,* total cellular protein was harvested from TPC-1 and K1 cells and co-immunopreciptitation assays were performed. When compared to no antibody controls, p53 was co-immunoprecipitated with PBF using our PBF polyclonal antibody, in both the presence and absence of γ-irradiation (see Figure 4.1). In the presence of γ-irradiation significantly more p53 co-immunoprecipitated with PBF (TPC-1, 2.7 +/- 0.38 fold. n=4, p<0.05; K1, 2.1 +/- 0.13 fold, n=4, p<0.01). p53 also co-immunoprecipitated with PBF in HCT116 cells in both the presence and absence of DNA damage (see Figure 4.2A). DNA damage also induced a significant increase in the yield of p53 in this cell line (1.9 +/- 0.13 fold, n=3, p<0.01, Figure 4.2B). Further to these experiments PBF was also co-immunoprecipitated with p53 using a specific monoclonal p53 antibody compared to no antibody controls (NAb) in HCT116 cells (see Figure 4.2C). Finally, an interaction

between PBF and p53 was observed through co-immunoprecipitation assays when exogenous p53 was transiently over-expressed in HCT116 cells (Figure 4.2D). These data demonstrate a specific and robust interaction between PBF and p53 under normal cellular conditions in both the presence and absence of DNA damage.

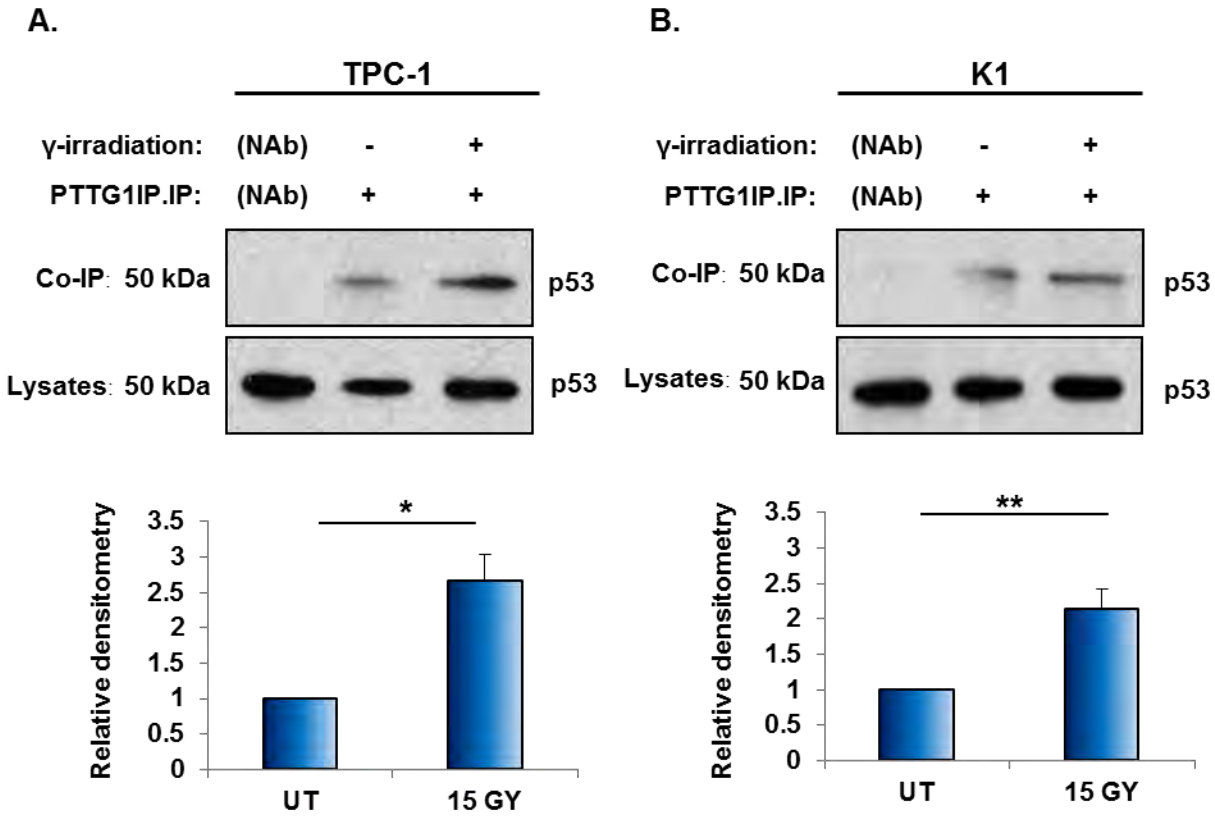


Figure 4.1: PBF binds to p53 in co-immunoprecipitation assays ***A:*** *Co-immunoprecipitation of p53 with PBF in TPC-1 cells in both the absence (-) and presence (+) of γ-irradiation (NAb = no antibody control). Scanning densitometry revealed that in the presence of γ-irradiation more p53 co-immunoprecipitated with PBF (2.7-fold, n=3, p<0.05) compared to untreated controls.* ***B:*** *Co-immunoprecipitation of PBF with p53 in K1 cells in the presence and absence of γ-irradiation compared to NAb controls. Scanning densitometry confirmed that significantly more p53 co-immunoprecipitates with PBF in the presence of γ-irradiation (2.1-fold, n=3, p<0.01) compared to untreated controls.*

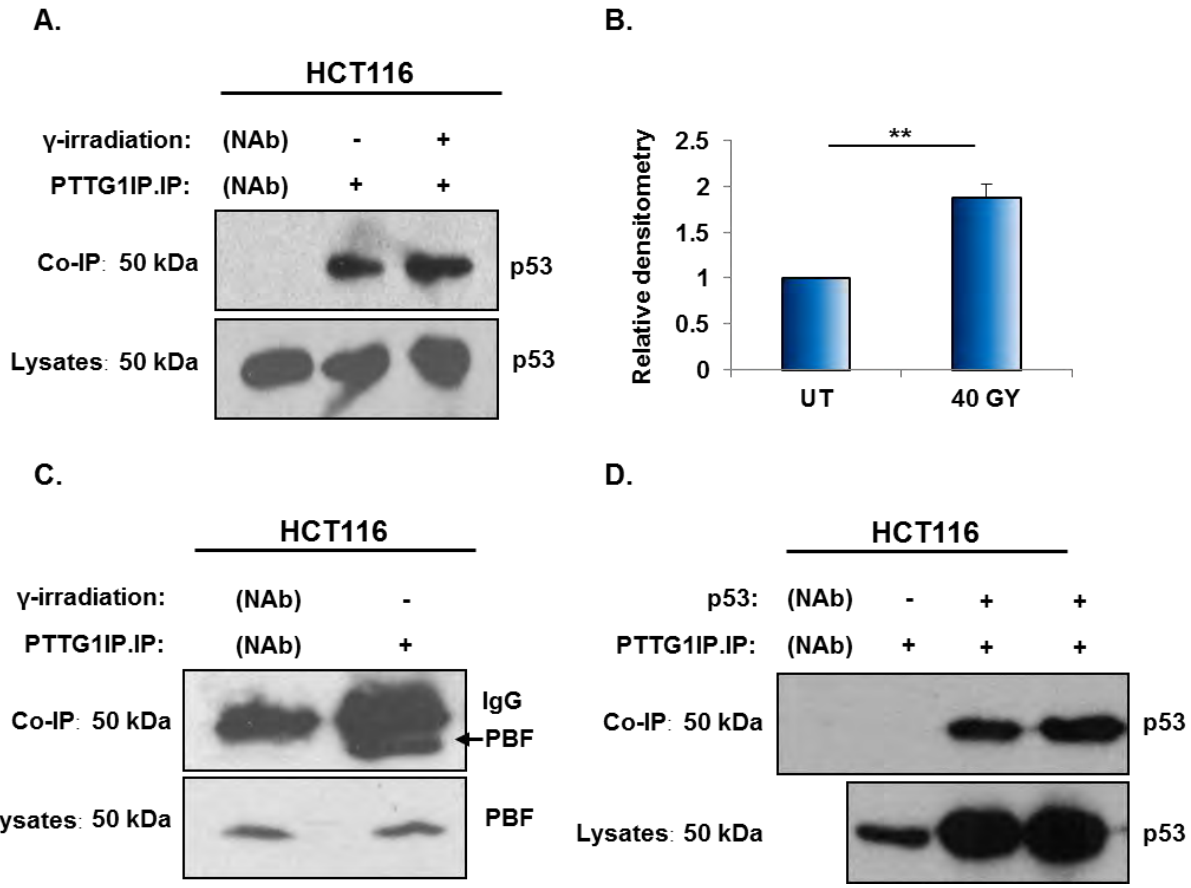


Figure 4.2: PBF binds to p53 in co-immunoprecipitation assays. ***A:*** *Co-immunoprecipitation of p53 with PBF in HCT116 cells in both the absence (-) and presence (+) of γ-irradiation (NAb =no antibody control).* ***B:*** *Scanning densitometry revealed that In the presence of γ-irradiation more p53 co-immunoprecipitated with PBF (1.9-fold, n=3, p<0.01) compared to untreated controls.* ***C:*** *Reverse co-immunoprecipitatiom of PBF with p53 in the absence of DNA damage. IgG =antibody contamination from the immunoglobulin G light-chain.* ***D:*** *Co-immunoprecipitation of exogenous p53 with PBF.*

4.3.2. PBF and p53 colocalisation

Fluorescence immunocytochemistry experiments determined the colocalisation of PBF and p53 in COS-7 cells, TPC-1 cells and HCT116 cells. Such studies were initially carried out and optimised in COS-7 cells due to their large size compared to the thyroid and colorectal cell lines. In COS-7 cells p53 was predominantly nuclear in both the presence and absence of DNA damage, indicative of a transformed cell line

(Hess & Brandner, 1997). Despite this, levels of p53 visibly increased in the nucleus following DNA damage (Figure 4.3). PBF expression appeared to be ubiquitously distributed throughout the cell (located within the cytosol, intracellular vesicles and the nucleus) However, in response to DNA damage, PBF nuclear expression visibly increased compared to untreated controls. PBF and p53 appeared to colocalise within the nucleus of COS-7 cells in both the presence and absence of DNA damage, with greater levels of colocalisation detected following DNA damage, in keeping with the apparent increase in the nuclear expression of each protein (Figure 4.3). Fluorescence immunocytochemistry experiments were repeated in both TPC-1 and HCT116 cells with similar results (Figure 4.4). In TPC-1 cells p53 was primarily located within the nucleus but with detectable levels within the cytoplasm (Figure 4.4A). Following DNA damage, nuclear p53 expression was visibly increased. Again in these cells PBF was distributed throughout the cell, with the majority of PBF localised to the nucleus, and detectable levels throughout the cytosol and intracellular vesicles. In response to DNA damage these cells did not display increased PBF protein expression within the nucleus or otherwise (consistent with the data described in Chapter 2). PBF and p53 appeared to colocalise in these cells in both the presence and absence of DNA damage. In the absence of DNA damage, PBF and p53 colocalisation was most apparent in the cytoplasm. However, following DNA damage PBF and p53 appear to colocalise within the cytoplasm and compared to untreated controls, to a greater degree within the nucleus. Finally in HCT116 cells, p53 expression was predominantly nuclear, with detectable levels within the cytosol, and after DNA damage, p53 nuclear expression was greatly increased (Figure 4.4B). PBF was located predominantly in the nucleus, but also localised to the cytosol and

intracellular vesicles. Following DNA damage PBF nuclear expression increased compared to untreated controls. PBF and p53 appeared to colocalise in the nucleus of these cells in the presence and absence of DNA damage; although colocalisation was striking following DNA damage, it was minimal in the absence of DNA damage.

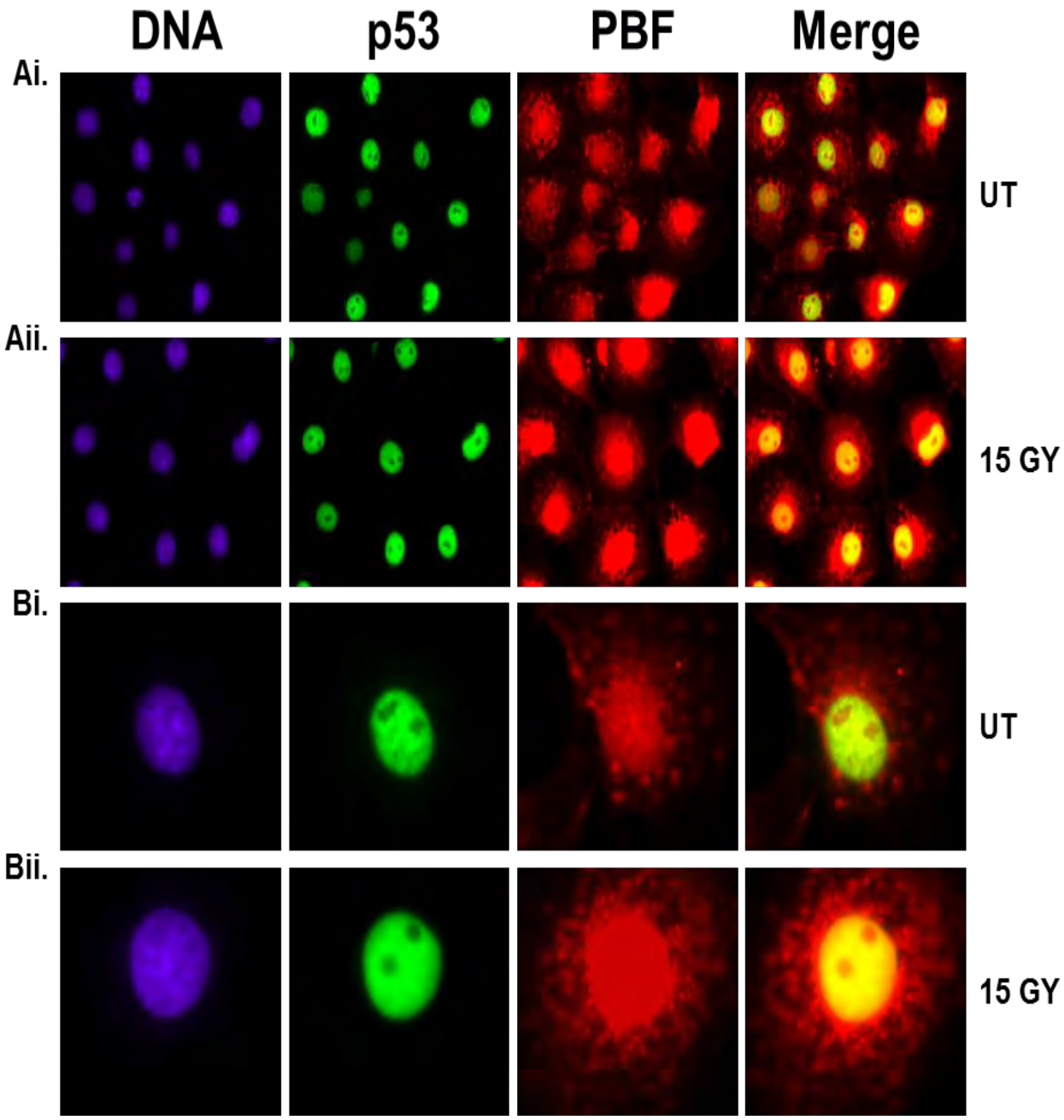


Figure 4.3: Localisation of PBF and p53 in COS-7 cells at 40x and 100x magnification. ***A:*** *p53 and PBF localisation in a cluster of COS-7 cells in the presence and absence of DNA damage. p53 (green) is predominantly nuclear, with PBF expression (red) located throughout the cytosol, intracellular vesicles and the nucleus. PBF and p53 displayed significant co-localisation within the nucleus as seen in the merged images (yellow). In the presence of DNA damage both p53 and PBF nuclear expression was increased, resulting in a significant increase in nuclear co-localisation (DAPI nuclear staining is shown in blue).* ***B:*** *A single COS-7 cell at 100x magnification displaying colocalisation of PBF and p53 in the nucleus in both the presence and absence of DNA damage.*

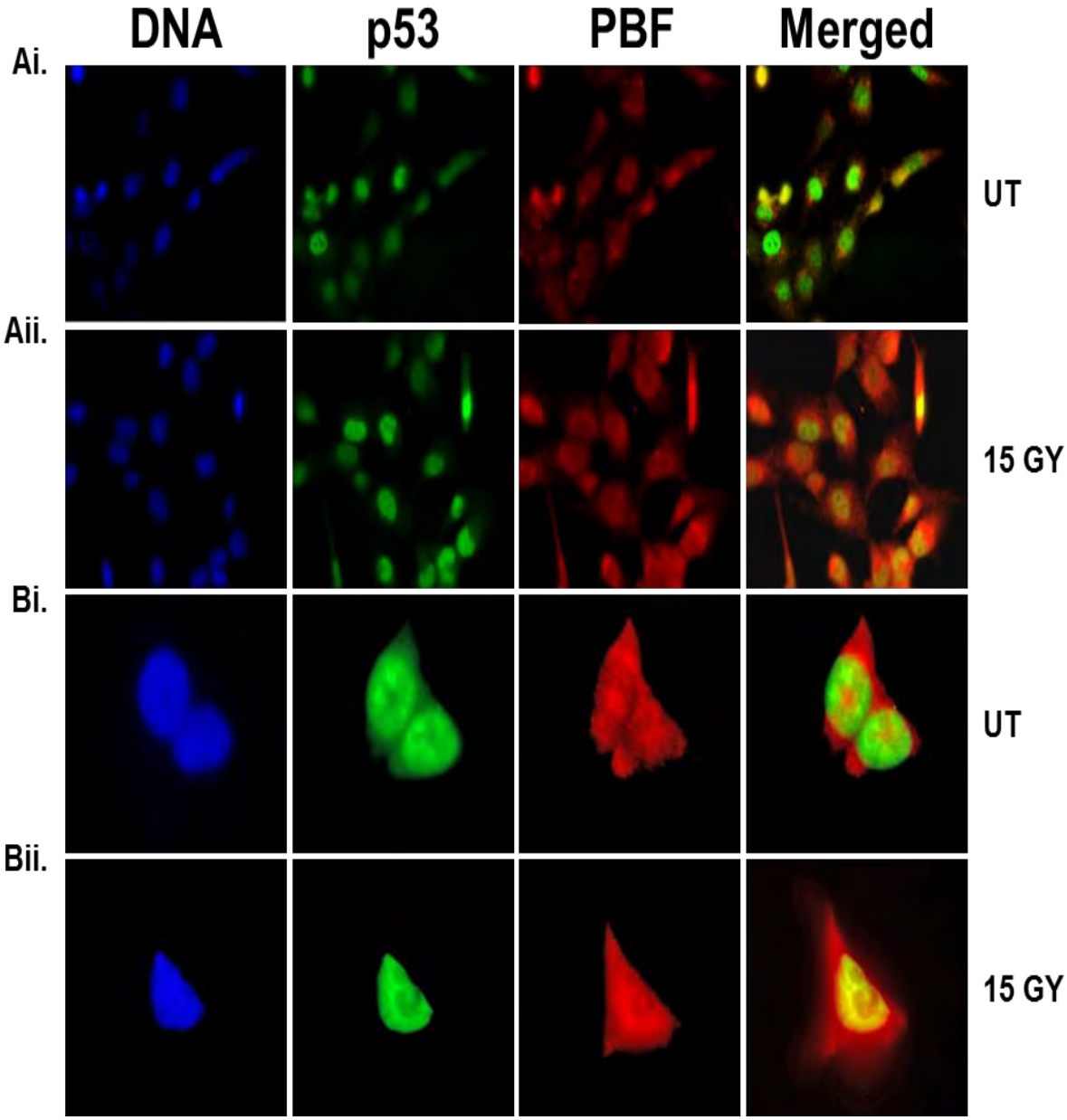


Figure 4.4. Localisation of PBF and p53 in TPC-1 cells and HCT116 cells at 40x and 100x magnification respectively. ***A:*** *p53 and PBF localisation in a cluster of TPC-1 cells in the presence and absence of DNA damage. p53 (green) was predominantly nuclear, with detectable levels within the cytosol. PBF (red) was ubiquitous throughout the cell. PBF and p53 displayed significant co-localisation within the nucleus as observed in the merged images (yellow). DNA damage resulted in greater p53 nuclear expression, resulting in a significant increase in nuclear co-localisation.* ***B:*** *p53 (green) and PBF (red) subcellular localisation in both the presence and absence of ionising radiation in HCT116 cells. Following treatment with ionising radiation p53 nuclear localisation significantly increased resulting in increased co-localisation of PBF and p53 in the nucleus (DAPI nuclear staining is shown in blue).*

4.3.3. PBF does not affect the subcellular localisation of p53

Previous data in this chapter demonstrated that PBF binds directly to p53, and that the 2 proteins colocalise within the cell both in the presence and absence of DNA damage. It was therefore of interest to determine whether PBF effects the subcellular localisation of p53. To do this PBF was overexpressed in thyroid papillary carcinoma cell lines and the colorectal cancer cell line, both in the presence and absence of DNA damage, and gross cytosolic and nuclear proteins were harvested. In the cytoplasmic fraction of TPC-1 cells, p53 levels remained constant when PBF was overexpressed compared to VO-transfected controls (Figure 4.5). p53 protein expression was increased following DNA damage as expected but there was no significant difference in p53 levels when PBF was overexpressed compared to controls. PBF overexpression had no significant effect on p53 levels within the nucleus, either in the presence or absence of DNA damage (Figure 4.5A; TPC-1 cells; n=3). HCT116 cells displayed similar results, where p53 levels in either fraction were unaltered in response to PBF overexpression in the presence or absence of DNA damage. (Figure 4.5B; HCT116 n=5).

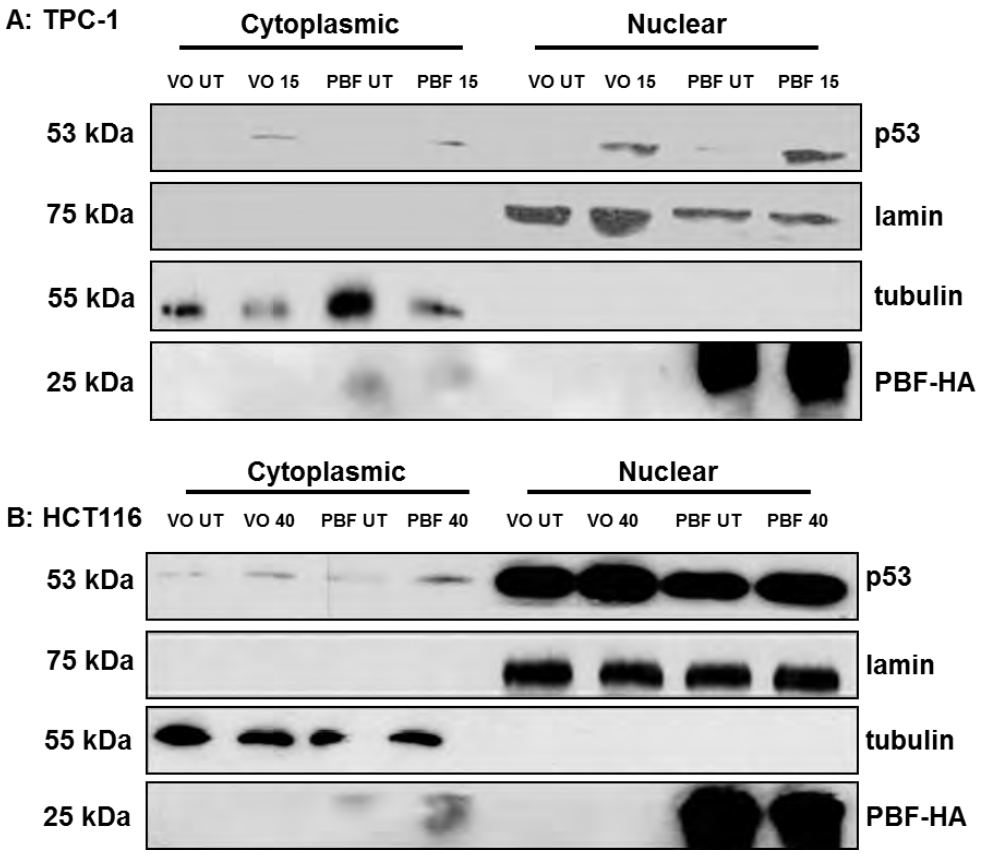


*Figure 4.5: The effects of PBF overexpression on p53 subcellular localisation in **A:** TPC-1 (n=3) and **B:** HCT116 cells (n=5) in the presence and absence of DNA damage (TPC-1 15 Gy, and HCT116 40 Gy). Assessment of relative total protein loading and purity of the Nuclear and cytoplasmic fractions were determined using specific lamin and α-tubulin antibodies respectively. (VO = vector only controls, UT = untreated).*

4.4. **Discussion**

4.4.1. PBF and p53 interact in vivo

Of the diverse range of viral and cellular oncogenes that have been characterised to functionally interact with p53 most elicit their functional properties via a direct physical interaction. Indeed, in thyroid papillary carcinomas, overexpression of HMGA factors in thyroid epithelial cells leads to cell transformation, by directly interacting with the C-terminal oligomerisation domain of p53, thereby functionally inactivating it (Frasca *et al*, 2006; Malaguarnera *et al*, 2007a; Pierantoni *et al*, 2007).

Whilst remaining a relatively uncharacterised protein, PBF has an emerging repertoire of cellular roles, providing evidence for its multi-functionality (Chien & Pei, 2000; Smith *et al*, 2009; Watkins *et al*, 2010a; Read *et al*, 2011). Additionally, oncogenic expression of PBF has been reported in thyroid tumours, with overexpression resulting in cell transformation *in vitro* and tumourigenesis *in vivo* (Stratford *et al*, 2005). We hypothesised that functional inactivation of p53 plays a role in transformation and progression of thyroid papillary carcinomas when p53 mutations are not present. Given the transforming and tumourigenic properties observed when PBF was overexpressed; we investigated the relationship between PBF and p53 in thyroid papillary carcinoma cells.

We recently demonstrated a potential interaction between PBF and p53 *in vitro* with the use of recombinant PBF and p53 protein. Initial GST pull-down assays demonstrated that L-α-[^{35}S]-methionine-labelled PBF interacts with the tumour suppressor protein p53 (Figure 1.13). Therefore to determine whether PBF and p53 interact within TPC-1, K1 and HCT116 cells, we performed co-immunoprecipitation

assays designed to explore potential interaction between endogenous PBF and p53. Numerous cellular conditions and protocols were used to optimise the conditions of the co-IP assays for ideal detection of the potential interaction between PBF and p53 (data not shown). Using the conditions described in Section 4.2.2, p53 was successfully co-immunoprecipitated with PBF when PBF was immunoprecipitated with our PBF antibody in TPC-1, K1 and HCT116 cells. Significantly more p53 was observed on Western blots 8 hours after cells were subjected to ionising γ-irradiation treatment. These data do not indicate that the stringency of interaction between PBF and p53 is increased following DNA damage, because more p53 is available for the interaction due to its stabilisation; however it does indicate that PBF and p53 interact within the intracellular environment in both the presence and absence of DNA damage. To provide further evidence that PBF binds directly to p53 we were able to co-immunoprecipitate PBF with p53 using the DO-1 p53 specific antibody, and also co-immunoprecipitate exogenous p53 with PBF. These data are in support of the GST-pulldown assays performed recently with our group. The interaction of viral and cellular proteins with p53 commonly results in the modulation of p53 function, and whilst evidence for the interaction between PBF and p53 is robust, the functional consequence of the interaction of PBF and p53 remains to be characterised.

4.4.2. PBF and p53 colocalisation

We demonstrated a direct interaction between PBF and p53 in TPC-1, K1 and HCT116 cells in both the presence and absence of DNA damage. Therefore to analyse the subcellular relationship between PBF and p53 we performed fluorescence immunocytochemistry experiments in both the presence and absence of γ-iradiation.

An increasing number of roles are being attributed to the PBF protein, and as such PBF is found throughout the cell, at the plasma membrane, within the cytosol, intracellular vesicles, and the nucleus (Chien & Pei, 2000; Smith *et al*, 2009, 2012a). We first analysed the subcellular relationship between PBF and p53 in COS-7 African green monkey kidney cells as these cells are much larger than TPC-1 or HCT116 cells, thus allowing optimal visualisation of subcellular localisation. In a manner typical of transformed cells lines, p53 was observed to be predominantly nuclear in COS-7, TPC-1 and HCT116 cells, with some cytosolic p53 within TPC-1 and HCT116 cells (Moro *et al*, 1995). Under normal cellular conditions (the absence of DNA damage) PBF and p53 appeared to colocalise within the nucleus of COS-7 cells and the nucleus and cytoplasm of TPC-1 cells. However, in contrast to the co-immunoprecipitation data, colocalisation between PBF and p53 was minimal in HCT116 cells in the absence of DNA damage. Following the induction of DNA damage, colocalisation of PBF with p53 was apparent and increased in all 3 cell lines, and this effect is most likely due to increased stability and accumulation of p53. The colocalisation of PBF with p53 in COS-7, TPC-1 and HCT116 cells supports the co-immunoprecipitation data generated in Section 4.3.1 and demonstrates that PBF and p53 colocalisation is predominantly nuclear following ionising radiation, indicating that PBF may functionally interact with p53 in the nucleus of these cells. It would be of great interest to observe the relationship between PBF and p53 in normal thyroid epithelial cells to determine the normal distribution and subcellular localisation of PBF and p53.

4.4.3. PBF does not affect the subcellular localisation of p53

Viral oncogenes, including the adenovirus E1B 55 kDa protein sequester p53 to cytoplasmic aggresomes by actively promoting nuclear export of p53, thereby inhibiting its function (Zhao & Liao, 2003; Mihara *et al*, 2003; Hartl *et al*, 2008). Aberrant expression of cellular oncogenes including the deubiquitinylating enzyme HAUSP also results in p53 sequestration in the cytoplasm due to impaired interaction with p53 (Moll *et al*, 1995; Becker *et al*, 2007).

We have characterised a robust interaction between PBF and p53 in our cell lines in both the presence and absence of γ-irradiation. Given the subcellular distribution of PBF and its characterised role in the subcellular distribution of important proteins (Chien & Pei, 2000; Smith *et al*, 2009, 2012a; Lewy *et al*, 2012) we aimed to assess the effects of PBF overexpression on the subcellular distribution of p53. To do this we performed subcellular fractionation studies aimed to isolate total cytoplasmic and nuclear proteins in TPC-1, K1 and HCT116 and determine total levels of p53 in each fraction following PBF overexpression in both the presence and absence of ionising radiation. After numerous independent observations we did not detect any difference in p53 subcellular localisation following PBF overexpression in the presence or absence of DNA damage in any of the cell lines. In keeping with the fluorescence immunocytochemistry experiments in Section 4.2.4, these data indicate, that whilst PBF and p53 colocalise and interact in TPC-1, K1 and HCT116 cells, PBF does not specifically alter p53 subcellular localisation despite having other characterised roles in the nuclear import of PTTG and the internalisation of NIS at the plasma membrane (Chien & Pei, 2000; Smith *et al*, 2009, 2012a). It would be useful to investigate whether specific mutants of PBF, such as one lacking the bipartite nuclear

localisation signal and which is sequestered within the cytoplasm, could alter p53 subcellular distribution.

4.4.4. Concluding statements

This chapter provides evidence that PBF interacts with both in the presence and absence of DNA damage within the intracellular environment. These findings support existing observations that recombinant PBF and p53 interact in cell-free GST-pulldown assays. The interaction of PBF with p53 suggests that in thyroid cancers where PBF is overexpressed, PBF may bind to p53, potentially abrogating its function in the absence of p53 mutations. Furthermore, PBF and p53 appear to colocalise within the nucleus of COS-7, TPC-1 and HCT116 transformed cells. Subcellular fractionation studies confirmed that the overexpression of PBF within TPC-1, K1 or HCT116 cells did not significantly alter p53 subcellular localisation, suggesting that the functional consequence of the interaction of PBF with p53 does not concern the subcellular redistribution of p53, as is the case with many cellular oncogenes. Data in the following chapters explore the functional consequences of the newly characterised interaction of PBF with p53 in thyroid cancer.

Chapter 5. **The effects of PBF on p53 mediated gene expression**

5.1. **Introduction**

p53 acts as a node or pivot for incoming stress signals, which are then transduced largely through the ability of p53 to act as a transcription factor to bring about a plethora of cellular responses that ultimately provide preventative measures against malignant transformation (Vogelstein *et al*, 2000; Meek, 2004; Brooks & Gu, 2006). For example, in its role as a master regulator, p53 transcriptional control expands across a large number of genes encompassing a diverse range of biological activities. These genes amongst others, control DNA metabolism, cell cycle arrest, angiogenesis, senescence and apoptosis (Vogelstein *et al*, 2000). However, in its function as a tumour suppressor the primary roles of p53 are in DNA repair, cell cycle arrest and apoptosis (Meek, 2004). As described extensively in Section 1.2, p53 is rapidly stabilised due to multi-site phosphorylation of both HDM2 and p53 following a genotoxic insult. In the case of DNA DSBs after exposure to ionising radiation, p53 is initially phosphorylated at ser-15 by the DNA damage kinase ATM. This event is crucial for the inhibition of p53 degradation and allows further post-translational modification of p53 to bring about its activity. Initial phosphorylation of Ser-15 primes the p53 protein for a subsequent cascade of post translational modifications (as many as 17 sites undergo phosphorylation and acetylation) which disrupt the interaction of p53 with HDM2. Furthermore, post-translational modification of p53 stimulates recruitment of transcription factors including p300 and CBP. These factors are not only responsible for the transcriptional stimulation of p53 target genes, but also acetylate critical lysine residues within the p53 protein that are normally ubiquitinated as part of the negative regulation of p53. Other residues are posttranslationally regulated in response to DNA DSBs. For example many residues

within the N and C-terminus of the protein are deemed important for specific promoter selectivity and for site-specific DNA binding.

Many viral and cellular oncogenes are responsible for the disruption of p53-mediated gene transcription. For instance aberrant expression or mutation of HDM2, MDMX, ARF and USP10 results in disruption of p53 activity by altering its stability (Lee & Gu, 2009; Gilkes *et al*, 2006; Yuan *et al*, 2010). ARF, by example, is a tumour suppressor protein with a role in p53-mediated gene transactivation. ARF activation results in the disruption of the E3 ligase activity of HDM2, causing p53 stabilisation and increasing p53 transcriptional activity. Furthermore, disruption of the p53 pathway and related transcriptional activities are associated with loss of ARF function (Sherr, 2006; Kamijo *et al*, 1998; Pomerantz *et al*, 1998). Additionally the oncogene MDMX directly associates with HDM2 to perform distinct yet co-operative functions in p53 inactivation (Shvarts *et al*, 1996; Hu *et al*, 2006). For example, MDMX augments the ubiquitin ligase activity of HDM2, and despite possessing no intrinsic ubiquitin ligase (E3) activity, can still inhibit p53 functionality (Okamoto *et al*, 2009). These findings were determined using the potent p53 activator Nutlin, where administration of Nutlin to transformed cells overexpressing HDMX was not sufficient to activate p53 (Hu *et al*, 2006). Such examples, coupled with the oncogenes described in Section 4.1, demonstrate that p53 inactivation can occur in the absence of p53 mutation, and significantly affect the ability of p53 to transcribe target genes in response to cellular stresses such as DNA DSBs.

PBF is a novel regulator of NIS expression and function, and despite functionally interacting with NIS to alter its subcellular distribution, also directly represses NIS mRNA expression, thereby abrogating the efficiency of radioiodide uptake when

overexpressed (Smith *et al*, 2009; Read *et al*, 2011; Smith *et al*, 2012a, 2011). Data in 0 demonstrate the physical interaction between PBF and p53 in TPC-1, K1 and HCT116 cells, and that this interaction may occur within the nucleus of these cells. To determine the effects that such an interaction might have on the activity of p53, the effects of PBF overexpression on the ability of p53 to drive the transcription of target genes was analysed. To do this we aimed to make use of a p53 signalling pathway PCR array (SA biosciences, Qiagen). This array profiles the expression of 84 p53 related genes involved in the processes of apoptosis, the cell cycle, cell growth, proliferation, differentiation and DNA repair using real-time PCR.

The overall aims of this chapter were therefore to determine the functional consequences of the interaction between PBF and p53 with regard to p53 transcriptional activity, both in the presence and absence of ionising radiation, using focused microarrays and *TaqMan* based assays.

5.2. **Materials and methods**

5.2.1. Cell lines and plasmids

TPC-1, K1 and HCT116 cells were routinely subcultured using the methods described in Section 2.1. For experiments requiring transient PBF overexpression, and /or γ-irradiation exposure, cells were treated using the procedures described in Section 3.3. For PBF siRNA knockdown experiments, K1 and TPC-1 cells were seeded into 6-well dishes at a density of $1x10^5$ cells per well and incubated for 24 hours prior to siRNA delivery. siRNA was delivered at a concentration of 100 nM (either scrambled, negative control #1 siRNA or PBF specific siRNA #14399 and #147350 mixed in equal quantities, Ambion, Grand Island NY, USA) in serum free

optiMEM™. Cells were incubated in 1 ml of serum free optiMEM™ 30 minutes prior to transfection. siRNA and Lipofectamine (6 µl Lipofectamine per reaction) were prepared in separate 100 µl volumes of serum free optiMEM™ for delivery into 6-well dishes. The 2 solutions were incubated at room temperature for 5 minutes before combining siRNA- optiMEM™ with lipofectamine 2000™- optiMEM™. After 15 minutes of incubation the siRNA-lipofectamine 2000™ complexes were delivered drop-wise on to cells in serum free optiMEM™. Cells were then incubated for a minimum of 72 hours to encourage maximal knockdown of PBF (Watkins *et al*, 2010a; Read *et al*, 2011).

5.2.2. SDS-PAGE/Western blotting

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see Section 2.6). Blocked membranes were subsequently incubated with primary antibodies. Antibodies used were our rabbit anti-human PBF-8 polyclonal (1:1000) (Stratford *et al,* 2005), rabbit anti-human HA (Covance) Mouse anti-human p53 (D0-1) monoclonal at 1:5000 (Santa Cruz, CA, USA), monoclonal anti-β-actin clone AC-15, (used at 1:10,000, Sigma-Aldrich, Poole, UK).

5.2.3. RNA extraction and QT-PCR

Total RNA was extracted and quantified from TPC-1, K1 and HCT116 cells as described in Section 2.4 for eventual use with *TaqMan* based assays. To ensure the quality and purity of RNA for use within focused microarrays, Total cellular RNA was isolated from TPC-1 cells using an RNeasy Minikit (Qiagen, UK), as per the manufacturer's instructions. This Kit incorporates and additional DNase step to irradicate possible genomic and plasmid DNA carry over, providing greater quality RNA samples.

Reverse transcription and QT-PCR techniques were as described in Sections 2.4 and 2.5. Primers and probes for *PBF* mRNA detection were designed as described above (see Section 2.5) and the sequences are given in Table 3-1. Gene-specific expression assays for CDKN1A, HDM2, E2F3, FAS, APAF-1, NF-1 and BRCA2 were purchased from Applied Biosystems (Warrington, UK).

5.2.4. Focused Microarrays

p53 pathway specific focused microarrays (SA Biosciences, Qiagen, UK) were performed with 1µg of cDNA per sample as per the manufacturer's instructions. 384-well focused microarray plates were analysed using the ABI PCR 7900 Sequence Detection System.

5.2.5. Statistical analysis

Data were analysed as described in Section 2.8.

5.3. **Results**

5.3.1. The effects of PBF overexpression on CDKN1A and HDM2 mRNA expression

In order to determine the effects of PBF overexpression on p53-mediated gene induction, TPC-1 cells were transiently transfected with our PBF-pCDNA3 plasmid for 24 hours to allow overexpression of PBF protein before treatment with 15 Gy of ionising radiation. Total cellular RNA was then harvested 8 hours post treatment for measurement of p53-mediated gene activation. SDS-PAGE/Western blotting was used to confirm that all samples were successfully overexpressing PBF protein and

that p53 protein expression was induced following DNA damage (Figure 5.1A); and the assessment of PBF overexpression on the induction of CDKN1A and HDM2 mRNA expression was then measured using QT RT-PCR. PBF overexpression in TPC-1 cells had no significant effect on CDKN1A mRNA expression in the absence of DNA damage (*n*=4; *p*=NS, Figure 5.1B). CDKN1A mRNA expression was significantly induced in VO-transfected TPC-1 cells 8 hours after treatment with 15 Gy γ-irradiation at levels similar to those observed in Figure 3.5 (5.17-fold; *n*=4; *p*<0.001; Figure 5.1B). Interestingly when PBF was overexpressed a repeatable 20 % reduction in CDKN1A mRNA expression was observed compared to VO-transfected controls (reduced to 4.15-fold; *n*=4; *p*=*NS*). However, this result did not reach statistical significance. Additionally the effects of PBF overexpression on the induction of HDM2 mRNA expression were assessed. Once again PBF overexpression alone did not significantly affect HDM2 gene activity in the absence of ionising radiation (*n*=4; *p*=NS, Figure 5.1C). HDM2 mRNA expression was significantly elevated in response to ionising radiation (3.9-fold; *n*=4; *p*<0.001). Transient overexpression of PBF had no effect on the induction of this gene, where cells overexpressing PBF exhibited a 3.6-fold induction of HDM2 mRNA expression in response to ionising radiation (*n*=4; *p*=NS; see Figure 5.1C).

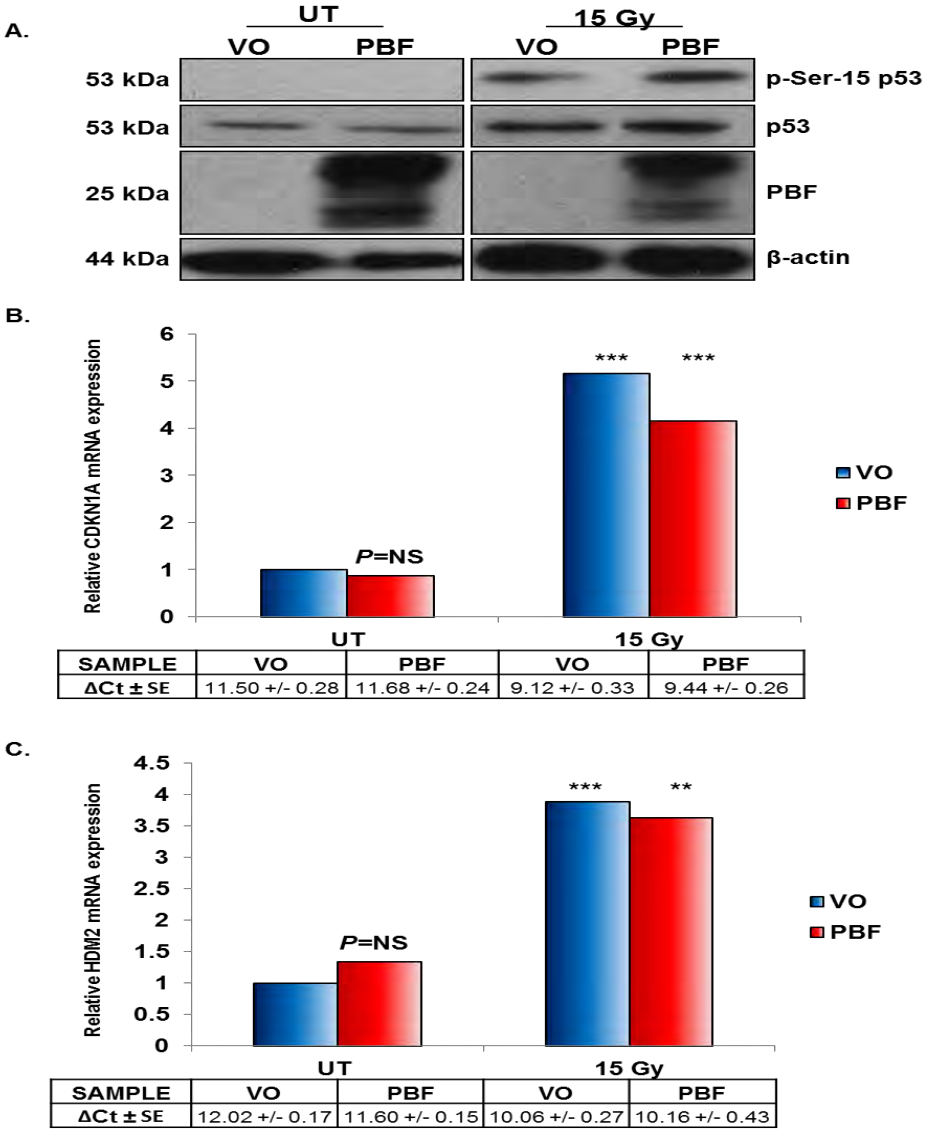
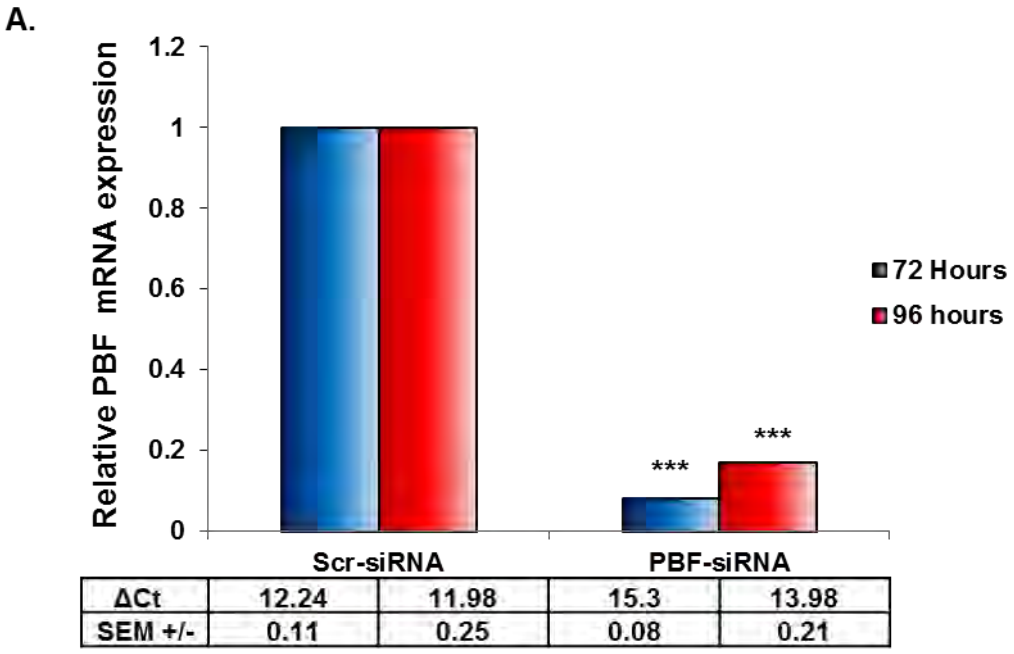


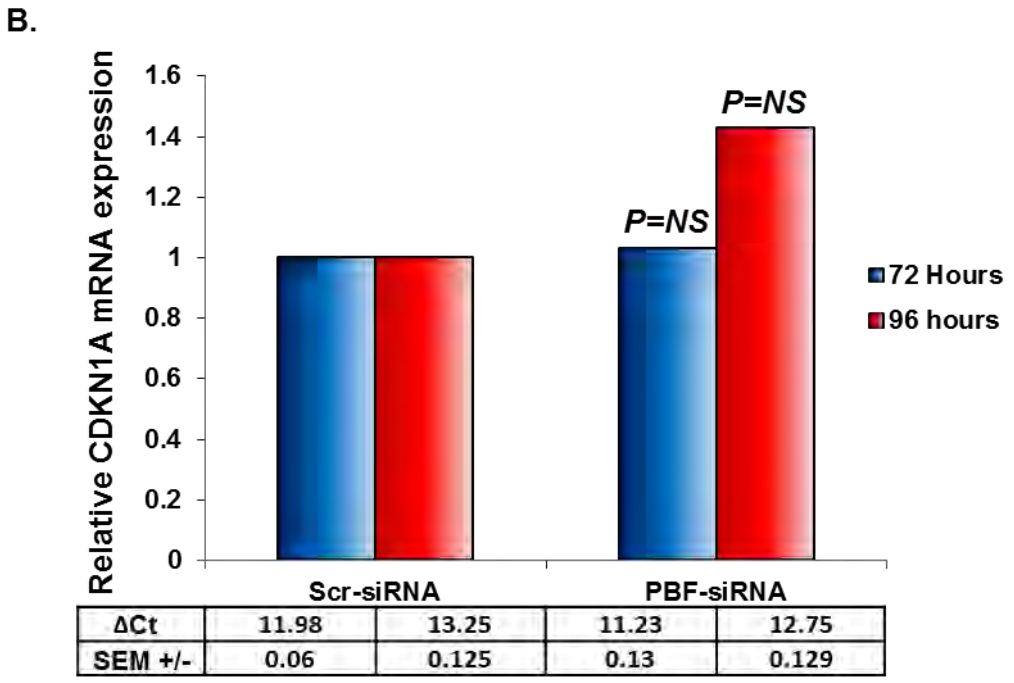
Figure 5.1: The effects of PBF overexpression on p53-mediated induction of CDKN1A and HDM2 mRNA expression in response to DNA damage in TPC-1 cells. ***A:*** *Representative Western blot demonstrating successful induction of p53 stability and phosphorylation at serine-15 in response 8 hours after treatment with 15 Gy γ-irradiation, and successful transient overexpression of PBF (n=4).* ***B:*** *The effects of PBF overexpression on the induction of CDKN1A mRNA expression following ionising radiation treatment. PBF overexpression alone had no effect on CDKN1A mRNA expression (n=4, P=NS). Treatment with ionising radiation significantly induced CDKN1A mRNA expression (5.17-fold; n=4; p<0.001), CDKN1A expression was reduced by ~20 % following PBF overexpression, but this effect did not reach statistical significance.* ***C:*** *The effects of PBF overexpression on the induction of HDM2 mRNA expression following ionising radiation treatment. Again, PBF overexpression alone had no effect on HDM2 mRNA expression (n=4; p<0.05). HDM2 expression was significantly induced following treatment with ionising radiation (3.9-fold; n=4; p<0.001). However, overexpression of PBF did not affect the induction of this gene (3.6-fold; n=4; p<0.01).*

5.3.1.1. *The effects of PBF siRNA knockdown on CDKN1A mRNA expression*

In addition to analysing the effects of PBF overexpression on the induction of CDKN1A mRNA expression in response to ionising radiation, the effects of PBF knockdown via delivery of specific siRNAs was assessed in HCT116 cells (Figure 5.2). Previous studies from our laboratory confirm that PBF protein is depleted significantly 72 hours after delivery of a combination of PBF-specific siRNAs (Read *et al*, 2011; Watkins *et al*, 2010a). CDKN1A mRNA expression was assessed both 72 hours and 96 hours after PBF siRNA delivery, and 8 hours after exposure to 40 Gy γ-irradiation. PBF mRNA expression was confirmed to be significantly reduced even 96 hours after siRNA delivery with 92 % knockdown of PBF after 72 hours (n=3; p<0.001) and 83 % at 96 hours (n=3; p<0.001) compared to cells transfected with a scrambled siRNA control (see Figure 5.2A) . Despite achieving desirable levels of PBF knockdown in HCT116 cells, there was no significant effect on p21 mRNA expression after DNA damage, where CDKN1A mRNA expression was increased to 1.02-fold (n=3*; p=NS*) after 72 hours and to 1.4-fold after 96 hours (n=3; *p=NS*) compared to Scr-siRNA treated controls (Figure 5.2B).



ΔCt	12.24	11.98	15.3	13.98
SEM +/-	0.11	0.25	0.08	0.21



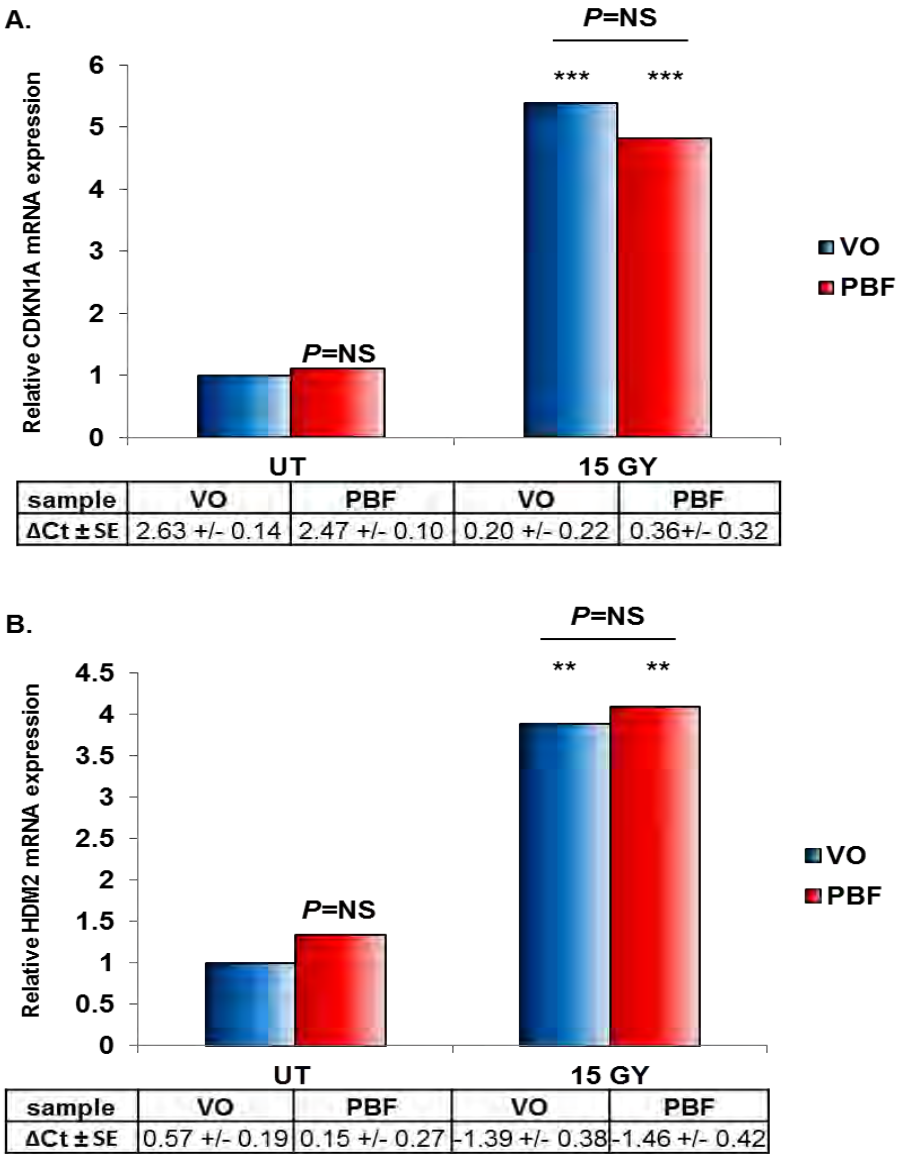
ΔCt	11.98	13.25	11.23	12.75
SEM +/-	0.06	0.125	0.13	0.129

Figure 5.2: The effects of PBF siRNA knockdown on the induction of p21 mRNA expression following treatment with 40 Gy of γ-irradiation in HCT116 cells. ***A:*** *PBF mRNA was significantly reduced following siRNA delivery 96 hours after transfection (92% reduction after 72 hours and 83 % at 96 hours; n=3; p<0.001).* ***B:*** *QT-PCR revealed that siRNA depletion of PBF did not have a significant effect on the induction of p21 mRNA expression in response to 40 Gy of γ-irradiation.*

5.3.2. Analysis of the effects of PBF overexpression on p53-mediated gene induction using focused microarrays

TPC-1 lysates were thus confirmed to be overexpressing PBF at the protein level, and to have significantly elevated levels of p53 protein expression in response to treatment with ionising radiation, along with the induction of ser-15 phosphorylation, a hallmark of p53 activity. Furthermore, analysis of CDKN1A and HDM2 mRNA expression in these samples revealed that both CDKN1A and HDM2 expression were significantly induced following treatment with ionising radiation, consistent with activation of p53 as a transcription factor. From these initial data and analysis of purified RNA, the quality of sample was deemed acceptable for use in a series of focused microarrays aimed at assessing the effects of PBF overexpression on the modulation of 84 p53-mediated genes in both the presence and absence of DNA damage (Section 5.1 and 5.2.4). CDKN1A and HDM2 were both present within the focused microarrays. Therefore we next gauged the technical consistency of our test samples using TaqMan gene expression assays and the focused microarrays (Figure 5.3). In keeping with the data in Figure 5.1, PBF overexpression had no effect on CDKN1A or HDM2 mRNA expression in the absence of DNA damage. Following treatment with 15 Gy γ-irradiation both CDKN1A and HDM2 mRNA expression were significantly induced to levels consistent with those detected using the TaqMan based assays (CDKN1A, 5.38-fold; n=3; $p<0.001$ and HDM2, 3.9-fold; n=3; $p<0.01$; Figure 5.3A+B). PBF overexpression did not significantly affect the magnitude of either CDKN1A or HDM2 mRNA expression in response to ionising radiation, where both genes remained significantly elevated (CDKN1A, 4.8-fold, n=3; $p<0.001$ and

HDM2, 4.09-fold, n=3; p<0.01; Figure 5.3A+B). These data indicate technical consistency between the cDNA array and *TaqMan* RT-PCR analysis.



sample	VO	PBF	VO	PBF
ΔCt ± SE	2.63 +/- 0.14	2.47 +/- 0.10	0.20 +/- 0.22	0.36+/- 0.32

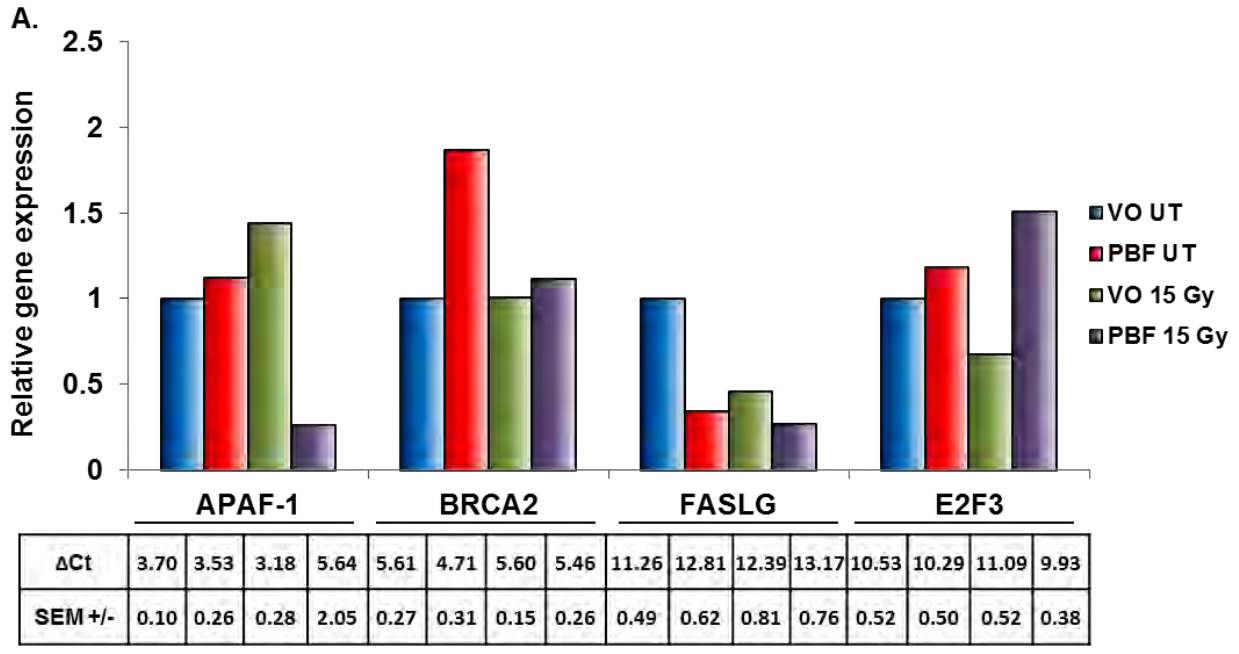
sample	VO	PBF	VO	PBF
ΔCt ± SE	0.57 +/- 0.19	0.15 +/- 0.27	-1.39 +/- 0.38	-1.46 +/- 0.42

Figure 5.3: The effects of PBF overexpression on the induction of CDKN1A and HDM2 mRNA expression using data generated from the focused microarray. ***A:*** *PBF alone had no effect on the expression of CDKN1A transcription. In response to ionising radiation, CDKN1A mRNA expression was significantly elevated (5.4-fold; n=3; p<0.001). However, when PBF was overexpressed the magnitude of CDKN1A mRNA induction was reduced (4.8-fold; n=3; p<0.001) but this effect was not significant.* ***B:*** *Again, PBF overexpression alone did not affect the expression of*

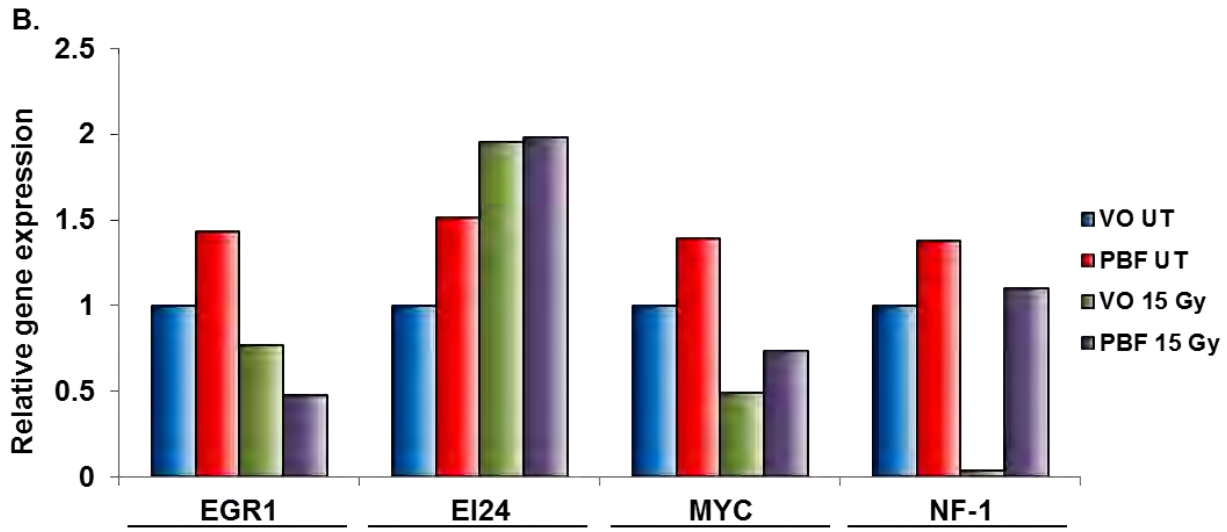
HDM2. In response to ionising radiation, HDM2 expression was significantly induced (3.88-fold; n=3; p<0.01). However, there was no significant effect on HDM2 induction following treatment with γ-irradiation when PBF was overexpressed, with HDM2 induced to the same magnitude as VO controls (4-fold; n=3; p<0.01).

5.3.2.1. *Analysis of focused microarray data*

With the integrity of TPC-1 samples persisting between both TaqMan gene expression assays and the focused microarray data, we analysed the effects of PBF overexpression on the induction of 84 important genes mediated directly by p53 in response to ionising radiation (Figure 5.4). Surprisingly, PBF overexpression had minimal effect on the 84 p53 mediated genes, and based on 3 separate focused microarray plates PBF had no significant effect on the magnitude of p53-mediated gene transcription. Whilst PBF had no significant effect on the 84, 8 genes (APAF-1, BRCA2, FASLG, E2F3, EGR1, EI24, MYC and NF-1) from the microarray are represented in figure 5.4. Of the 84 genes assessed, these 8 genes appeared to be the most altered in response to PBF overexpression (Figure 5.4).



ΔCt	3.70	3.53	3.18	5.64	5.61	4.71	5.60	5.46	11.26	12.81	12.39	13.17	10.53	10.29	11.09	9.93
SEM +/-	0.10	0.26	0.28	2.05	0.27	0.31	0.15	0.26	0.49	0.62	0.81	0.76	0.52	0.50	0.52	0.38



ΔCt	6.82	6.30	7.20	7.89	4.10	3.51	3.14	3.12	2.21	1.74	3.24	2.66	4.60	4.13	9.38	4.46
SEM +/-	0.30	0.44	0.16	0.28	0.08	0.22	0.23	0.43	0.12	0.30	0.40	0.17	0.33	0.15	4.21	0.35

Figure 5.4 Expression data representing the most altered genes based on the average of 3 separate focused microarrays in TPC-1 cells. ***A:*** *The effects of PBF overexpression on the gene expression of APAF-1, BRCA2, FASLG and E2F3 in the presence and absence of ionising radiation* ***B:*** *The effects of PBF overexpression on the gene expression of EGR1, EI24, MYC and NF-1 in the presence and absence of ionising radiation. The effects of PBF on the expression of these genes did not reach statistical significance (n=3).*

APAF-1 (apoptotic peptidase activating factor) is a central component of the intrinsic pathway of apoptosis. This protein is released along with cytochrome C from the mitochondrial membrane during the onset of apoptosis, resulting in its oligomersiation to form a multi-protein complex known as the apoptosome (Li *et al*, 1997; Robles *et al*, 2001; Soengas *et al*, 1999). Whilst the effects of PBF on APAF-1 expression did not reach statistical significance, a reduction in APAF-1 expression was observed when PBF was overexpressed following exposure to γ-irradiation by an average of 70 % compared to VO controls, although the standard error for these samples was high. PBF overexpression alone had no apparent effect on APAF-1 mRNA expression compared to negative controls (see Figure 5.4A).

BRCA2 (breast cancer 2 susceptibility protein), first identified in 1994 (Wooster *et al*, 1994), has an established role in tumourigenesis. For instance germline mutations in BRCA1 or BRCA2 account for 20-60% of breast cancer cases in families where multiple individuals are affected, equating to ~2-6% of all cases reported (Nathanson *et al*, 2001). BRCA2 is a reported transcriptional target of p53; however, in TPC-1 cells exposed to 15 Gy of γ-irradiation, BRCA2 mRNA expression remained similar to those of untreated controls, indicating that BRCA2 gene activity is unaltered at this dose of ionising radiation. Interestingly, when PBF was overexpressed a 1.9-fold induction of BRCA2 mRNA expression was detected, in the absence of DNA damage, indicating that PBF overexpression alone may influence BRCA2 mRNA expression (Figure 5.4A).

FASLG, a member of the tumour necrosis factor (TNF) family, encodes a type II transmembrane protein (FASL) with an established role in the regulation of the immune system through induction of apoptosis. The death receptor FAS (also known

as CD95) is activated by interaction with FASL, leading to the induction of pro-apoptotic responses. Interestingly in TPC-1 cells, FASL was repressed when PBF was overexpressed, compared to VO-transfected controls in the absence of DNA damage. This repression persisted following treatment with γ-irradiation, suggesting that PBF may alter the transcription of this gene (Figure 5.4A).

The E2F family of transcription factors including the E2F3 protein have been characterised with an important role in the control of cellular proliferation by directly regulating the activity of genes that mediate progression through the cell cycle, specifically via regulation of the G1/S phase transition (Sharma *et al*, 2006; Shan & Lee, 1994). PBF overexpression alone did not modify E2F3 gene transcription; however, ionising radiation treatment resulted in non-significant repression of this gene by ~33%. This effect was completely reversed following overexpression of PBF, where a 1.5-fold induction of E2F3 was observed, suggesting that normal repression of E2F3 activity in response to DNA damage may be perturbed by PBF overexpression (Figure 5.4A).

EGR-1 is a p53-mediated gene implicated in tumour progression. For instance, in prostate cancer (the most common malignancy in men, and frequent cause of cancer death) EGR-1 is frequently overexpressed and increasing expression levels correlate strongly with malignant progression (Scharnhorst *et al*, 2000, -1; Virolle *et al*, 2003, -1). TPC-1 cells were observed to express detectable levels of EGR-1 mRNA. PBF overexpression in these cells resulted in a minor increase in EGR-1 mRNA expression compared to VO-transfected controls in the absence of DNA damage (~1.4-fold; n=3; p=NS), thus indicating little or no effect of PBF on EGR-1 expression under normal conditions. Following induction of DNA damage, EGR-1 mRNA

expression was reduced by ~25 % in response to DNA damage in VO-transfected controls, and by 53 % in those cells overexpressing PBF (Figure 5.4B).

Etoposide inducible factor 2.4 is a DNA damage response gene, discovered following the characterisation of NIH3T3 cells treated with etoposide to induce apoptotic pathways (Lehar *et al*, 1996). NIH3T3 cells express wild-type p53 and as such undergo p53-mediated apoptosis following treatment with etoposide or γ-irradiation. Treatment with such DNA damaging agents revealed a clear association between p53 and EI24 expression (Gu *et al*, 2000). As expected EI24 mRNA expression was increased following induction of DNA damage via γ-irradiation treatment in both cells overexpressing PBF and in VO-transfected cells, to a similar magnitude (~2-fold; n=3). However in the absence of DNA damage, PBF overexpression resulted in a 1.5-fold increase in EI24 expression (n=3; *p*=NS; Figure 5.4B).

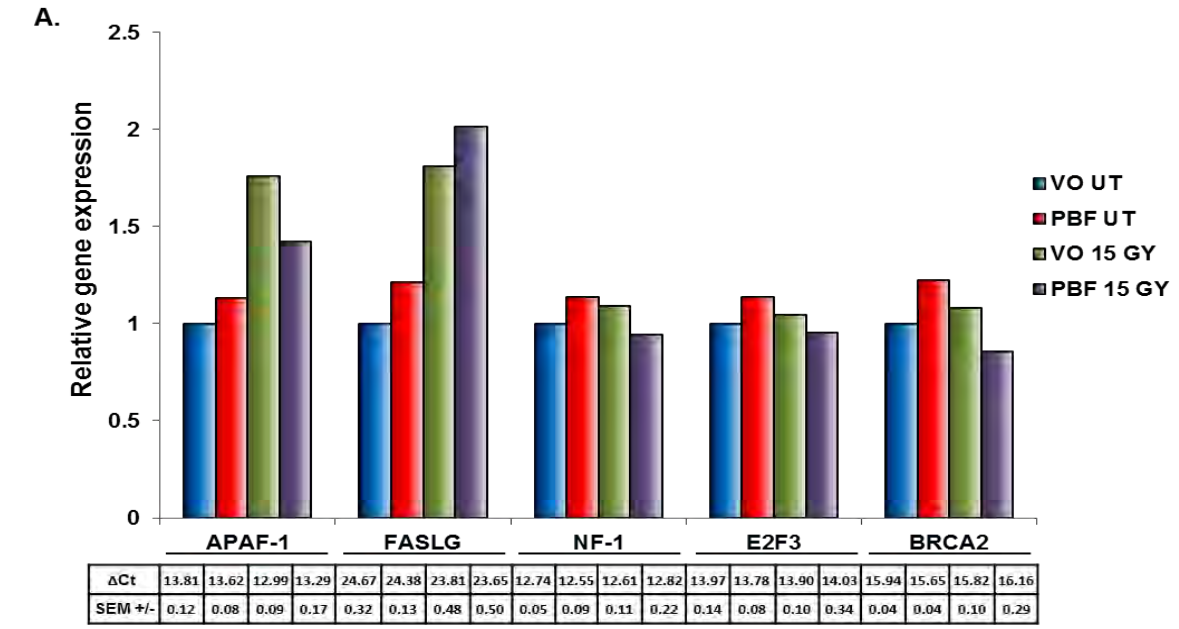
C-MYC is a proto-oncogene reported to be negatively regulated by p53. This proto-oncogene is a transcription factor with established roles in important cellular processes such as cell proliferation and apoptosis (Liao & Dickson, 2000). C-MYC mRNA expression was reduced by ~50 % in VO-transfected TPC-1 cells following DNA damage (n=3; p=NS). In cells overexpressing PBF, C-MYC expression was elevated in the absence of DNA damage to 1.4-fold and subsequent repression of C-MYC expression on response to DNA damage was attenuated to only 27 % (n=3; p=NS; Figure 5.4B).

Finally, the NF-1 tumour suppressor gene was analysed with regard to PBF overexpression in TPC-1 cells. PBF overexpression resulted in a 1.4-fold increase in NF-1 mRNA expression compared to controls, in the absence of DNA damage.

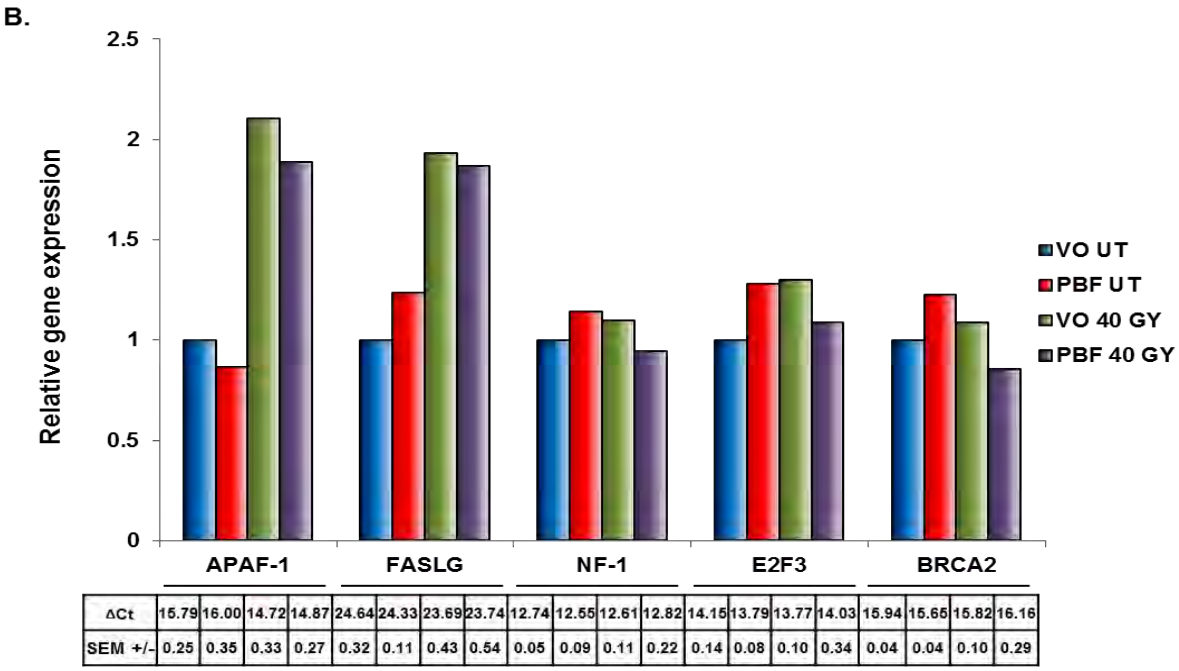
However, following genotoxic insult, VO-transfected cells displayed a ~90% reduction in NF-1 mRNA expression; by contrast, no repression of NF-1 mRNA expression was observed in cells overexpressing PBF (n=3; Figure 5.4B).

5.3.2.2. *Validation of focused microarray results*

Despite successfully overexpressing PBF in TPC-1 cells and inducing DNA damage by administration of 15 Gy of γ-irradiation, we observed no significant effects of PBF overexpression on any of the 84 p53-mediated genes within the focused microarrays in the presence or absence of ionising radiation, based on 3 separate focused microarrays. To confirm that PBF overexpression had no effect on p53-mediated gene transcription in our system, we chose 5 of the 8 genes presented in Figure 5.4 based on the magnitude of the effect of PBF and the importance of the p53-mediated gene in cell transformation and tumourigenesis, and assessed the validity of the findings from the microarray using specific *TaqMan* gene expression assays in TPC-1, K1 (not shown) and HCT116 cells (Figure 5.5). Those genes that were investigated further were APAF-1, FASLG, NF-1, E2F3 and BRCA2. These genes were analysed both 8 and 24 hours after induction of DNA damage in TPC-1 and HCT116 cells. Data representing the effects of PBF overexpression on the 5 array genes 8 hours post γ-irradiation treatment are presented in Figure 5.5. No significant effects of PBF overexpression were found.



ΔCt	13.81	13.62	12.99	13.29	24.67	24.38	23.81	23.65	12.74	12.55	12.61	12.82	13.97	13.78	13.90	14.03	15.94	15.65	15.82	16.16
SEM +/-	0.12	0.08	0.09	0.17	0.32	0.13	0.48	0.50	0.05	0.09	0.11	0.22	0.14	0.08	0.10	0.34	0.04	0.04	0.10	0.29



ΔCt	15.79	16.00	14.72	14.87	24.64	24.33	23.69	23.74	12.74	12.55	12.61	12.82	14.15	13.79	13.77	14.03	15.94	15.65	15.82	16.16
SEM +/-	0.25	0.35	0.33	0.27	0.32	0.11	0.43	0.54	0.05	0.09	0.11	0.22	0.14	0.08	0.10	0.34	0.04	0.04	0.10	0.29

Figure 5.5: Assessment of 5 of the 8 most altered genes from the focused microarray using specific Taqman-based assays. ***A:*** *TPC-1 cells (n=3) and* ***B:*** *HCT116 cells (n=3). No gene was validated to statistical significance.*

5.4. **Discussion**

The data in this chapter demonstrate an investigation in to the effects of PBF overexpression on the induction of p53-mediated gene expression in the presence and absence of ionising radiation. Overexpression of PBF and induction of p53 following treatment with γ-irradiation was confirmed in samples via SDS-PAGE/Western blotting, and the induction of the p53-mediated genes CDKN1A and HDM2 were observed using *TaqMan* based assays. Samples displaying significant induction of PBF overexpression following transfection, and consistent induction of p53 activity following γ-irradiation treatment, were chosen for use within a p53 signalling pathway PCR array (SA Biosciences, Qiagen) so that the global effects of PBF overexpression on p53 transcriptional activity could be measured.

5.4.1. The effects of PBF overexpression on CDKN1A and HDM2 mRNA expression

Many interacting partners of p53 when expressed at oncogenic levels serve to functionally inactivate p53 by inhibition of p53-mediated gene transcription (Lee & Gu, 2009; Gilkes *et al*, 2006; Yuan *et al*, 2010a). For example, overexpression of HGMA1 results in repression of CDKN1A transcription that would normally result in successful G1/S phase arrest after treatment with DNA damaging agents, thus serving to functionally inactivate p53 (Frasca *et al*, 2006; Malaguarnera *et al*, 2007a). In light of previous cellular examples, the functional consequences of the interaction between PBF and p53 on p53-mediated gene transcription were interrogated. Firstly the effects of PBF overexpression on the induction of CDKN1A and HDM2 mRNA expression were investigated in TPC-1 cells, as these genes were significantly induced at a dose of 15 Gy γ-irradiation (see Figure 3.5A), and are amongst the most

common genes transcribed by p53 in response to DNA damage. CDKN1A mRNA expression was significantly elevated in response to 15 Gy γ-irradiation (consistent with levels reported in Section 3.3.2). PBF overexpression resulted in a ~20 % repression of CDKN1A mRNA expression following DNA damage, but this effect did not reach statistical significance. PBF also had no effect on p53-mediated HDM2 induction. When specific PBF siRNAs were used to deplete PBF protein in HCT116 cells, PBF knockdown did not significantly alter the magnitude of CDKN1A induction in response to ionising radiation. These data suggest that despite interacting with p53, PBF has no significant effect on CDKN1A or HDM2 mRNA expression under the conditions reported. To thoroughly examine the effects of PBF overexpression on global p53 transcriptional activity, focused microarrays were used to test the induction of 84 p53 mediated genes when PBF was overexpressed.

5.4.2. The effects of PBF overexpression on p53-mediated gene induction

Samples for use within the p53 signalling array were tested for genomic DNA contamination using spectrophotometry, and viable samples were then selected for use within separate focused microarrays. Many genes were altered following a 15 Gy dose of ionising radiation, but the induction of only a small number of p53 related genes was affected by PBF in TPC-1 cells. The most altered of these genes were APAF-1, BRCA2, E2F3, EGR1, EI24, MYC and NF-1. However, statistical analysis of the effects of PBF overexpression on the activity of these p53-mediated genes confirmed that these changes were not statistically significant.

Given the importance of the transcriptional activities performed by p53 in the maintenance of cellular and genetic stability (Levine *et al,* 2001), 5 of the most altered genes from the focused microarray were chosen to be assessed using

TaqMan based assays; in an attempt to determine whether these effects were real. We therefore assessed the effects of PBF overexpression on APAF-1, BRCA2, FASL, E2F3 and NF-1 mRNA expression using *TaqMan* based expression assays.

APAF-1 is a critical downstream effector of p53-mediated apoptosis and silencing of the APAF-1 gene would appear to support oncogenesis and malignant progression. in fact, loss of APAF-1 has been reported in approximately half of all metastatic melanomas (Furukawa *et al*, 2005; Soengas *et al*, 2001). Furthermore in the context of thyroid cancer, a study by Aratake *et al* revealed that APAF-1 immunostaining was significantly reduced in histological samples within the more aggressive undifferentiated regions of thyroid cancer specimens compared to those areas that were well-differentiated, indicating that APAF-1 may play a role in thyroid tumour aggressiveness (Aratake *et al*, 2006). In TPC-1 thyroid papillary carcinoma cells, expression of APAF-1 was readily detectable, and therefore in response to ionising radiation it may be expected that APAF-1 mRNA expression would be significantly induced. However, in VO-transfected TPC-1 cells, APAF-1 mRNA expression was non-significantly elevated to ~1.5-fold, suggesting that 15 Gy of γ-irradiation does not significantly induce this gene in TPC-1 cells. APAF-1 expression is repressed in thyroid cancers and correlates with tumour aggressiveness (Aratake *et al*, 2006; Soengas *et al*, 2001). Therefore, given the important role of APAF-1 as a central component of the apoptotic pathway, loss or inhibition of p53-mediated APAF-1 upregulation would lead to impaired apoptotic responses to genotoxic insult. Interestingly, in cells overexpressing PBF, APAF-1 mRNA expression was reduced in response to ionising radiation treatment, suggesting that PBF overexpression may result in the repression of this gene. However, subsequent investigations using

TaqMan based assays confirmed that PBF overexpression had no significant effect on the modulation of APAF-1 gene activity in TPC-1, HCT116 or K1 cells. These results imply that PBF overexpression does not significantly affect the ability of p53 to modulate APAF-1 gene activity following treatment with 15 Gy of γ-irradiation.

.We next investigated the effects of PBF overexpression on the gene expression of the important DNA repair protein BRCA2. The function of BRCA2 was elucidated following the observation of BRCA2 deficient cells in culture; these cells were found to display increased sensitivity to ionising radiation. Furthermore, BRCA2-deficient cells accumulate chromosomal breaks and aberrant mitotic exchanges during culture (Marmorstein *et al*, 1998a; Moynahan *et al*, 2001). BRCA2 has also been observed to functionally interact with other well characterised DNA repair proteins, including RAD51, providing further evidence that BRCA2 plays a central role in DNA repair following the induction of DSBs, for example after exposure to ionising radiation (Marmorstein *et al*, 1998; Venkitaraman, 2001). As one would expect, VO-transfected TPC-1 cells displayed a ~2-fold increase in BRCA2 expression following the induction of DNA DSBs through administration of 15 Gy of γ-irradiation. However following PBF overexpression, the induction of BRCA2 in response to ionising radiation was not observed, indeed levels were similar to that of untreated controls. This result would imply that PBF represses p53 induction of BRCA2 expression following genotoxic insult. Further investigations into the effect of PBF overexpression on BRCA2 mRNA modulation following DNA damage were performed using specific BRCA2 *TaqMan* assays. However, no significant effects were observed in TPC-1, HCT116 or K1 cells. Again, these results imply that PBF

overexpression does not significantly affect the modulation of BRCA2 gene activity in response to ionising radiation.

Furthermore*, TaqMan* based assays confirmed that PBF had no effect on, FASL, E2F3 or NF-1 expression in the presence and absence of ionising radiation, in TPC-1, K1 and HCT116 cells both 8 and 24 hours post irradiation treatment. These results would suggest that the effects of PBF on p53-mediated gene expression are minimal, and that the interaction between PBF and p53 does not alter p53 transcriptional activity. Before utilising samples for analysis of the effects of PBF overexpression on p53-mediated gene expression, PBF was confirmed to be overexpressed using SDS-PAGE/Western blotting and the activation of p53 was visualised using the total p53 antibody DO-1. Furthermore samples for use within focused microarrays were assessed for the both PBF overexpression and p53 stabilisation, along with assessment of CDKN1A and HDM2 induction. Given that CKN1A and HDM2 were present within the focused microarray technical consistency between samples was therefore easily independently verified confirming the integrity and reliability of samples. This suggests that the results were reliable; and to further confirm that PBF had no significant effects on p53-mediated gene transcription 5 of the most altered genes from the focused microarray were assessed using *TaqMan* based assays. Taken together these results demonstrate a thorough investigation in to the effects of PBF on the induction of p53-mediated genes in both the presence and absence of ionising radiation. To further characterise the effects of PBF on p53-mediated gene expression it would be of interest to repeat these p53 signalling arrays in normal thyrocytes to determine the effects of PBF in untransformed cells. Furthermore,

assessment of the effects of PBF on p53-mediated gene expression in cell stably overexpressing PBF would alleviate the problem of transient transfection efficiency.

Whilst the principal molecular function of p53 concerns its action as a transcription factor, there are nonetheless well characterised and important transcriptional independent roles of p53, and aberrant control of these has significant implications in human cancers (Vousden & Prives, 2009; Speidel *et al*, 2006; Moll *et al*, 2005). Most, but not all, transcriptional independent roles appear to be heavily involved with the onset of apoptosis (Moll *et al*, 2005; Galy *et al*, 2001). For instance, p53 mutants lacking the ability to influence gene transcription have been characterised to promote apoptosis; most notably, p53 can directly activate the pro-apoptotic protein BAX (Brady & Attardi, 2010; Chipuk *et al*, 2004). These examples suggest that the interaction between PBF and p53, whilst having no effect on p53-mediated transcription, may prompt further study of the transcriptional independent roles of p53.

5.4.3. Concluding statements

Despite confirming that PBF was overexpressed in TCP-1 samples through Western blotting, and confirming p53 activity through Western blotting and *TaqMan* gene expression assays, PBF overexpression had no significant effect on 84 p53-mediated genes in the presence or absence of DNA damage (TPC-1; $n=3$), Furthermore, 5 of the most altered genes in the focused microarray were investigated further through *TaqMan* based assays in TPC-1, HCT116 and K1 cells, and no significant effects were observed. These results indicate that PBF overexpression does not significantly affect p53-mediated gene expression in TPC-1, HCT116 or K1 cells, and that the functional consequence of the interaction of PBF and p53 in these cells does not

aberrantly affect p53 transcriptional function. The next chapters explore the effects of PBF on p53 metabolism and assess the effects of PBF on transcription independent p53 functions

Chapter 6. **The effects of PBF overexpression on p53 biology**

6.1. **Introduction**

p53 is by necessity an intrinsically unstable protein, undergoing perpetual degradation by E3 ubiquitin ligases including HDM2. Continually transcribed and translated, p53 rapidly accumulates following a genotoxic insult due to inhibition of its degradation by HDM2 and other E3 ligases (Chen *et al*, 1993; Oliner *et al*, 1993; Kubbutat *et al*, 1997). HDM2 engages in a high affinity interaction with p53, by virtue of its large N-terminal p53 binding domain (see Figure 1.6). The functional interaction between the HDM2 and p53 proteins is necessary for p53 inactivation, where apart from ubiquitinating both itself and p53 (bringing about the proteasomal degradation of each protein), HDM2 masks the DNA binding domain of the p53 protein, also preventing p53 from acting as a transcription factor (Vogelstein *et al*, 2000; Brooks & Gu, 2006). HDM2 is considered the major physiological antagonist of p53 activity, although other proteins are emerging with characterised physiological role in p53 regulation such as HDMX/MDMX (or HDM4/MDM4), ARF and USP10. Clearly dysregulation of p53 metabolism has drastic consequences for cell physiology. Indeed oncogenic expression or mutation of HDM2, MDMX, ARF and USP10 results in disruption of p53 activity by altering its stability (Lee & Gu, 2009; Gilkes *et al*, 2006; Yuan *et al*, 2010).

Interestingly, in thyroid papillary carcinomas oncogenic expression of HDM2 itself has been reported to interfere with wild-type p53 function (Malaguarnera *et al*, 2007a). For example, in a large study involving a series of well-differentiated thyroid papillary cancer specimens, HDM2 was highly overexpressed, and expression levels correlated with poor clinical outcome (Horie *et al*, 2001; Jennings *et al*, 1995).

Oncogenic expression of HDMX (a potent agonist of p53 activity) has been reported in up to 20 % of colon and breast cancers and 65 % of retinoblastomas (Toledo & Wahl, 2007). Furthermore in glioblastoma multiforme (GBM), the most common form of primary brain tumour in adults, HDMX amplification occurs solely in the absence of p53 mutations, resulting in the functional inactivation of p53 by preventing p53 transactivation and targeting it for degradation via the ubiquitin pathway. That HDMX amplification occurs solely in the absence of p53 mutations in GBM demonstrates the potency of this oncogene in the functional inactivation of p53 in these tumours (Jin *et al*, 2010). More recently the protein USP10 has been characterised to interact directly with p53 and bring about its stabilisation. USP10 causes increased p53 stability by virtue of its ubiquitin specific protease activity. This activity causes the deubiquitination of p53 in the cytoplasm, thus bringing about the reversal of HDM2 mediated p53 degradation. USP10 overexpression was associated with increased p53 accumulation and decreased p53 ubiquitination (measured using anisomycin half-life assays and MG132 ubiquitin assays respectively). USP10, in fact, has been observed to be downregulated in a high percentage of clear cell carcinomas and overexpression leads to reduced cell growth in tumours expressing wild-type p53 (Yuan *et al*, 2010). This pertinent example demonstrates that novel cellular proteins are still emerging that bind to and modulate p53 function and when aberrantly expressed negatively affect p53 stability resulting in increased susceptibility to cellular transformation and tumourigenesis. The results from such studies have led to increased speculation that oncogene inactivation of p53 may, in fact, represent an earlier event in the progression of some cancers. However, an improved understanding of the biological consequences following disruption of p53 by different

oncogenes is still required before the sequence of molecular events in tumourigenesis can be better defined, especially in thyroid papillary carcinomas where mutations in p53 are rare.

We have previously determined that PBF is overexpressed in thyroid papillary cancers, and that oncogenic expression of PBF is transforming *in vitro*; and tumourigenic *in vivo* (Stratford *et al*, 2005). Data in Chapter 4 provide evidence to demonstrate that PBF acts as a novel binding partner of p53 (through co-immunoprecipitation assays and fluorescence immunocytochemistry assays) in human thyroid papillary carcinoma cell lines and a human colorectal carcinoma cell line. To follow on from these observations we aimed to characterise the effects of PBF on p53 metabolism. Thus, we attempted to assess the effects of PBF overexpression on p53 using classical anisomycin p53 half-life assays, and attempted to determine the effects of PBF overexpression on p53 ubiquitination using MG132 ubiquitin assays. Furthermore, given the close relationship between p53 and its master regulator HDM2, we performed co-immunoprecipitation assays to investigate the specific relationship between PBF and HDM2 in thyroid papillary carcinoma cell lines.

6.2. **Materials and methods**

6.2.1. Cell lines, plasmids and siRNAs

TPC-1, K1 and HCT116 cells were routinely subcultured using the methods described in Section 2.1. For experiments requiring transient PBF overexpression, and / or γ-irradiation exposure, cells were treated using the procedures described in

Section 3.3. For PBF siRNA experiments, PBF specific siRNA was delivered into TPC-1 and K1 cells using the methods described in Section 5.2.1.

6.2.2. p53 half-life assays

TPC-1, K1 and HCT16 cells were seeded into 6 well dishes at a density of $7.5x10^4$, $1x10^5$ and $2.5x10^5$ cells/well respectively. Cells were then transiently transfected with pCDNA3-PBF-HA, pCDNA3-PBF, pCDNA3-PBF-M13 (PBF-M13 contains a Y174A substitution thereby inhibiting the action of the NLS region) and pCDNA3 only or PBF specific siRNA or scrambled controls. Half-life assays to determine the effects of PBF on p53 stability were then performed 24 hours after transient overexpression of PBF or 72 hours after PBF specific siRNA delivery, to allow for maximal overexpression and knockdown of PBF respectively. Serum containing media was then removed and replaced with Anisomycin, delivered at a final concentration of 100 mM in 1 ml of serum free optiMEM™ per well at specific time-points (0, 60, 90 and 120 minutes). At the end of the 2 hour time-course, total cellular protein was promptly harvested and samples prepared for analysis via SDS-PAGE/Western blotting.

6.2.3. p53 ubiquitin assays

TPC-1, K1 and HCT16 cells were seeded into 6 well dishes at a density of $7.5x10^4$, $1x10^5$ and $2.5x10^5$ cells/well respectively. These cells were then transiently transfected with pCDNA3-PBF-HA and pCDNA3 to allow for the potential effects of PBF overexpression on p53 ubiquitination to be measured. Cells were subsequently treated with 20 μM MG132 (Sigma-Aldrich Poole, Dorset, UK), over a 120 minute time-course in a similar fashion to the anisomycin half-life assays described in Section 6.2.2. Protein was then promptly harvested and prepared for analysis via

SDS-PAGE/Western blotting in the manner described in Section 2.6. Ubiquitinated p53 was detected using the mouse anti-human p53 specific antibody DO-1.

6.2.4. Co-immunporecipitation assays

TPC-1, K1 and HCT116 cells growing in T25 culture flasks were harvested in 500 ul RIPA buffer with 60 µl/ml protease inhibitor (100 mM sodium chloride, 0.1 % Triton X-100 and 50 mM Tris (pH8.3)). Co-immunprecipitation assays were performed in a similar manner to Section 4.2.2. HDM2 was immunoprecipitated using a mouse anti-human HDM2 polyclonal antibody (HDM2 2αB, Courtesy of Dr Andrew Turnell, University of Birmingham, School of Cancer Sciences) at a concentration of 1:50 (antibody to lysate volume). The presence of co-immunoprecipitated PBF was detected via SDS-PAGE/Western blotting using our PBF specific antibody. Proteins from pre immunoprecipitated lysates were used to identify co-immunoprecipitated protein bands detected by the PBF antibody.

6.2.5. SDS-PAGE/Western blotting

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see Section 2.6). Blocked membranes were subsequently incubated with primary antibodies against p53 (DO-1, 1:1000), PBF (our PBF-8 polyclonal rabbit anti-human PBF specific antibody 1:1000) and HDM2 (2αB, courtesy of Dr Andrew Turnell, School of Cancer Sciences, University of Birmingham). After washing in TBS-T, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidise (Dakocytomation, UK) for 1 hour at room temperature before being visualised by the techniques described in Section 2.6.

6.2.6. RNA extraction and QT-PCR

Total RNA was extracted from TPC-1, K1 and HCT116 cells as described in Section 2.4. Reverse transcription and QT-PCR techniques were as described in Sections 2.4 and 2.5. Primers and probes for *PBF* mRNA detection were designed as described above (see Section 2.5) and the sequences are given in Table 3-1. Gene-specific expression assays for p53 *(Hs01034249_m1)* were purchased from Applied Biosystems (Warrington, UK).

6.2.7. Statistical analysis

Statistical analysis was performed using the methods described in Section 2.8.

6.3. **Results**

6.3.1. The effects of PBF on p53 protein expression and turnover

Given that a direct interaction between PBF and p53 was observed through the use of GST-pulldown and co-immunopreciptitation assays (Chapter 4), we considered the effects of PBF on p53 protein stability by performing p53 half-life assays. PBF was transiently transfected into TPC-1, K1 and HCT116 cells (with pCDNA3 vector-only controls) for 24 hours before replacing basal media with optiMEM™ containing 100 μM anisomycin (an inhibitor of *de novo* protein synthesis). Protein was then harvested from cells overexpressing PBF or vector-only 0, 60, 90 and 120 minutes after delivery of anisomycin, and the levels of total cellular p53 were observed via SDS-PAGE and Western blotting. VO-transfected TPC-1 cells displayed detectable levels of p53 across the 120 minute time-course, with levels of p53 decreasing at each time-point (Figure 6.1A). However, overexpression of PBF resulted in significantly increased turnover of p53 protein in TPC-1 cells, where levels of p53

were visibly lower on Western blots from 90 minutes of this time-course compared to vector only controls. Scanning densitometry confirmed a significant difference in p53 levels at 120 minutes, with cells overexpressing PBF having 10-fold lower p53 expression (n=4; p<0.05) than time-matched VO controls (Figure 6.1A). p53 expression was markedly different in K1 cells, appearing to remain constant throughout the 120 minute time course, suggesting that p53 may be more stable in this cell line. Again, Western blotting revealed that p53 turnover was increased following PBF overexpression, with levels reduced from 90 minutes. Scanning densitometry confirmed that p53 levels were significantly lower in cells overexpressing PBF after 120 minutes (~17-fold; n=4; p<0.01) when compared to VO controls (Figure 6.1B).

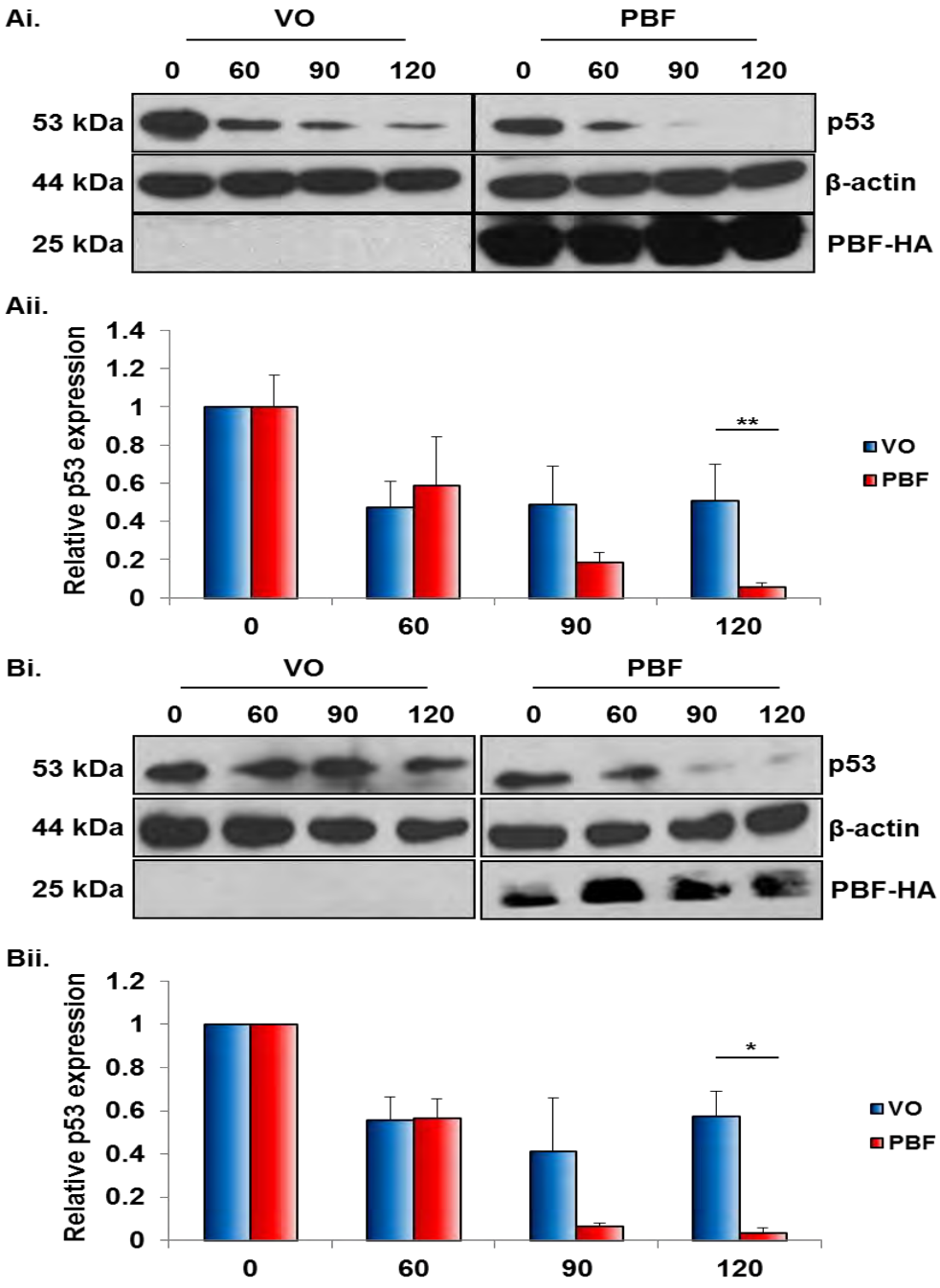
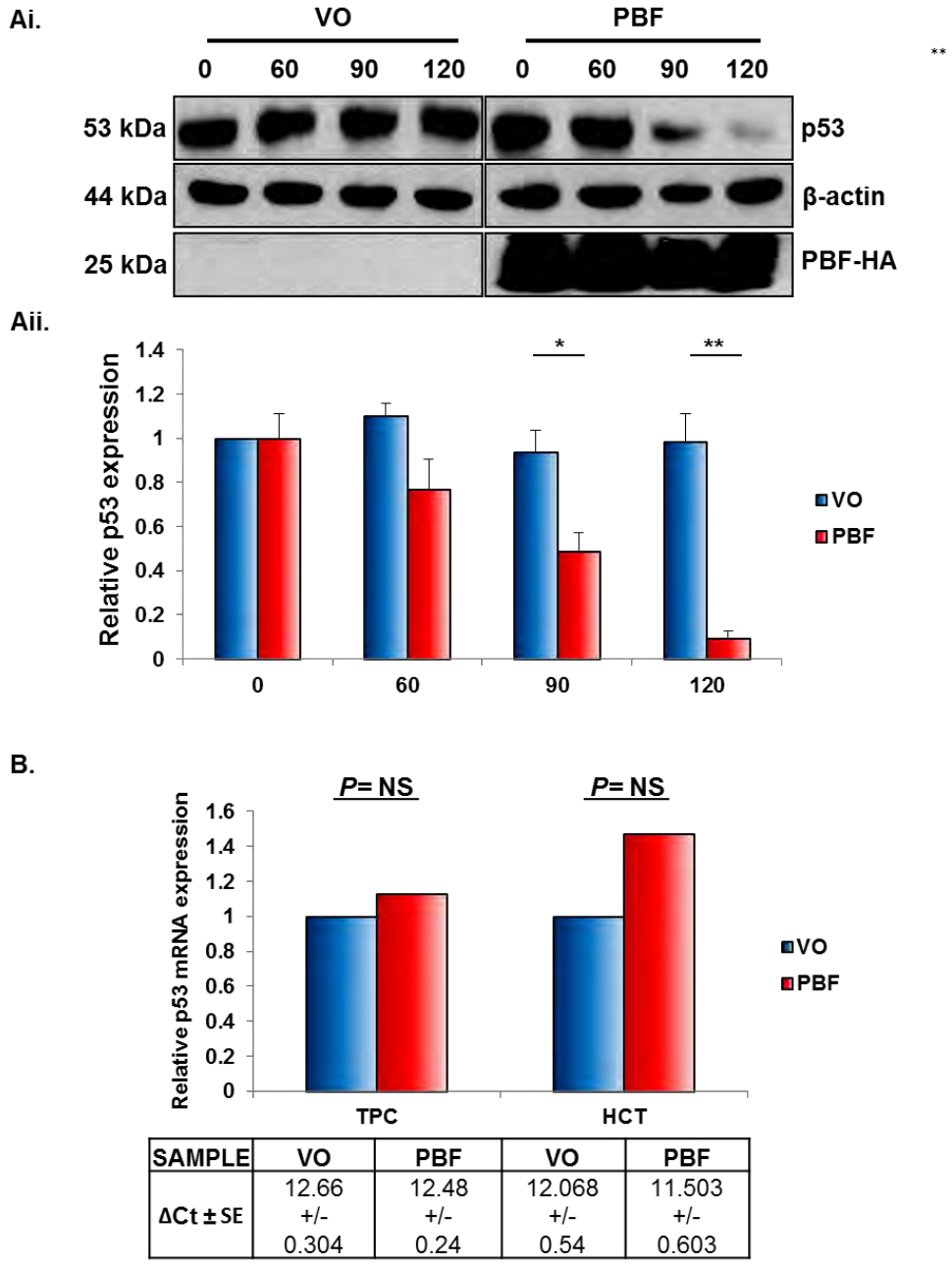


Figure 6.1: Western blot analysis and scanning densitometry to determine the effects of PBF overexpression on p53 half-life following treatment of cells with anisomycin to block de novo protein synthesis. ***Ai:*** *Western blot representing the effects of PBF overexpression on p53 stability in TPC-1 cells compared to vector only controls.* ***Aii:*** *Scanning densitometry revealed that PBF significantly reduced p53-half-life 120 minutes after administration of anisomycin (~10-fold; n=4; p<0.05).* ***Bi:*** *Western blots representing the effect of PBF overexpression on p53 half-life in K1 cells.* ***Bii.*** *Scanning densitometry confirmed that PBF overexpression also reduces p53 half-life in these cells after 120 minutes (~17-fold; n=4; p<0.01).*

The effects of PBF overexpression on p53 stability in HCT116 cells were also analysed (Figure 6.2). In a similar manner to K1 cells, p53 levels did not drop significantly over the 120 minute time-course, suggesting that p53 in these cells is also more stable than in TPC-1 cells. However, following PBF overexpression, p53 levels were rapidly reduced from 90 minutes of this time-course. In these cells scanning densitometry revealed that when PBF was overexpressed p53 levels were significantly reduced from 90 minutes (~2-fold at 90 minutes, n=4; p<0.05, and ~10-fold at 120 minutes, n=4; p<0.01) compared to time-matched VO controls (Figure 6.2). To investigate whether the effects of PBF overexpression were a result of reduced protein stability, or altered mRNA repression, the levels of p53 mRNA expression were observed following PBF overexpression in TPC-1 and HCT116 cells. QT RT-PCR did not reveal any significant difference in p53 mRNA expression when PBF was overexpressed in either cell line (TPC-1, 1.13-fold; n=3; p=NS, HCT116, 1.47-fold; n=3; p<0.05 and Figure 6.2A) compared to VO controls, indicating that PBF overexpression does indeed effect p53 half-life at the level of protein stability, and not mRNA expression (see Figure 6.2).

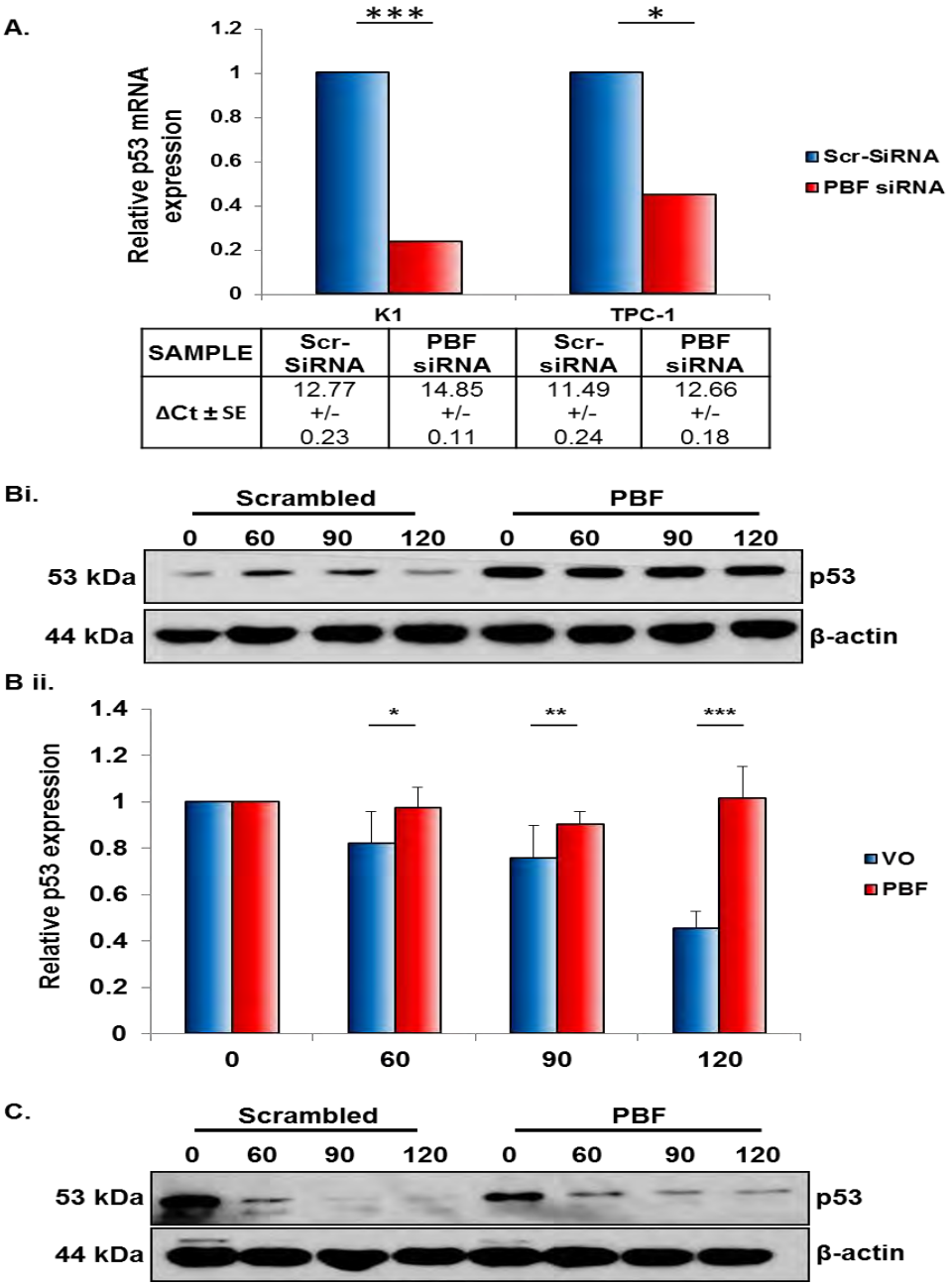


SAMPLE	VO	PBF	VO	PBF
ΔCt ± SE	12.66 +/- 0.304	12.48 +/- 0.24	12.068 +/- 0.54	11.503 +/- 0.603

Figure 6.2: Western blot analysis and scanning densitometry to determine the effects of PBF overexpression on p53 half-life following treatment of HCT116 cells with anisomycin to block de novo protein synthesis, and real-time PCR to determine the effects of PBF overexpression on p53 mRNA expression. ***Ai:*** *Western blot representing the effects of PBF overexpression on p53 stability in HCT116 cells compared to vector only controls.* ***Aii:*** *Scanning densitometry revealed that PBF significantly reduced p53-half-life from 90 minutes after administration of anisomycin (~2-fold at 90 minutes,* n=4*;* p<0.05*, and ~10-fold at 120 minutes,* n=4*;* p<0.01*) compared to time-matched VO controls.* ***B:*** *QT RT-PCR did not reveal any significant difference in p53 mRNA expression following PBF overexpression in either TPC-1 or HCT116 cells (TPC-1, 1.13-fold;* n=*3;* p=*NS, HCT116, 1.47-fold;* n=*3;* p=*NS).*

To interrogate the effects of PBF on p53 half-life further, we depleted PBF using specific PBF-siRNAs and assessed the stability of p53 in order to observe whether the inverse relationship held true (Figure 6.3). The most significant levels of PBF knockdown were achieved in K1 cells, where PBF was consistently knocked down by ~75 % or more even after 72 hours (n=4; p<0.001; Figure 6.3A). K1 cells were transfected with PBF-specific siRNAs before performing anisomycin half-life as described earlier in this Section. Interestingly, K1 cells transfected with PBF siRNAs displayed an increase in p53 protein expression at every stage of this time-course compared to scrambled siRNA treated controls (Figure 6.3B). Scanning densitometry revealed that PBF depletion was associated with a significant increase in p53 half-life from 60 minutes (n=4; p<0.05), resulting in a >3-fold increase in p53 levels (n=4; P<0.01) compared to scrambled controls after 120 minutes.

PBF specific siRNA delivery into TPC-1 cells resulted ~60 % depletion in PBF mRNA expression after 72 hours (n=4; p<0.05; Figure 6.3A). In keeping with the reduced potency of PBF knockdown, TPC-1 cells transfected with PBF-specific siRNAs did not display an obvious increase in p53 expression at time 0 (Figure 6.3C). However, PBF knockdown in TPC-1 cells did result in an observable increase in p53 half-life. For instance in TPC-1 cells treated with scrambled siRNA controls, p53 levels decreased rapidly over the 120 minute time course, in keeping with our previous data (Figure 6.3A). Nevertheless, TPC-1 cells treated with PBF specific siRNAs displayed increased levels of p53 from 90 minutes of this time-course, with levels remaining highly detectable even after 120 minutes (n=3;Figure 6.3C). These experiments were also repeated in HCT116 cells with similar results obtained (n=1, data not shown).



SAMPLE	Scr-SiRNA	PBF siRNA	Scr-siRNA	PBF siRNA
ΔCt ± SE	12.77 +/- 0.23	14.85 +/- 0.11	11.49 +/- 0.24	12.66 +/- 0.18

Figure 6.3: the effects of PBF siRNA knockdown on p53 half-life in K1 and TPC-1 cells measured through the use of anisomycin half-life assays. ***A:*** QT RT-PCR revealed that PBF specific siRNA knockdown in K1 and TPC-1 resulted in significant knockdown of PBF mRNA expression even after 72 hours (K1, ~75 %; n=4; p<0.001, TPC-1, ~60 %; n=3; p<0.05). **B:** *Representative Western blot demonstrating the effects of PBF knockdown on p53 stability in K1 cells.* ***Bii:*** *scanning densitometry revealed a significant increase in p53 half-life from 60 minutes resulting in > 3-fold increase in p53 levels after 120 minutes (*n=4; p<0.001). **C:** representative Western blot displaying the effects of PBF knockdown on p53 stability in TPC-1 cells (n=3).

Finally, we investigated the effects of a specific mutant of PBF (PBF-M13) to further describe the effects of PBF on p53 stability. PBF-M13 (courtesy of Dr Vicki Smith, School of Clinical and Experimental Medicine, University of Birmingham), contains a single amino acid substitution at tyrosine 174 (Figure 6.4A). This mutation prevents the phosphorylation of PBF by specific tyrosine kinases and causes sequestration of PBF at the plasma membrane, an area we did not associate with p53 co-localisation (Dr Vicki Smith unpublished data and Section 4.3.2). Our previous data strongly suggests that PBF binds directly to p53 and affects its half-life. Therefore, we repeated anisomycin half-life assays in TPC-1 cells following overexpression of PBF-M13, and observed its effects on p53 stability compared to overexpression of WT PBF and VO controls. Overexpression of WT PBF resulted in a visible decrease in p53 half-life, compared to VO controls; with p53 levels once more appearing reduced 90 minutes after treatment with anisomycin (Figure 6.4B). Overexpression of PBF-M13 however, had no effect on the turnover of p53, when compared to VO controls, where p53 levels were reduced at each time-point in a similar fashion to VO controls (Figure 6.4C). When the p53 expression levels of cells overexpressing WT PBF were observed against those of cells overexpressing PBF-M13 significant differences were observed. p53 turnover was highly increased in cells overexpressing WT PBF compared to those overexpressing PBF-M13. Indeed, at the exposure presented in Figure 6.4D, p53 levels were undetectable from 60 minutes when PBF was overexpressed, whilst overexpression of PBF-M13 resulted in detectable levels of p53 at each time-point. These data suggest that sequestration of PBF away from its sites of interaction with p53 prevents the effects of PBF on p53 turnover (Figure 6.4A-D).

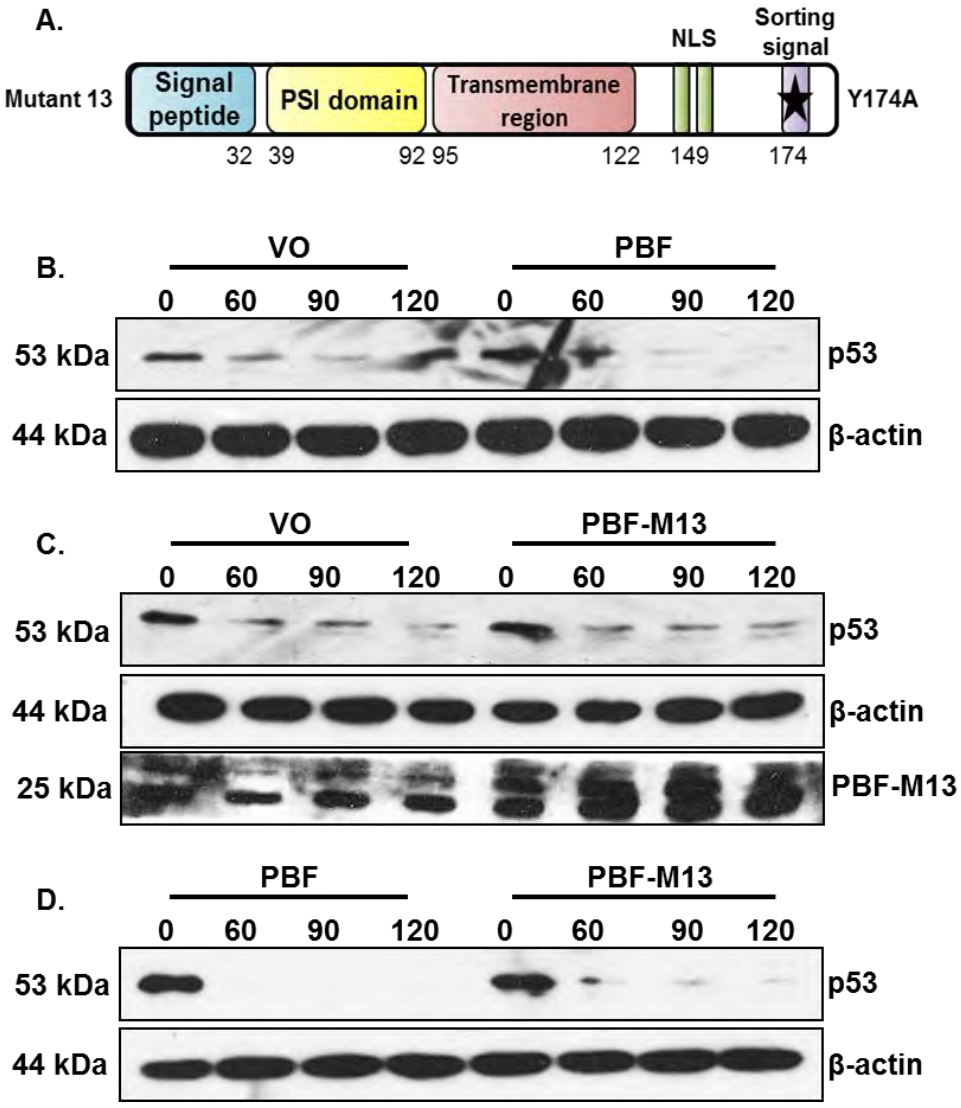


Figure 6.4: Western blot analysis of the effects of PBF-M13 on the turnover of p53 measured through the use of anisomycin half-life assays across a 120 minute time-course. ***A:*** *Schematic showing the location of the Y174A within the C-terminal sorting signal of the PBF protein.* ***B:*** *Representative Western blot demonstrating that PBF overexpression is associated with increased p53 turnover.* ***C:*** *The effects of PBF-M13 overexpression on p53 stability compared to VO-transfected controls* ***D:*** *The effects of WT PBF overexpression vs PBF-M13 overexpression on p53 stability.*

6.3.2. PBF overexpression results in increased p53 ubiquitination

Given that modulation of PBF expression was associated with significantly altered p53 stability, we next examined p53 ubiquitination in cells treated with the proteasome inhibitor MG132 (MG132 facilitates the accumulation of ubiquitinylated

proteins by preventing their degradation). Treatment of TPC-1 and HCT116 cells with 20 μM MG132 resulted in a stepwise accumulation of p53 ubiquitin isoforms over a 120 minute time-course (data not shown). When PBF was overexpressed, the level of p53 ubiquitintation was increased in both TPC-1 cells and HCT116 cells Figure 6.5). Immunoprecipitation of p53 and subsequent Western blot analysis revealed that accumulation of p53 ubiquitin chains was greater in cells overexpressing PBF immediately after administration of MG132, with maximal levels of p53 ubiquitination occurring from 90 minutes, compared to VO controls (Figure 6.5Ai). p53 ubiquitin chains are routinely detected via SDS-PAGE/Western blotting using the p53 specific antibody DO-1 and appear as laddered bands above the classical 53 kDa band of native p53 (see Section 6.2.3). We were able to reliably repeat the observation that PBF overexpression correlates with increased p53 ubiquitination 90 minutes after treatment of TPC-1 cells with 20 μM MG132 via p53 immunoprecipitation and subsequent Western blot analysis (TPC-1; *n*=4; Figure 6.5A+B). In light of the observations that overexpression of PBF resulted in a detectable increased in p53 ubiquitination immediately after MG132 administration, PBF was transiently overexpressed for 24 hours in TPC-1 cells and total cellular protein was harvested. Western blotting revealed that PBF overexpression resulted in increased accumulation of ubiquinated p53 in a dose dependent manner, compared to VO-transfected controls (Figure 6.5B). A significant increase in p53 ubiquitination was also observed when PBF was overexpressed in HCT116 cells 90 minutes after treatment with MG132 (Figure 6.5C, HCT116; *n*=3).

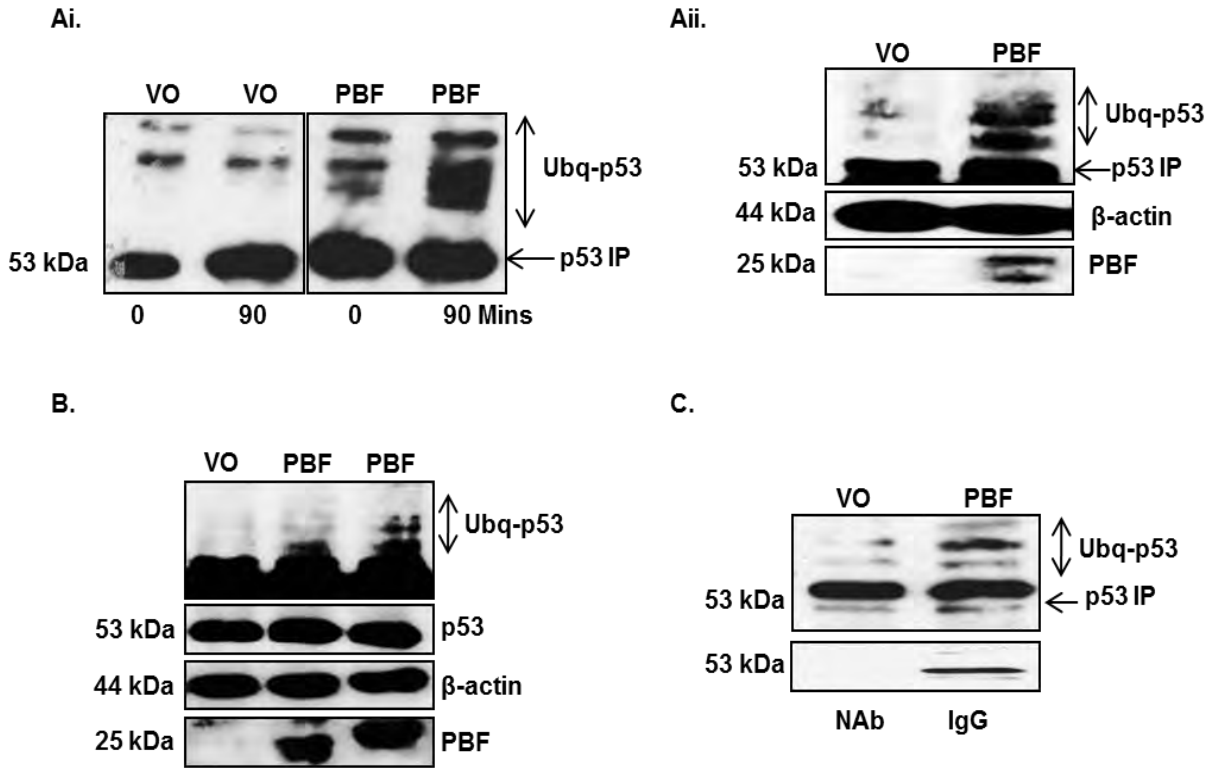


Figure 6.5: PBF increases ubiquitination of p53. ***Ai+ii:*** *Extracts from TPC-1 cells transfected with the indicated vectors for 24 hours and treated with 20 μM MG132 were immunoprecipitated with an anti-p53 antibody, then analysed by Western blotting with the same antibody. PBF and β-actin were determined by Western blotting on the pre-immunoprecipitated lysates.* ***B:*** *Western blots representing the detection of p53 ubiquitin isoforms following PBF overexpression on whole-cell lysates in the absence of MG132 in TPC-1 cells.* ***C:*** *Extracts from HCT116 cells transfected with the indicated vectors for 24 h and treated with 20 μM MG132 were analysed by Western blotting with the indicated antibodies.*

6.3.3. The relationship between PBF and HDM2

The previous data in this chapter describes a role for PBF in the modulation of p53 stability. Specifically, when wild-type PBF is overexpressed (in thyroid or colorectal cell lines) p53 turnover is significantly increased as measured by anisomycin half-life assays, and these findings correlate with increased p53 ubiquitination. As previously described, p53 protein expression is controlled not by the rate of gene transcription, but by the rate of its protein degradation. Under normal physiological conditions, p53

is continually transcribed and translated; however, levels remain low due to the action of the E3 ubiquitin ligase HDM2. HDM2 binds directly to p53 and ubiquitinates both itself and p53, thus targeting each protein for degradation in the proteasome. It was initially hypothesised that PBF may be an E2 ubiquitin ligase, but experiments performed by our collaborator, Dr Pascal Lopez, Lyon, France, disproved this hypothesis, as *in vitro* ubiquitin assays confirmed that PBF was unable to ubiquitinate itself in isolation (personal communication). From these observations, it is unlikely that PBF is directly responsible for the increased p53 ubiquitination observed following its overexpression. Therefore given the important role of HDM2 in the negative regulation of p53 protein expression under physiological conditions, and given the direct interaction between PBF and p53, we sought to determine the relationship between PBF and HDM2 in our cell lines through the use of co-immunoprecipitiation assays. Endogenous HDM2 was successfully immunoprecipitated using the antibody 2αB (the MDM2 2AB antibody is a mouse polyclonal antibody which detects total MDM2 and HDM2, courtesy of Dr Andrew Turnell, University of Birmingham) in TPC-1, K1 and HCT116 cells. Subsequent probing for the presence of co-immunoprecipitated PBF in TPC-1 cells using our PBF specific antibody revealed the presence of specific bands in co-immunoprecipitation samples, that were not present in either the no antibody controls (NAb), or IgG controls, indicating that PBF may indeed be interacting with HDM2 (Figure 6.6). However, further analysis of pre-immunoprecipitated lysates suggests that the bands detected in the Co-IP samples are apparently of a slightly lower molecular weight when compared with these lysates (Figure 6.6A). Using our PBF antibody to assess the presence of co-immunoprecipitated PBF in K1 cells did not result in detectable

bands in the co-IP samples, suggesting that whilst HDM2 was successfully immunoprecipitated in these cells, PBF was not co-immunoprecipitated and therefore no interaction was apparent (Figure 6.6B). Finally, in HCT116 cells, successful immunoprecipitation of HDM2, and subsequent immuno-detection of PBF revealed specific bands within our co-IP test samples, with no detectable bands present in the negative controls (NAb control, or IgG control), indicating a possible interaction between the 2 proteins. Subsequent analysis of PBF from the pre-immunoprecipitated lysates again revealed that bands detected in the co-IP test samples ran at a slightly lower molecular weight on SDS-PAGE/Western blots (Figure 6.6C).

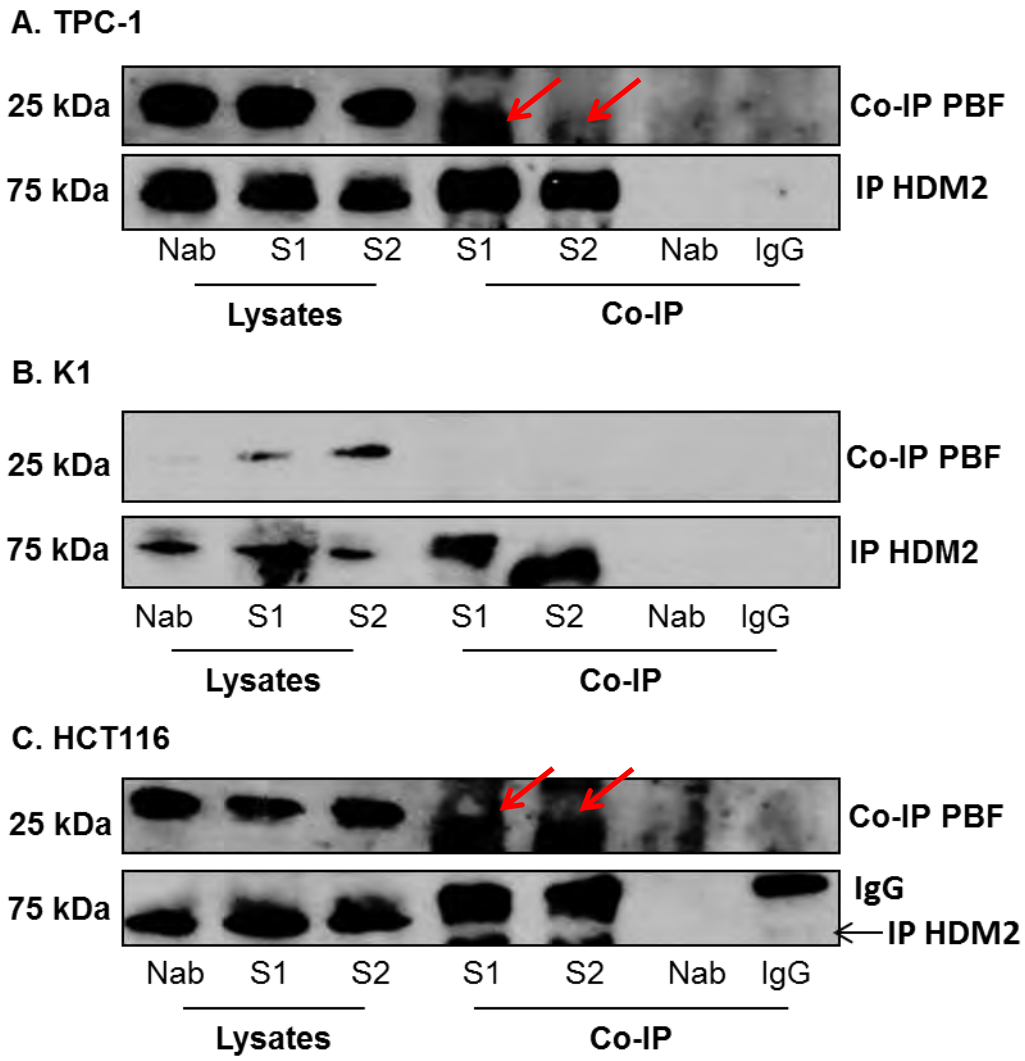


Figure 6.6: Representative Western blots to assess whether PBF co-immunoprecipitates with HDM2 in ***A:*** *TPC-1,* ***B:*** *K1 and* ***C:*** *HCT116 cells. Cell-lines were tested for successful immunoprecipitation of HDM2 and protein from the pre immunoprecipitated lysates was used for the identification of Co-immunoprecipitated proteins. No antibody controls (NAb) and igG controls were used as negative controls to assess the ability of proteins to bind in a non-specific manner to sepharose G beads and antibody fragments respectively. (S1 +S2 represent 2 separate co-IP expermients).*

6.4. **Discussion**

Data in Chapter 4 probed the relationship of PBF and p53; specific co-immunoprecipitation assays confirmed that PBF is a novel interacting partner of p53 in both the presence and absence of ionising radiation (inactive or active p53) in 2 thyroid papillary carcinoma cell lines (TPC-1 and K1 cells) and the HCT116 colorectal carcinoma cell line. These data correlated with colocalisation of PBF and p53 within the nucleus of TPC-1, COS-7 and HCT116 cells. Despite the physical interaction between PBF and p53, PBF had no effect on the subcellular distribution of p53 using specific subcellular fractionation assays. Data in Chapter 5 therefore were aimed at characterising the functional outcome of the PBF:p53 interaction on p53-mediated gene transcription using p53 specific focused microarrays and TaqMan based assays. Regardless of successful PBF overexpression through transient transfection, and the induction of p53 activity following treatment with ionising radiation, PBF had no significant effect on p53-mediated gene transcription within our cell lines. The data in 6 therefore were aimed at determining the effects of PBF on p53 biology.

p53 is a potent tumour suppressor, bringing about a plethora of important cellular effects (for example growth arrest or apoptosis) in response to genotoxic insults (Vogelstein *et al*, 2000; Meek, 2004; Meek & Anderson, 2009; Vousden & Prives, 2009). By necessity, such influential effects must be tightly regulated, to prevent aberrant regulation of such important cellular processes (Brooks & Gu, 2006; Meek & Hupp, 2010; Meek & Anderson, 2009). Furthermore, p53 is rendered inactive despite being transcribed and translated at relatively high levels at all times. This effect is mediated through the action of HDM2, the E3 ligase. In addition to the action of HDM2 novel cellular proteins are emerging that bind directly to p53 and effect p53

metabolism, and when aberrantly expressed contribute to its functional inactivation in human cancers (Lee & Gu, 2009; Sherr, 2006; Gilkes *et al*, 2006; Yuan *et al*, 2010). A recent paper by Yuan *et al* described the effects of the deubiquitinylating enzyme USP10 on p53 stability, reporting its interaction with p53 and increased rate of down regulation in clear cell carcinomas (Yuan *et al*, 2010). With the observed overexpression of PBF in thyroid and other cancers, the established oncogenic nature of PBF, and the recently characterised interaction between PBF and p53, we characterised the effects of PBF overexpression on p53 metabolism, and assessed the functional association of PBF with the master regulator of p53, HDM2.

6.4.1. The effects of PBF overexpression on p53 metabolism

Treatment of TPC-1 cells with anisomycin to block *de novo* protein synthesis revealed that despite being a transformed cell line (where increased p53 stability is observed compared to normal cells) p53 levels decreased over the 120 minute timecourse. However, following the transient overexpression of PBF, p53 expression levels were significantly reduced from 120 minutes. Furthermore, in K1 cells, where p53 appeared to be much more stable across the 120 minute time course than TPC-1 cells, PBF still had a potent effect on p53 turnover, with p53 levels also significantly reduced from 90 minutes. To test whether this observation was thyroid specific, anisomycin half-life assays were performed in HCT116 cells. In this cell line p53 was particularly stable across the 120 minute time course, with no visible decrease in p53 levels even after 120 minutes. Nonetheless, PBF overexpression significantly increased the turnover of p53 from 90 minutes. The direct interaction of PBF with p53 may therefore increase the rate of p53 degradation, an effect that has profound implications on the ability of p53 to function normally in response to genotoxic insult.

The ability of PBF to bring about significant alterations in p53 metabolism strongly suggests that PBF should join a growing number of cellular oncogenes that negatively affect the stability of p53 when aberrantly expressed.

Given the potential importance of the observation that PBF is a novel mediator of p53 metabolism, the effects of PBF on p53 half-life were characterised further. Firstly, the mRNA expression of p53 was monitored in the presence and absence of PBF overexpression, where we found that there was no significant difference in p53 mRNA expression following PBF overexpression in either TPC-1 or HCT116 cells. These data suggest that the increase in p53 turnover observed in Figure 6.1 and Figure 6.2 occurs solely as a function of post-translational events and not as a consequence of *TP53* gene repression. Secondly, given the probable overexpression of PBF in the cell lines used for study, investigation into the effects of transient PBF overexpression on p53 function may be, if at all, less sensitive than the depletion of PBF through siRNA knockdown in these cells. To that end, we used specific siRNAs to deplete PBF in K1 and TPC-1 cells and determined whether this would reverse the finding that PBF overexpression reduces p53 half-life. siRNA depletion in TPC-1 and K1 cells resulted in a significant repeatable increase in p53 half-life, further strengthening the observation that PBF is a novel mediator of p53 metabolism. Such an increase in p53 half-life on depletion of PBF protein may point to the importance of PBF in the maintenance of p53 homeostasis under normal conditions. However, when expressed at oncogenic levels PBF may cause the functional inactivation of p53. We found it difficult to directly implicate the physical interaction of PBF and p53 with the turnover of p53. However, we were able to provide evidence that PBF must be in close proximity to p53 to affect its half-life with the use of a specific mutant of

PBF which is sequestered at the plasma membrane. Transient overexpression of this mutant did not alter the rate of p53 turnover; indeed the rate of p53 metabolism closely matched those of time-matched VO-transfected controls, providing evidence (albeit indirect) that PBF and p53 must physically interact to bring about the modulatory effect on p53 metabolism. That PBF can bind to and modulate the rate of p53 turnover indicates that PBF is a novel mediator of p53 stability, and when overexpressed may play a pivotal role in the functional inactivation of p53 in thyroid papillary carcinomas and other tumours where p53 mutations are rare.

6.4.2. PBF overexpression results in increased p53 ubiquitination

p53 turnover is mediated by ubiquitination (Brooks & Gu, 2006; Chen *et al*, 1993; Lee & Gu, 2009), and therefore observation of p53 ubiquitination following PBF overexpression was necessary to provide greater meaning to the p53 half-life studies performed in Section 6.3.2. p53 ubiquitination was measured in TPC-1 cells and HCT116 cells, and in keeping with the anisomycin half-life assays, MG132 ubiquitin assays confirmed that PBF overexpression correlated with increased p53 ubiquitination. These data indicate that PBF modulates p53 physiology by increasing p53 turnover specifically via the ubiquitin proteasomal route. Oncogenic expression of PBF therefore has the potential to disrupt p53 regulation by altering its stability in thyroid and colorectal carcinomas.

Despite the prominent effects of PBF on p53 metabolism, specific ubiquitin assays performed by our collaborator Dr Lopez (Lyon), confirmed that PBF was unable to incorporate ubiquitin, and was thus unlikely to possess ubiquitin ligase activity. This result suggests that PBF is not directly responsible for p53 ubiquitination, and therefore we hypothesised that PBF may bind directly to p53, influencing the ability of

HDM2 to bind to p53 and modulate its turnover. Recent work within our laboratory has been aimed at discerning the mechanism by which PBF influences p53 ubiquitination. For example, experiments performed by Mr Gavin Ryan (University of Birmingham) using the HDM2 antagonist/ p53 activator Nutlin-3 (a compound that prevents HDM2 activity by preventing the direct interaction between p53 and HDM2 (Vassilev *et al*, 2004)), revealed that PBF was unable to influence p53 turnover in the absence of HDM2 activity in TPC-1 cells. These data provide independent verification of the effects of PBF on p53 half-life in TPC-1 cells and demonstrate that that PBF influences p53 half-life in an HDM2 dependent manner.

The data revealing that PBF can bind to, and negatively regulate, p53 expression by increasing its ubiquitination in an HDM2 dependent manner are suggestive that PBF may play the role of a co-factor, for example facilitating the docking of HDM2 or increasing the stability of the p53:HDM2 interaction to bring about optimal ubiquitination of p53 and HDM2. There are examples of co-factors of p53 and HDM2 that serve to negatively regulate p53 homeostasis. For example the E3/E4 ligase UBE2B directly associates with p53 and HDM2, increasing its p53 degradation. Interestingly this protein has a characterised role in brain tumours (for example medulloblastoma) where amplification of UBE2B expression results in the functional inactivation of p53 (Wu *et al*, 2011). Furthermore, in line with the absence of ubiquitin ligase activity exhibited by PBF, the transcription factor Ying Yang 1 (YY1) has no intrinsic ubiquitin ligase activity, but is able to negatively influence p53 protein expression and bring about its ubiquitination. The mechanism whereby YY1 induces the turnover of p53 has been characterised by Sui *et al*, 2004. Briefly YY1 Is able to induce HDM2-mediated degradation of p53 (in the absence of YY1 transcriptional

activation of target genes), and the basis for the regulation of p53 turnover by YY1 is primarily due to the ability of YY1 to facilitate or stabilise the interaction between p53 and HDM2 (Sui *et al*, 2004; Atchison *et al*, 2011; Zhang *et al*, 2011). These relatable examples indicate that PBF may indeed facilitate HDM2 mediated degradation of p53 by virtue of its direct interaction with p53 and HDM2.

6.4.3. The relationship between PBF and HDM2

With the data described in the previous Sections, it was important to assess the potential interaction of PBF with HDM2, as it may be that PBF binds to p53 and facilitates the action of HDM2. Co-immunoprecipitation assays were therefore performed to assess the potential interaction between PBF and HDM2 in TPC-1, K1 and HCT116 cells. Co-immunoprecipitation reactions were inconclusive, but provided potential evidence for the interaction between PBF and HDM2. For example, in TPC-1 and HCT116 cells, successful immunoprecipitation of HDM2 was followed by the detection of bands of ~23 kDa in size using our PBF specific antibody, that were not present in the NAb or IgG controls, indicating that the bands detected were not a result of antibody fragments or of proteins binding non-specifically to various components of the co-immunoprecipitation assay. However, in both cells lines the bands detected were of a different size to those which appeared on the pre-immunoprecipitated lysates. The discrepancy in band size detracts from the validity of these findings, although provides potential supporting evidence of the GST-pulldown assays that initially revealed an interaction between recombinant PBF and HDM2 *in vitro* (Dr Andrew Turnell, School of Cancer Sciences, University of Birmingham –unpublished data). We were unable to detect the interaction between HDM2 and PBF in K1 cells using the conditions described in Section 6.2.4.

Interestingly there are numerous lines of evidence that PBF exists in a number of distinct isoforms within the cell. For instance specific use of mutants lacking putative glycosylation sites are found to run at different sizes to endogenous WT PBF.

6.5. **Concluding statements**

The data within this chapter provide evidence that PBF is a novel regulator of p53 metabolism. For example oncogenic expression of PBF in thyroid cancer cells results in a marked increase in p53 turnover, correlating with increased ubiquitination of p53. A specific mutant of p53, which remains sequestered at the plasma membrane away from the observed interaction with p53, was unable to influence p53 half-life. These data suggest that the interaction of PBF with p53 is necessary for the increase in p53 turnover observed. Furthermore, independent experiments confirm that PBF decreases p53 half-life in an HDM2 dependent manner, and our preliminary data indicate that PBF may also interact with HDM2. These findings demonstrate that PBF may be a novel co-factor serving to promote HDM2-mediated degradation of p53.

In conclusion this chapter provides evidence for a novel potential mechanism of tumourigenesis in thyroid papillary carcinomas, whereby PBF binds directly to p53, and oncogenic expression of PBF perturbs p53 function by increasing its turnover in an HDM2 dependent manner.

Chapter 7. **The biological effects of PBF on cell survival, apoptosis and growth**

7.1. **Introduction**

As previously described, the functional inactivation of p53 has been extensively linked to the initiation and progression of human cancer. PBF overexpression may therefore result in a biological change in p53 biology that might predispose the cell to malignant transformation and tumourigenic progression. Whilst the data in chapter 5 demonstrate that PBF had no significant effect on p53-mediated gene transcription (as measured through the use of p53-specific focused microarrays), p53 also has well characterised roles as a tumour suppressor independently of its role as a transcription factor. For instance, p53 directly mediates apoptosis induction following genotoxic insult by rapidly translocating to the mitochondria (Moll, Wolff, Speidel, & Deppert, 2005). P53 mitochondrial translocation occurs very rapidly, and precedes cytochrome c release, the collapse of the mitochondrial membrane potential and caspase-3 activation (Marchenko, Zaika, & Moll, 2000). The majority of mitochondrial p53 localizes to the outer membrane (Sansome, Zaika, Marchenko, & Moll, 2001), although a subfraction is found in complex with the major mitochondrial import proteins mthsp70 and mt hsp60 in the mitochondrial matrix (Dumont, Leu, Pietra, George, & Murphy, 2003; Mihara et al., 2003). The importance of p53 in the initiation of the release of cytochrome C and subsequent caspase cascade were highlighted using p53 fusion proteins. Specifically, Targeting of p53 to mitochondria by fusing it with mitochondrial address peptides, thereby bypassing the nucleus, was found to be sufficient to launch acute apoptosis and chronic colony suppression directly from the mitochondrial platform (Mihara et al., 2003; Moll et al., 2005; Sansome et al., 2001). Such studies highlight that the p53 tumour suppressor can directly initiate apoptosis,

and demonstrate that the potential effects of PBF on p53 function may involve the suppression of p53 transcription independent functions.

The data in chapter 6 describes the effect of PBF on p53 turnover, where oncogenic expression of PBF results in significant reduction of p53 half-life, causing increased degradation of p53 via the ubiquitin-proteasomal pathway. To investigate the gross effects of PBF on cell physiology, we used MTT cell viability assays to determine the overall effects of PBF overexpression on relative cell survival following exposure of ionising radiation to induce DNA DSBs. Furthermore, given the importance of the apoptotic pathway in the induction and progression of tumours, we assessed the effects of PBF overexpression on the mRNA and protein expression of 3 important regulators of apoptosis, BAX, PUMA and BCl-2, which are controlled directly by p53. We aimed to make use of specific caspase 3/7 cleavage assays to determine the overall levels of apoptosis following overexpression of PBF in thyroid papillary carcinoma cells.

Recently a murine model of targeted PBF overexpression in the thyroid was generated and characterised. Mice overexpressing PBF in the thyroid were found to exhibit normal thyroid function but presented with a striking enlargement of the thyroid gland associated with hyperplastic and macrofollicular lesions (Read *et al*, 2011). Thyroid cell growth is governed by a series of well-defined kinase pathways, which are commonly disrupted in thyroid neoplasia; therefore investigations into the expression of proteins involved in the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways were undertaken to gain mechanistic insights to the growth effects observed. Whilst MAPK was found to be unaltered in PBF transgenic mice when compared to wild-type counterparts, the important PI3K

target Akt was significantly induced by phosphorylation at ser-473 (a critical residue for AKT activation). In accordance with PBF overexpression causing the activation of AKT, the total levels of AKT in murine transgenic thyroids was unaltered (Read *et al*, 2011). These data indicate that PBF may induce AKT activation, a critical mediator of cellular proliferation and novel mediator of p53 turnover and ubiquitination (Gottlieb *et al*, 2002; Boehme *et al*, 2008; Ogawara *et al*, 2002). In light of these recent data, we also aimed to assess the effects of PBF overexpression on AKT in human primary thyrocytes and thyroid papillary carcinoma cells.

The overall aims of this Chapter were therefore to observe the gross biological effects of PBF overexpression, and potentially elucidate the potential mechanism whereby PBF affects p53 function.

7.2. **Materials and methods**

7.2.1. Cell lines and plasmids

TPC-1, K1 and HCT116 cells were routinely subcultured using the methods described in section 2.1. For experiments requiring transient PBF overexpression, and / or γ-irradiation exposure, cells were treated using the procedures described in section 3.2. Human primary thyrocytes were cultured as described in Section 2.3 (Thyrocytes were transfected using Fugene as directed in Section 3.2)

7.2.2. SDS-PAGE/Western blotting

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see Section 2.6). Blocked membranes were subsequently

incubated with primary antibodies. Antibodies used were our rabbit anti-human PBF-8 polyclonal (1:1000) (Read *et al*, 2011), rabbit anti-human HA (Covance) Mouse anti-human p53 (D0-1) monoclonal at 1:5000 (Santa Cruz, CA, USA), monoclonal anti-β-actin clone AC-15, (used at 1:10,000, Sigma-Aldrich, Poole, UK). Antibodies used for detection of apoptotic proteins were, rabbit anti-human BAX (#2774), BCL-2 (#2870) and PUMA (#4976) purchased from Cell-Signaling Technology (New England Biolabs, Ipswich, Massachusetts, USA). Observation of AKT and p-AKT ser-473 protein expression was determined using rabbit anti-human Akt Antibody #9272, and Phospho-Akt (Ser473) (D9E) XP® antibody #4060 purchased from Cell-Signaling Technology (New England Biolabs, Ipswich, Massachusetts, USA).

7.2.3. RNA extraction and QT-PCR

Reverse transcription and QT-PCR techniques were as described in Sections 2.4 and 2.5. Specific primers and probes for assessment of BAX, BCL-2 and PUMA mRNA expression were purchased from Applied Biosystems (Warrington, UK).

7.2.4. MTT cell-viability assays

The rate of cellular proliferation of TPC-1, K1 and HCT116 cells following transient PBF overexpression and treatment with ionising γ-irradiation was assessed by internalisation and reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded into 6-well dishes, and incubated for 24 hours before transient overexpression of PBF, using the methods described in section 3.3. After 24 hours thyroid cells lines and the colorectal cell line were treated with 15 Gys and 40 Gys γ-irradiation respectively and returned to incubation. Following treatment with γ-irradiation cells were trypsinised, counted and seeded into 96-well dishes at a density of 7500 cells/well and incubated for 24 hours. Cells were incubated with 0.8

mg/ml MTT (Sigma-Aldrich, UK) for the last 3 hours of culture incubation. Cells were then washed with cold PBS before formazan crystals were solubilised by addition of 100 µl dimethyl sulfoxide for 15 minutes. The absorbance at 540 nm was measured using a Wallac 1420 Victor plate-reader (Perkin-Elmer, Massachusetts, USA).

7.2.5. Caspase 3/7 cleavage assays

The effects of PBF overexpression on the gross levels of apoptosis were assessed using specific caspase 3/7 cleavage assays (CaspaseGlo 3/7 assay, Promega). The luminogenic substrate within the assay is cleaved by caspases 3 and 7 to yield a substrate for luciferase. Relative levels of apoptosis were then measured using a luminometer. Cells were seeded into 6-well dishes and treated as above (7.2.4), and were seeded into 96-well plates at a density of $1x10^5$ per well, incubated for 24 hours before application of CaspaseGlo 3/7 substrate as per manufacturer's instructions. Luminescence was measured using a Wallac 1420 Victor plate-reader (Perkin-Elmer, Massachusetts, USA).

7.2.6. Statistical analysis

Statistical analysis was performed using the methods described in Section 2.8.

7.3. **Results**

7.3.1. The biological effects of PBF overexpression

To investigate the physiological relevance of our finding that PBF decreases p53 stability by targeting it for ubiquitination, we examined the influence of manipulating PBF expression on cell survival in response to ionising radiation (I.R). We used MTT

assays to assess the effects of PBF overexpression on cell viability 24 hrs after treatment with γ-irradiation. VO transfected TPC-1 cells displayed a significant, repeatable reduction in cell viability (17.9%+/-1.3 %; $p<0.05$; $n=3$; Figure 7.1A). However, TPC-1 cells overexpressing PBF alone did not show any significant reduction in cell viability, resulting in a significant difference in cell viability following exposure to ionising radiation (Figure 7.1A; $n=3$; $p<0.001$). A similar result was observed in K1 cells where VO-transfected cells displayed a ~20 % reduction in cell viability compared to cells overexpressing PBF, which displayed only a 7 % decrease in cell viability compared to untreated controls. The increase in cell survival observed when PBF was overexpressed in K1 cells following treatment with γ-irradiation did not reach statistical significance ($n=4$; $p=NS$; Figure 7.1B). MTT cell viability assays were also assessed following the transient overexpression of PBF in HCT116 cells. In this cell line VO controls displayed a repeatable ~25 % reduction in cell viability in response to γ-irradiation. However when PBF was overexpressed no reduction in cell viability was observed, and hence PBF overexpression resulted in a significant difference in cell viability in response to γ-irradiation (Figure 7.1C, $n=4$; $p<0.001$).

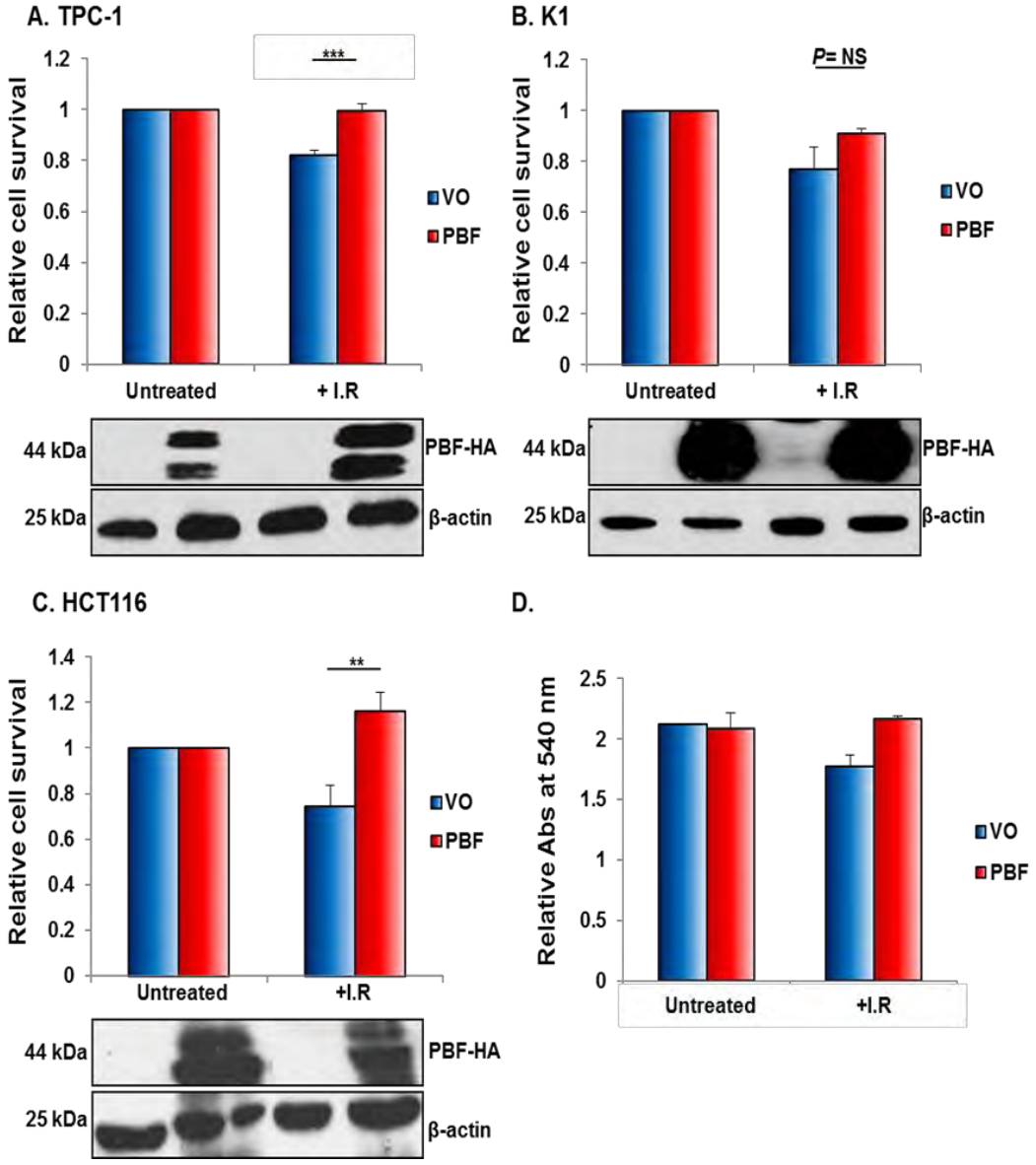


Figure 7.1: MTT cell viability assays representing the effects of transient PBF overexpression on the relative change in cell viability 24 hrs after treatment with γ-irradiation compared to untreated controls. ***A:*** *TPC-1 cells transiently overexpressing PBF displayed a significant increase in cell viability in response to 15 Gy γ-irradiation treatment (*n*=3;* $p<0.0001$*).* ***B:*** *K1 cells transiently overexpressing PBF showed a 13 % reduction on cell viability in response to 15 Gy γ-irradiation (*n*=4;* p*=NS).* ***C:*** *HCT116 cells transiently overexpressing PBF displayed a significant increase in cell viability in response to 40 Gy γ-irradiation treatment (*n*=4;* $p<0.001$*). Representative Western blots demonstrating p53 induction in response to ionising radiation and levels of transient overexpression of PBF were obtained from lysates from cells treated in parallel with cells seeded for the MTT viability assays.* ***D:*** *Raw data exemplar of absorbance readings at 540 nm for HCT116 MTT cell viability data (n=3).*

In order to assess whether the effects of PBF overexpression were directly linked to the effects of PBF on p53 metabolism, MTT cell viability assays were repeated in the p53 null SW1736 anaplastic thyroid cell line. Preliminary data suggest that the significant differences observed in cell viability in TPC-1 cells and HCT116 cells are lost in p53 null SW1736 cells. In this cell line DNA damage induced a ~10 % reduction on cell viability in VO-transfected controls, with cells overexpressing PBF displaying a similar decrease in cell survival of ~12 % (preliminary data; n=2; Figure 7.2), suggesting that the effects of PBF overexpression on cell survival may indeed be p53 specific.

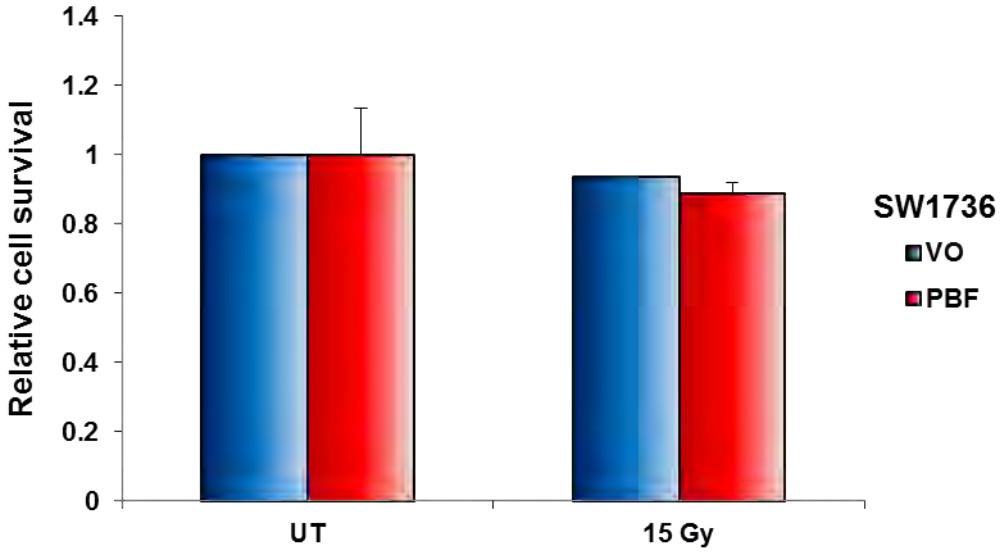


Figure 7.2: Preliminary MTT assay data demonstrating the effects of PBF overexpression on cell viability/ survival following exposure to ionising radiation in p53 null SW1736 anaplastic thyroid carcinoma cells (n=2).

7.3.2. The effects of PBF on the p53-mediated apoptotic pathway

Given the potential survival advantage conferred by PBF overexpression in response to ionising radiation, we aimed to define the biological pathway responsible. In this

Section we specifically focused on 2 key pro-apoptitic factors, BAX and PUMA and 1 important anti-apoptotic factor BCL-2, and determined the effects of PBF overexpression on the gene and protein expression of these factors following induction of DNA damage by ionising radiation. Furthermore, specific caspase 3/7 cleavage assays were performed to assess the effects of PBF on the levels of apoptosis following DNA damage.

7.3.2.1. *The effects of PBF on BAX and PUMA expression*

BAX and PUMA (described more fully in Section 3.1) are both pro-apoptotic genes, directly induced by p53 activity in response to cellular stress. The protein products of these genes directly interact with the anti-apoptotic protein BCL-2 (the expression of which is also mediated by p53 at the level of transcription), to bring about the onset of the apoptosis pathway. For instance, the presence of BCL-2 homodimers constitute an anti-apoptotic environment, but on activation of BAX, BCL-2:BCL-2 interactions are displaced by BCL-2:BAX heterodimers, resulting in the initiation of apoptosis. PUMA also interacts with BCL-2 and specifically inhibits its function, paving the way for BAX activity, and ultimately the onset of apoptosis via the mitochondrial cytochrome C pathway.

The effects of PBF overexpression on the induction of BAX, BCL-2 and PUMA expression were analysed in both the presence and absence of ionising radiation in TPC-1 cells and HCT116 cells (Figure 7.3). BAX mRNA expression was not affected by PBF alone (n=3; p=*NS*; Figure 7.3A), but was significantly induced by the administration of 15 Gy γ-irradiation in cells transiently overexpressing PBF and those expressing the vector only compared to untreated cells (elevated to 1.84-fold and 1.88-fold respectively, n=3; p<0.05). However, there was no significant

difference in BAX induction following PBF overexpression. The expression of the anti-apoptotic BCL-2 was not effected by PBF overexpression alone. Administration of γ-irradiation resulted in a predictable repression of BCL-2 expression in both the presence and absence of PBF overexpression (both by ~40%, *n*=3; *p=NS;* Figure 7.3A), indicating that PBF also has no effect on the activity of this gene in this cell line. Again, PBF overexpression had no effect on PUMA mRNA expression in either the presence or the absence of ionising radiation, although PUMA mRNA expression was significantly elevated in response to ionising radiation (~3-fold; *n*=3; *p*<0.001; Figure 7.3A).

Experiments were repeated in HCT116 cells and similar results were obtained (Figure 7.3B), BAX was non-significantly elevated following treatment with ionising radiation and PBF overexpression had no significant effect on BAX expression in the presence or absence of ionising radiation (*n*=3). Again, BCL-2 was non-significantly repressed in response to ionising radiation, with PBF having no effect on the induction of this gene (*n=3; p=NS*). Finally, PUMA mRNA expression was significantly elevated in response to ionising radiation (~3.2-fold: *n*=3; *p*<0.001), but PBF overexpression did not significantly affect the induction of this gene (see Figure 7.3B).

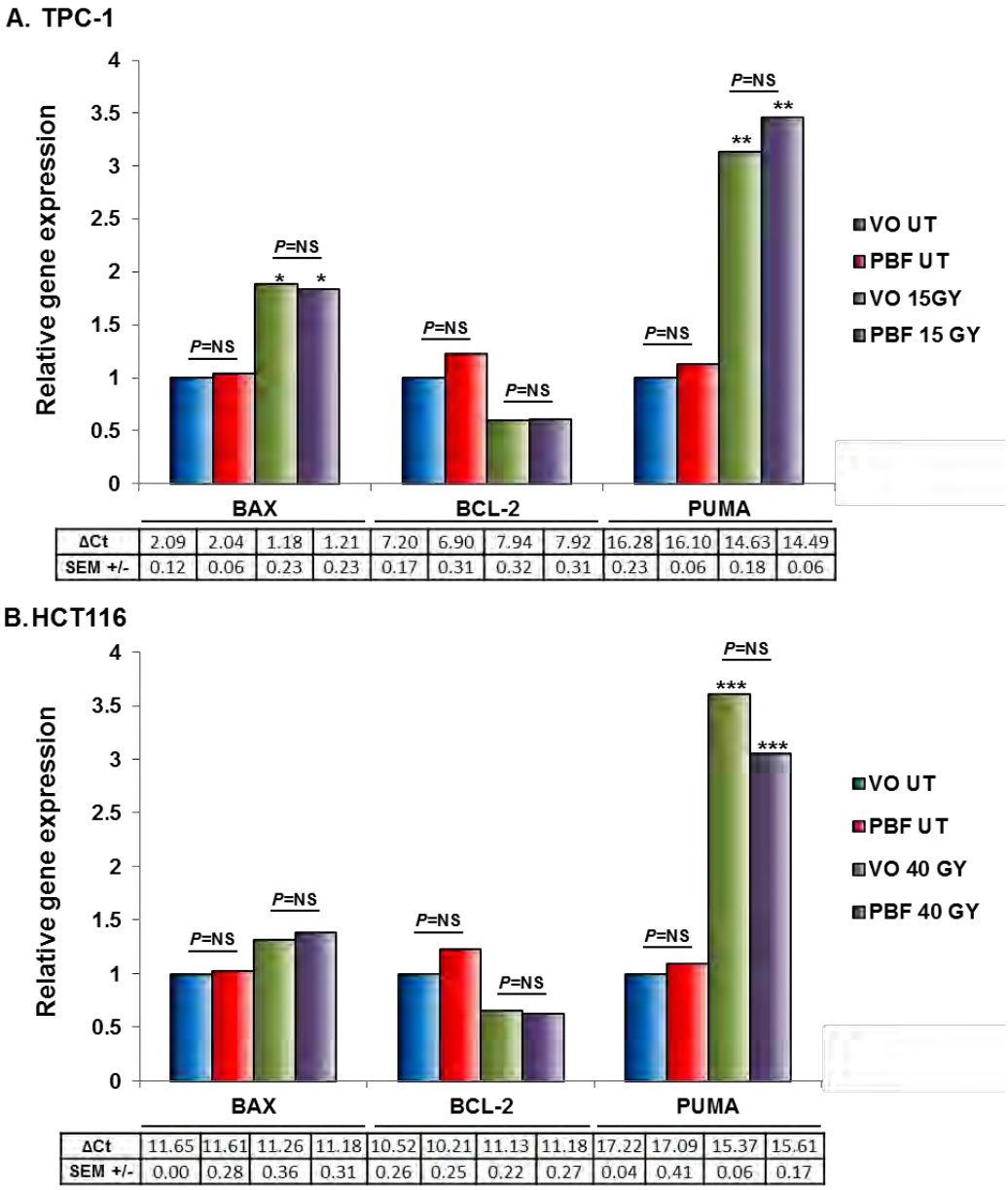


Figure 7.3: The effects of PBF overexpression on the mRNA expression of the apoptotic factors BAX, PUMA and BCL-2 in the presence and absence of DNA damage in HCT116 and TPC-1 cells ***A:*** *In TPC-1 cells, BAX and PUMA mRNA expression were significantly induced by ionising radiation, but PBF had no effect on this. BCL-2 expression was not significantly affected by ionising radiation, and was not significantly affected by PBF overexpression.* ***B:*** *Within HCT116 cells, PUMA was the only gene significantly elevated in response to ionising radiation, but PBF had no significant effect (n=3, *p<0.05, **p<0.01, ***p<0.001).*

7.3.2.2. *The effects of PBF overexpression on the protein expression of BAX, PUMA and BCL-2 in the presence and absence of ionising radiation*

In support of the data described in Section 7.3.2.1, no significant differences in the protein expression of PUMA or BCL-2 were detected in response to PBF overexpression, either in the presence or absence of genotoxic insult, in TPC-1 or HCT116 cells (Figure 7.4). We were unable to detect BAX protein expression in TPC-1 or HCT116 cells using the antibody described in Section 7.2.2 (Figure 7.4A+B). An increase in PUMA protein expression was observed in both TPC-1 and HCT116 cells following γ-irradiation, in support of the mRNA data generated by QT-PCR, demonstrating that PUMA mRNA expression was significantly upregulated in response to DNA damage in these cell lines (Figure 7.4).

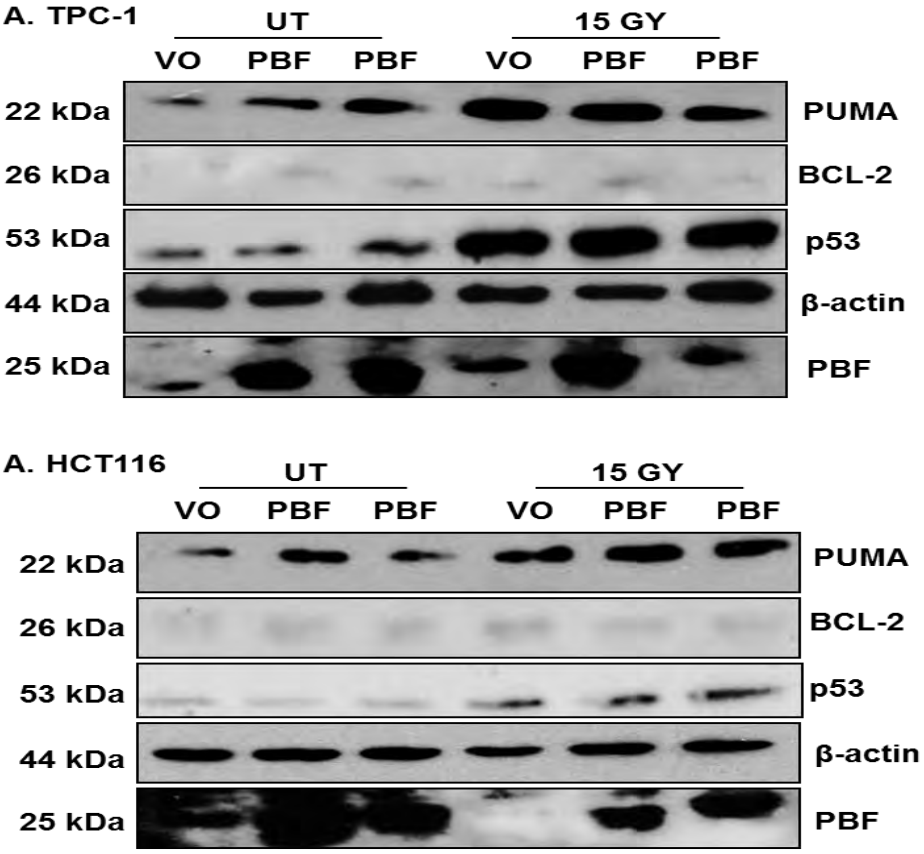


Figure 7.4: The effects of PBF overexpression on the protein expression of BAX, PUMA and BCL-2 in the presence and absence of ionising radiation in TPC-1 and HCT116 cells. ***A:*** *Representative Western blots displaying the effects of PBF overexpression on PUMA and BCL-2 protein expression in TPC-1 cells. PBF had no effect on the expression of these proteins (n=3)* ***B:*** *Representative Western blot displaying the effects of PBF overexpression on PUMA and BCL-2 protein expression in HCT116 cells. PBF also had no effect on the expression of these proteins (n=3). Immunoblots for p53 and PBF were included to represent successful induction of DNA damage and PBF overexpression, with β-actin used as a loading control. We were unable to detect BAX protein using the antibody described in Section 5.2.2.*

7.3.2.3. *The effects of PBF overexpression on gross levels of apoptosis in the presence and absence of ionising radiation*

PBF overexpression had no effect on the induction of 3 important mediators of apoptosis, BAX, BCL-2 and PUMA, providing evidence that PBF does not affect these important apoptotic factors in thyroid cancer. However, given the reported transcriptional independent roles of p53 in the induction of apoptosis, total levels of apoptosis were measured following PBF overexpression in the presence and absence of DNA damage compared to mock transfected controls.

The relative levels of apoptosis can be accurately measured in transformed cells in culture by assessing the activity of caspase 3 and 7 enzyme activity. Apoptosis assays incorporate the use of a pro-luminescent caspase 3/7 substrate containing the tetrapeptide sequence DEVD, which is cleaved to aminoluciferin, a substrate of luciferase, by the activity of caspase 3 and 7. Relative levels of apoptosis of cells in culture can be accurately measured by measuring the levels of luminescent signal produced using a luminometer.

TPC-1, K1 and HCT116 cells were seeded into 96-well dishes and PBF was overexpressed for 24 hours using the methods described in Section 2.1. Thyroid carcinoma cells and HCT116 cells were then treated with 15 Gy and 40 Gy γ-irradiation respectively and returned to incubation for 24 hours, with caspase 3/7 GLO substrate added in the final 3 hours of culture incubation, and measurement of apoptosis was performed after 24 hours (Figure 7.5). TPC-1 cells overexpressing PBF displayed a repeatable decrease in apoptosis, which was statistically significant (18 % +/- 4.5 % decrease; n=3; p<0.01; Figure 7.5A), suggesting that PBF causes a decrease in apoptosis in this cell line in the absence of DNA damage. Surprisingly,

treatment with 15 Gy γ-irradiation did not significantly elevate the rate of apoptosis in TPC-1 cells (Figure 7.5A), despite the earlier observation that 15 Gy ionising radiation significantly elevates the mRNA and protein expression of pro-apoptotic factors in this cell line. It would appear, as mentioned in Chapter 3, that the predominant response of TPC-1 cells to 15 Gy γ-irradiation is growth-arrest, rather than the induction of apoptosis. When the levels of apoptosis were measured in HCT116 cells PBF was observed to have no effect on the levels of apoptosis in the absence of DNA damage, in contrast to the data generated in TPC-1 cells (see Figure 7.5A+B). The levels of apoptosis were also unaltered in HCT116 cells treated with 40 Gy γ-irradiation (Figure 7.5B). Again, the main biological response of HCT116 cells treated with such a dose of ionising radiation appears to be growth arrest, rather than apoptosis, despite the apparent induction of proapoptotic factors in response to this treatment. Furthermore, as described in TPC-1 cells, PBF had no apparent effect on apoptosis in the presence of DNA damage (Figure 7.5B). Similar results were obtained in K1 cells, where PBF was observed to have no effect on apoptosis in either the presence or absence of ionising radiation (n=2, data not shown).

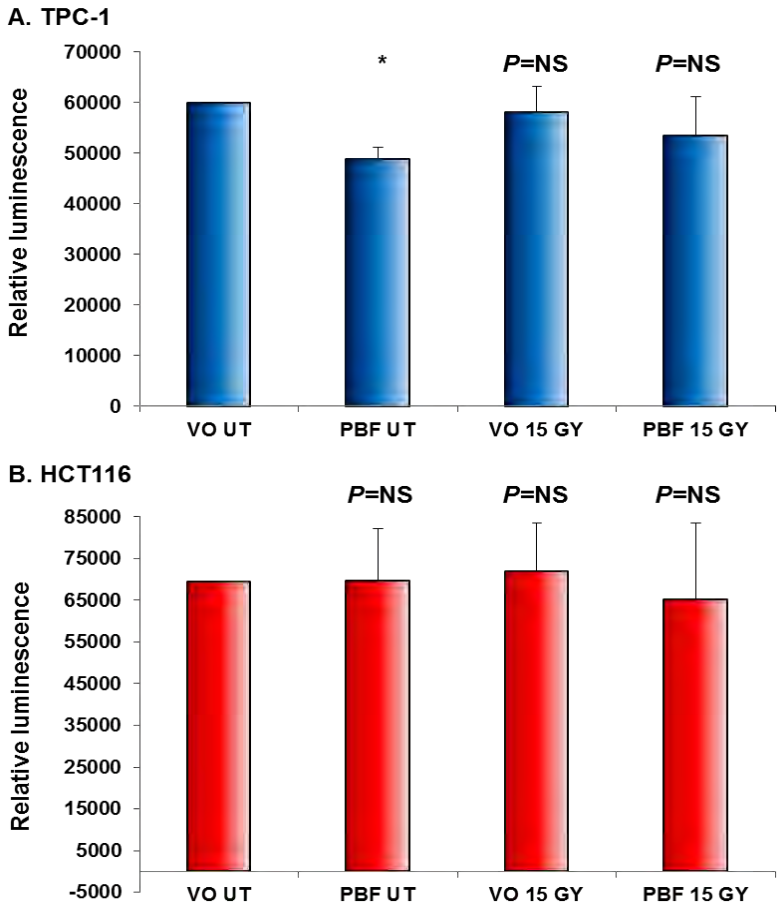


Figure 7.5: The effects of PBF overexpression on the relative levels of apoptosis in TPC-1 and HCT116 cells in the presence and absence of ionising radiation. ***A*** *PBF overexpression significantly repressed apoptosis in the absence of DNA damage (~18 % +/- 4.5 %; n=3; p<0.05). Ionising radiation did not affect the basal levels of apoptosis in this cell line, and PBF had no effect on apoptosis in the presence of DNA damage.* ***B:*** *The relative levels of apoptosis in HCT116 cells; PBF overexpression did not affect apoptosis levels in the presence or absence of ionising radiation. Furthermore, treatment of HCT116 cells with 40 Gy γ-irradiation did not significantly affect the induction of apoptosis (n=3; p=NS).*

7.3.3. PBF and PKB/AKT activity

With PBF overexpression linked with increased turnover and ubiquitination of p53, and increased cell survival following short-term exposure to ionising radiation, the effects of PBF overexpression on p-AKT induction were investigated in human primary thyrocytes and thyroid papillary carcinoma cells. In keeping with the findings

of Read *et al*, 2011, human primary thyrocytes transiently transfected with pCDNA3 for 24 hours demonstrated significant phosphorylation (and therefore activation) of AKT at Ser-473 compared to VO-transfected controls (Figure 7.6). In addition, those samples that displayed significant induction of AKT phosphorylation at Ser-473 did not display any significant increase in total AKT expression compared to VO-transfected controls (Figure 7.6).

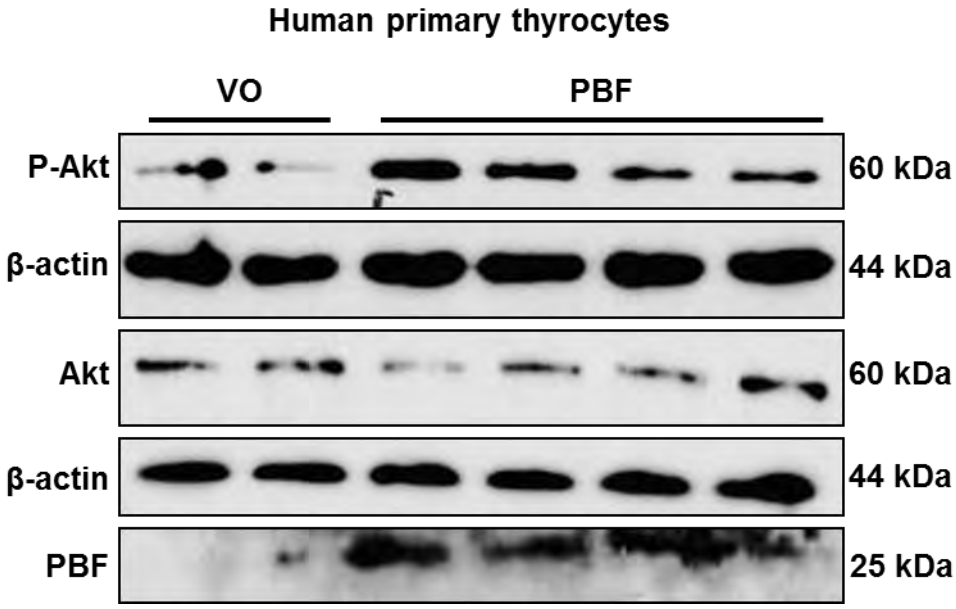


Figure 7.6: Representative Western blot demonstrating the effects of PBF overexpression on the induction of p-AKT in human primary thyrocytes. β-actin was used as a total protein loading control, and total AKT levels were monitored to assess the effects of PBF overexpression on total AKT expression.

The effects of PBF overexpression on AKT were investigated using TPC-1, K1 and HCT116 cell lines. In agreement with the data obtained using human primary thyrocytes, no significant difference in total AKT protein expression was observed in any of our cell lines (Figure 7.7A). Moreover, the effects of PBF overexpression on p-

AKT (Ser-473) were investigated in TPC-1 cells in the presence and absence of ionising radiation. Preliminary data suggested that PBF overexpression results in an increase in p-AKT levels in the absence of ionising radiation, to a similar level observed in normal thyrocytes. In the presence of ionising radiation, phosphorylation of AKT appears to be induced in both the presence and absence of PBF overexpression compared to time matched untreated controls (Figure 7.7B). However, the phosphorylation of AKT appeared to be greatest in cells overexpressing PBF (Figure 7.7B, preliminary data).

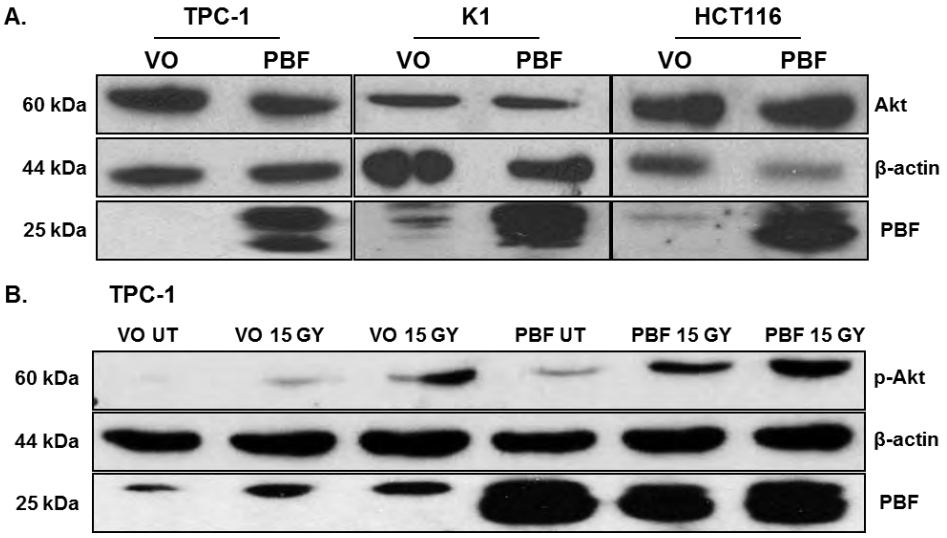


Figure 7.7: The effects of PBF overexpression on AKT expression: ***A:*** *Representative Western blots to assess the effects of PBF overexpression on total AKT protein expression in TPC-1, K1 and HCT116 cells (n=3).* ***B:*** *Preliminary data displaying the effects of ionising radiation and PBF overexpression on the expression of p-ser-473 AKT in the presence and absence of ionising radiation in TPC-1 cells. Detection of PBF confirmed successful overexpression of PBF following transient overexpression, and β-actin was used as a protein loading control.*

7.4. **Discussion**

7.4.1. The biological effects of PBF overexpression

In light of the observed effects of PBF on p53 biology, we sought to determine the gross biological effects elicited by PBF overexpression in the presence and absence of ionising radiation in TPC-1, K1 and HCT116 cells through the use of MTT cell viability/survival assays. PBF overexpression alone did not have a significant effect on cell viability or survival in the absence of DNA damage. However, following treatment with ionising radiation to induce DNA damage, TPC-1 and HCT116 cells overexpressing PBF demonstrated a significant, repeatable increase in cell survival 24 hours after the induction of DNA damage by ionising radiation treatment, compared to VO-transfected controls. MTT assays performed in K1 cells demonstrated a similar trend, where PBF overexpression resulted in an overall ~13 % increase in cell survival/viability following DNA damage, but this trend was not found to reach statistical significance. Data in Chapter 4 provide evidence for a direct interaction between PBF and p53 in the presence and absence of ionising radiation and Sections 6.3.1 and 6.3.2 present data to suggest PBF overexpression negatively influences p53 half-life by increasing its turnover via the ubiquitin proteasomal route. In accordance with this data, MTT cell viability assays revealed that PBF overexpression correlates with a significant increase in cell survival following exposure to γ-irradiation. The MTT survival data cannot be directly associated with the effects of PBF on p53 metabolism, but provides evidence that PBF overexpression has a gross biological effect that potentially promotes tumourigenesis. To address this issue we attempted to make use of the p53-null SW1736 anaplastic thyroid carcinoma cell line. These cells are functionally p53 null

by virtue of a striking down regulation of p53 mRNA expression (Blagosklonny *et al*, 1998). Our preliminary data indicate that PBF does not affect cell viability following DNA damage in these p53-null cells, implying that PBF affects cell physiology by modulating the activity of p53. To confirm that the cell viability effects are due to the functional interaction of PBF with p53, future experiments would assess the effects of PBF on cell viability in SW1736 cells expressing exogenous wild-type p53. These data suggest that PBF overexpression may inhibit mechanisms of growth arrest or apoptotic pathways, usually activated by p53 in response to DNA damage.

7.4.2. The effects of PBF overexpression on the p53-mediated apoptotic pathway

Given the overwhelming evidence linking the aberrant control of apoptosis to tumour progression (Cotter, 2009), the effects of PBF overexpression on the induction of 3 prominent mediators of apoptosis (BAX, PUMA and BCL-2) were analysed. BAX and PUMA are both pro-apoptotic factors, and aberrant expression of these proteins has been extensively characterised in cell transformation and tumourigenesis (Basu & Haldar, 1998; Ionov *et al*, 2000; Frenzel *et al*, 2009), along with BCL-2, the antiapoptotic factor (Oltval *et al*, 1993; Frenzel *et al*, 2009). As expected, following treatment of TPC-1 cells with γ-irradiation the 2 pro-apoptotic factors BAX and PUMA were significantly upregulated, and the antiapoptotic factor BCL-2 non-significantly repressed by ~50 %. These findings imply that 15 Gy γ-irradiation is sufficient to influence p53-mediated expression of these genes, as reported in Section 3.3.2. However, following PBF overexpression there was no observed effect on the expression of BAX, BCL-2 and PUMA expression. In accordance with these observations, PBF overexpression had no effect on any of the 3 apoptotic genes in

HCT116 cells treated with 40 Gy of γ-irradiation. Furthermore, Western blotting data suggested that PBF has no effect on PUMA protein expression. We were unable to detect BCL-2 protein expression via Western blotting in either the presence or absence of ionising radiation, despite readily detecting BCL-2 mRNA expression, although this may reflect the avidity of the antibody used.

7.4.2.1. *The effects of PBF on the gross levels of apoptosis*

In light of the evidence to demonstrate that p53 performs transcription independent roles in the response to DNA damage, the effects of PBF overexpression on the gross levels of apoptosis in the presence and absence of DNA damage were assessed (Vousden & Prives, 2009; Speidel *et al*, 2006; Moll *et al*, 2005) . There were no significant effects of PBF overexpression on the levels of apoptosis following treatment with DNA damage in TPC-1 or HCT116 cells. Interestingly, despite significant induction of the proapoptotic factor PUMA (at both the mRNA and protein level, Section 7.3.2), there was no significant induction of apoptosis following DNA damage in ether VO-transfected or PBF transfected cells. These data suggest that, as described in 0, the predominant response of TPC-1 and HCT16 cells to 15 and 40 Gy γ-irradiation respectively may be that of growth arrest and not apoptosis. In the absence of DNA damage however, TPC-1 cells displayed a significant 18 % decrease in apoptosis levels in response to PBF overexpression compared to those of VO-transfected controls. Such findings indicate that PBF may influence the levels of apoptosis in the absence of DNA damage, and therefore the presence of inactive p53. However, in light of the fact that PBF overexpression had no effect on apoptosis in the presence of DNA damage, this finding was deemed to be of no consequence. Furthermore, there was no effect of PBF overexpression on apoptosis in the absence

of DNA damage in K1 or HCT116 cells. These data demonstrate that it is unlikely that apoptosis is responsible for the survival effects determined through the use of MTT cell viability assays.

Separate from the transcription independent control of apoptosis orchestrated by p53, there are reports of transcription independent modulation of cell growth. For example a study by Galy *et al*, 2001 confirmed that p53 is able to inhibit human fibroblast growth factor 2 in the absence of transcriptional activity. This report provides evidence that transcription independent functions of p53 are emerging, and demonstrate that the effects of PBF on p53 may be entirely valid. It is interesting to note that PBF is required for PTTG activation of FGF-2 (Ishikawa *et al*, 2001; Chien & Pei, 2000). Therefore it may be of interest to assess the effects of PBF p53-mediated FGF-2 inhibition in future investigations.

7.4.3. PBF and the PKB/AKT pathway

Targeted overexpression of PBF in murine thyroids results in striking enlargement of the thyroid gland, with associated hyperplastic and macrofollicular lesions. Further analysis revealed that PBF transgenic thyroids displayed significant repeatable induction of p-AKT (Ser-473) compared to their wild-type counterparts (Read *et al*, 2011). These findings were relevant, as AKT is strongly associated with the p53 pathway. For instance, AKT, whilst having no effect on the transcription of p53, directly promotes p53 turnover via the ubiquitin proteasomal pathway (Ogawara *et al*, 2002). The mechanism whereby AKT promotes p53 turnover and inactivation appears to be due to HDM2 activation, and several independent studies have confirmed that AKT directly activates HDM2 by phosphorylation of Ser-186 (Ogawara *et al*, 2002; Mayo & Donner, 2001; Zhou *et al*, 2001). Following transient

overexpression of PBF in human primary thyrocytes, PBF expression correlated with activation of AKT at Ser-473. These data hint that PBF and AKT may co-operate to bring about the observed effects of PBF overexpression on p53 biology. We attempted to assess the effects of PBF overexpression on the induction of p-AKT in our cell lines, and confirmed that PBF overexpression had no effect on the expression of total AKT protein expression in TPC-1, K1 or HCT116 cells. Preliminary data suggest that ionising radiation induces Ser-473 phosphorylation of AKT in TPC-1 cells, consistent with previous studies (Li *et al*, 2009; Viniegra *et al*, 2005; Jung *et al*, 2010), and that PBF overexpression may increase the levels of p-AKT. Further studies to verify or refute these initial findings may point to or eliminate a novel potential mechanism of p53 functional inactivation based on the co-operation of PBF and AKT to increase p53 degradation.

7.5. **Concluding statements**

Experiments in this chapter attempted to assess the gross biological effects of PBF overexpression in thyroid cancer cells. Such data revealed that PBF overexpression correlated with an increase in cell viability following treatment with ionising radiation, implying that PBF might confer a survival advantage under these conditions. However, PBF had no effect on the gross levels of apoptosis, suggesting that the mechanism of increased cell survival following PBF was unlikely to have targeted the apoptotic pathway. Preliminary data suggest that PBF expression positively correlates with phosphorylation of Akt at ser-473, leading to its activation, further characterisation of the effects of PBF on the Akt pathway in thyroid cancer cell lines may provide a mechanism whereby PBF affects cell survival. At present the pathway targeted by PBF to bring about the observed effects remains to be elucidated.

Chapter 8. **Final discussion and future work**

The research described within this thesis demonstrates investigations into the relationship between the poorly characterised proto-oncogene PBF and the tumour suppressor protein p53. Specific research included assessing the potential interaction between PBF and p53 and determining the effects of PBF on p53 homeostasis and function in thyroid papillary carcinoma cells. Investigations were founded on the observations that p53 mutations are rare in thyroid cancer, yet the thyroid gland is particularly sensitive to the carcinogenic effects of ionising radiation (Olivier *et al*, 2002; Malaguarnera *et al*, 2007b; Nikiforov *et al*, 1996; Klugbauer *et al*, 1995). We therefore hypothesised that p53 functional inactivation is common in thyroid cancers lacking inactivating p53 mutations, and that PBF overexpression (as observed in thyroid cancers) leads to the functional inactivation of p53 by directly interacting with and abrogating its tumour suppressor function in response to cell stresses such as DNA double strand breaks (DSBs).

8.1. **PBF directly interacts with p53**

Primary research aimed to assess the potential interaction of PBF and p53 using co-immunoprecipitation assays. Initial data within the McCabe laboratory demonstrated that PBF may interact with p53 *in vitro*. Such data were generated through GST-pulldown assays, which are known to provide artificial proximity of proteins, thus allowing for the potential interaction of proteins in a physiologically irrelevant manner. Through co-immunoprecipitations assays, we observed PBF to interact directly with p53 in 2 thyroid papillary carcinoma cell lines (TPC-1 and K1) expressing wild-type p53, both in the presence and absence of DNA damage. Therefore the interaction of PBF with p53 in these cells occurred both when p53 was inactivated, and when p53

was activated by DNA DSBs. Furthermore, the interaction of PBF and p53 was observed HCT116 cells, suggesting that the interaction was not specific to the thyroid. Such data were consistent with subcellular colocalisation of PBF and p53 using fluorescence immunocytochemistry assays. The interaction of PBF and p53 may point to a functional consequence of PBF on p53 function. Oncogenic expression of viral or cellular proteins has been reported to sequester p53 away from sites of interaction and to therefore inhibit its function (Hartl *et al*, 2008; Becker *et al*, 2007). In light of the established roles of PBF in the subcellular redistribution of important cellular proteins (Chien & Pei, 2000; Smith *et al*, 2009, 2012), we hypothesised that p53 subcellular distribution might be affected by PBF. However, using subcellular fractionation studies, we were unable to detect significant subcellular redistribution of p53 following overexpression of PBF. These data indicate that whilst PBF binds to p53, oncogenic expression of PBF does not result in p53 subcellular redistribution, and hence, this does not appear to be a mechanism whereby PBF might functionally inactivate p53. To further characterise the effects of PBF on p53 subcellular localisation, future studies could make use of specific mutants of PBF. For instance, a mutant lacking the bipartite NLS, and observed to remain within the cytoplasm, could be assessed with regard to p53 subcellular localisation. Conversely, a mutant p53 lacking a functional NLS could be employed to observe the effect of WT PBF on p53 localisation, and thus determine whether PBF can affect the nuclear localisation of p53. It would be of interest to utilise human primary thyrocytes to assess the relationship between PBF and p53 in normal, untransformed cells. This could be achieved through use of proximity ligation assays (PLAs). PLAs allow protein:protein interactions to be detected and quantified using a

small number of cells. This technique also reveals the subcellular localisation of such interactions (Weibrecht *et al*, 2010).

8.2. **PBF does not alter p53-mediated gene expression**

p53 is a potent transcription factor, and this functional role of p53 has been extensively characterised with regard to tumour suppression (Vogelstein *et al*, 2000; Wu & Levine, 1997; Meek, 2004). We used focused microarrays to assess the effects of PBF on the expression 84 p53-mediated genes. Surprisingly, PBF did not significantly alter p53-mediated transcriptional activity in the presence or absence of DNA damage. These data contrast with preliminary data demonstrating the effects of PBF on p53-mediated gene transcription using specific luciferase reporter assays (Dr Martin Read, University of Birmingham). Such data demonstrate that PBF significantly represses p53 as a transcription factor. Retrospectively, transfection efficiencies in TPC-1 cells may have been sub-optimal. Furthermore, given that PBF is overexpressed in thyroid cancers, the potential effect of PBF on p53 transcription may have been saturated. Future work would aim to further investigate the effects of PBF on p53-mediated gene transcription, as p53 primarily functions as a transcription factor. The sensitivity of detection could be improved by generating cell lines to stably overexpress PBF, and thus discard transfection issues. Furthermore, these focused microarrays could be performed in normal untransformed thyrocytes to prevent problems arising from high background PBF expression.

8.3. **PBF affects p53 homeostasis**

Apart from its role as a transcription factor, p53 is also capable of tumour suppression by transcription independent mechanisms (Vousden & Prives, 2009;

Speidel *et al*, 2006; Moll *et al*, 2005). Many viral or cellular oncogenes functionally inactivate p53 by direct interaction, causing disruption of p53 homeostasis and metabolism (Lee & Gu, 2009; Gilkes *et al*, 2006; Yuan *et al*, 2010b) . Overexpression of PBF led to a significant increase in p53 turnover in thyroid and colorectal carcinomas. Critically, this effect was reversed when PBF was depleted through the administration of specific siRNAs. These findings indicate that, under normal conditions, PBF might play a physiological role in maintenance of p53 homeostasis (Figure 8.1). For example, given that the binding of PBF and p53 may increase following γ-irradiation, PBF may be responsible for mediating p53 expression levels by increasing p53 turnover following successful maintenance of genome integrity in response to DNA damage. The mechanism by which PBF affects p53 turnover appears to involve the E3 ligase HDM2. For instance, specific ubiquitin assays revealed that PBF overexpression correlated with increased ubiquitination. Additionally, other ubiquitin assays indicate that PBF does not possess ubiquitin ligase activity, and could not therefore cause p53 ubiquitination directly. Moreover, recent data point to an interaction between PBF and HDM2, through co-immunoprecipitation assays, and continuation of this work by Mr Gavin Ryan (University of Birmingham), revealed that the effect of PBF on p53 turnover was impaired when HDM2 activity was inhibited following administration of Nutlin. These data imply that PBF may function as a novel co-factor of p53, binding to p53 and facilitating the interaction of HDM2 thereby increasing the efficiency of p53 turnover (Figure 8.1). Therefore, PBF might increase the efficiency of the interaction between p53 and HDM2, allowing p53 or HDM2 docking (Figure 8.1). Oncogenic expression of PBF may perhaps enhance this function, leading to increased p53 degradation,

and potential impairment of p53 function in thyroid cancer. Also, PBF could interact with specific regions within the p53 molecule that result in uncoupling of p53 and HDM2 following genotoxic insult, thereby allowing PBF and HDM2 to re-associate (Figure 8.1).

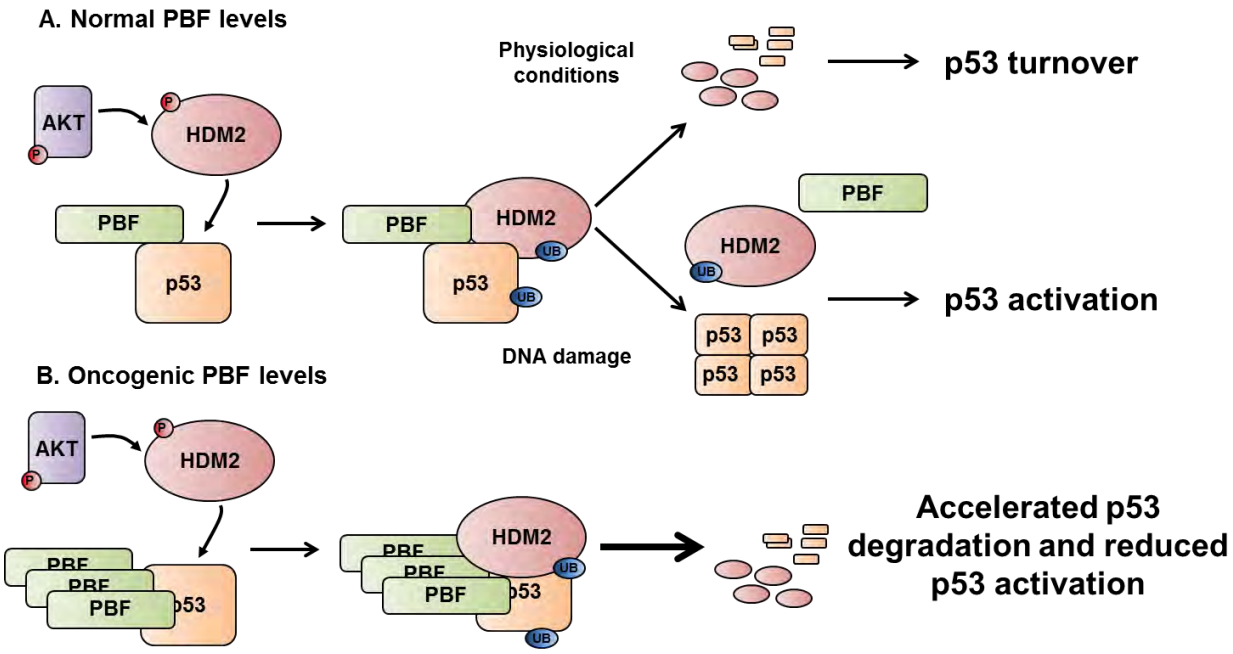


Figure 8.1: PBF influences p53 protein turnover. ***A:*** *PBF potentially binds to p53 and HDM2 and facilitates HDM2-mediated degradation of p53, serving to keep p53 levels low in the absence of cell stress. p-AKT may also play a role in this process.* ***B:*** *Oncogenic expression of PBF causes increased p53 turnover, potentially bringing about the impairment of p53 responses to cellular stress.*

8.4. **PBF confers a survival advantage after genotoxic insult**

Given the effects of PBF on p53 expression, we observed the effects of PBF overexpression on cell fate. MTT assays revealed that PBF overexpression correlated with increased cell viability or survival after DNA damage, which may be not due to inhibition of apoptosis. These findings imply that oncogenic expression of

PBF prevents normal cellular responses to DNA damage, thereby potentially allowing the incorporation genetic alterations within the genome (Figure 8.2).

Recently, in a murine model of targeted PBF overexpression within the thyroid, mice presented with striking enlargement of the thyroid with significant hyperplastic regions. Such findings are indicative of a PBF-mediated growth effect *in vivo* (Read *et al*, 2011). These mice however, did not develop thyroid tumours following PBF overexpression alone (Read *et al*, 2011, and McCabe laboratory unpublished data). Therefore, future experiments could be aimed at assessing tumour formation in these PBF transgenic mice following exposure to ionising radiation.

Interestingly, a potential mechanism for the altered cell growth in these transgenic mice was elucidated following the observation that PBF overexpression correlated with increased phosphorylation of AKT at ser-473 and hence activation of the AKT cell growth pathway (Read *et al*, 2011). We observed increased activation of AKT through serine-473 phosphorylation in human primary thyrocytes and assessed AKT activity in thyroid cancer. Our preliminary data suggest that PBF overexpression may also induce AKT activity in the presence and absence of ionising radiation. These data hint at a potential mechanism for the effect of PBF on cell survival in thyroid and colorectal cancer cells. Future studies would continue to assess the effects of PBF on AKT activation in thyroid carcinoma cells, to determine whether this effect was real. Furthermore, p-AKT appears to play an important role in p53 homeostasis by promoting p53 degradation through direct interaction with, and phosphorylation of, HDM2 (Ogawara *et al*, 2002). Therefore, investigating the relationship between PBF, p53, HDM2 and AKT, via 2-step co-immunoprecipitation assays, may serve to

elucidate the exact mechanisms whereby PBF effects p53 protein expression (Figure 8.1).

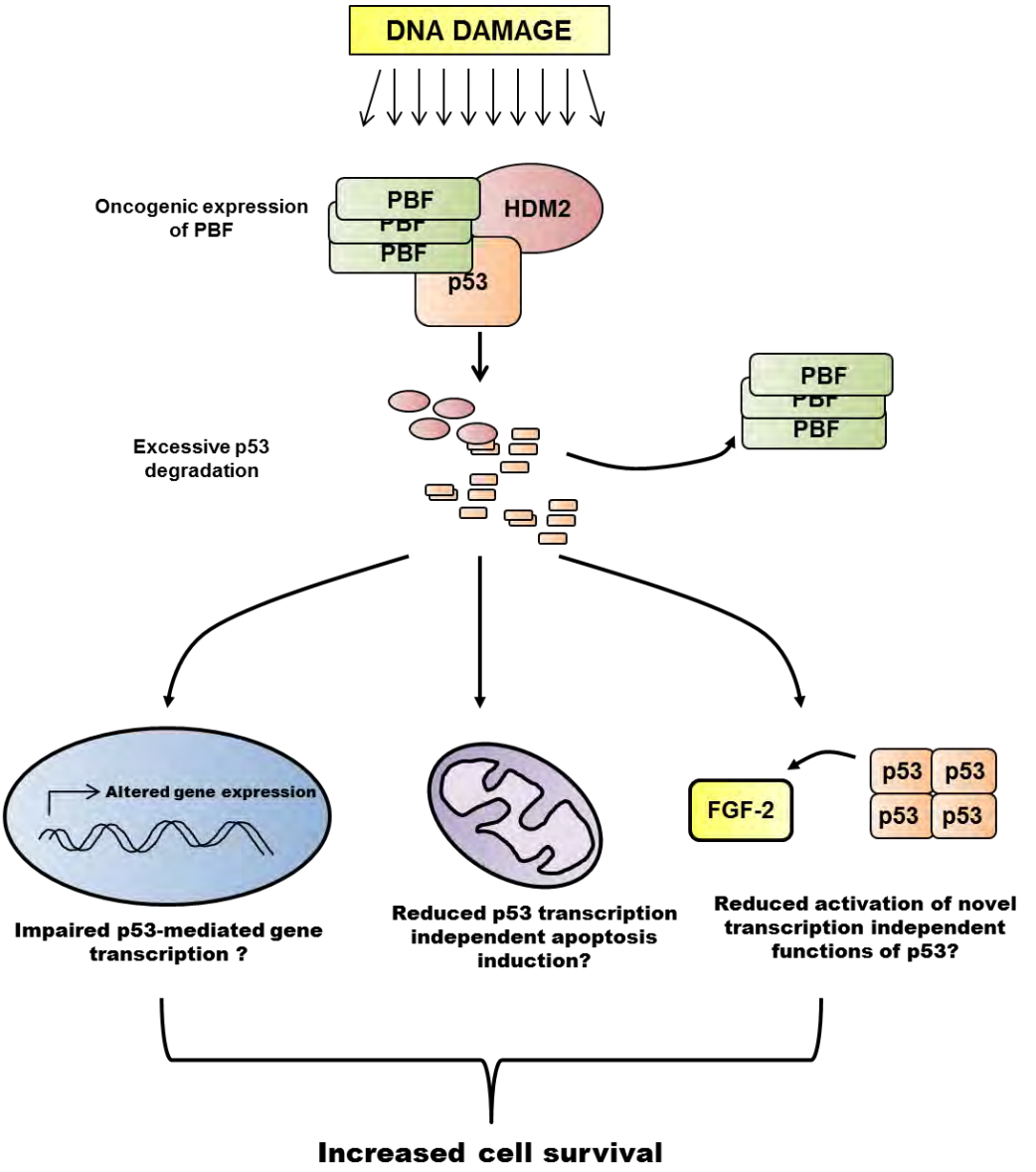


Figure 8.2: Oncogenic expression of PBF causes increased cell survival. PBF overexpression increases the HDM2-mediated degradation of p53. This finding correlates with an increase in cell survival following genotoxic insult. However, the exact functional consequence of PBF overexpression on the p53 pathway in thyroid cancer cells remains to be elucidated.

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8.5. **Concluding statements**

PBF binds to p53 and reduces its half-life in thyroid and colorectal cancer cell lines. Lacking intrinsic ubiquitin ligase activity, PBF appears to reduce p53 protein expression by bringing about its ubiquitination in an HDM2 dependent manner. The mechanism whereby PBF affects p53 expression may in part be due to activation and recruitment of p-AKT. Therefore, PBF may serve as a novel co-factor of p53 and HDM2 under normal conditions. However, oncogenic expression of PBF may lead to aberrant regulation of p53 turnover in thyroid cancer. Surprisingly, our data indicated that these effects of PBF on p53 homeostasis did not significantly affect the transcriptional functions of p53 in thyroid cancer cell lines. Nonetheless, overexpression of PBF conferred a significant survival advantage following DNA damage, indicating that oncogenic expression of PBF results in a gross biological effect that might potentially facilitate neoplastic growth and tumourigenesis. p53 tumour suppressor functions encompass a wide range of biological functions, and do not always involve gene transcription. PBF may therefore serve to promote p53 functional inactivation independently of its function as a transcription factor. However, the exact consequences of increased p53 degradation in response to oncogenic expression of PBF on p53 function remain to be elucidated.

In conclusion, oncogenic expression of PBF may result in a novel mechanism of p53 functional inactivation whereby PBF increases the degradation of p53 utilising the master regulator of p53, HDM2. These events may serve to increase cell survival following DNA damage, ultimately promoting cell transformation and tumorigenesis in thyroid cancers expression wild-type p53.

Chapter 9. **References**

Abou-El-Ardat K, Derradji H, Bekaert S, De Meyer T, Van Criekinge W & Baatout S (2008) Genrisk-T project: response of the TPC-1 papillary thyroid carcinoma cell line to a range of low to high doses of irradiation. *Commun. Agric. Appl. Biol. Sci.* **73:** 79–83

Albores-Saavedra J, Henson DE, Glazer E & Schwartz AM (2007) Changing patterns in the incidence and survival of thyroid cancer with follicular phenotype--papillary, follicular, and anaplastic: a morphological and epidemiological study. *Endocr. Pathol.* **18:** 1–7

Ambesi-Impiombato FS, Parks LA & Coon HG (1980) Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc. Natl. Acad. Sci. U.S.A.* **77:** 3455–3459

Appella E & Anderson CW (2000) Signaling to p53: breaking the posttranslational modification code. *Pathol. Biol.* **48:** 227–245

Appella E & Anderson CW (2001) Post-translational modifications and activation of p53 by genotoxic stresses. *European Journal of Biochemistry* **268:** 2764–2772

Aratake Y, Nomura H, Kotani T, Marutsuka K, Kobayashi K, Kuma K, Miyauchi A, Okayama A & Tamura K (2006) Coexistent anaplastic and differentiated thyroid carcinoma: an immunohistochemical study. *Am. J. Clin. Pathol.* **125:** 399–406

Ardley HC & Robinson PA (2005) E3 ubiquitin ligases. *Essays Biochem.* **41:** 15–30

Ashcroft M & Vousden KH (1999) Regulation of p53 stability. *Oncogene* **18:** 7637–7643

Astakhova LN, Anspaugh LR, Beebe GW, Bouville A, Drozdovitch VV, Garber V, Gavrilin YI, Khrouch VT, Kuvshinnikov AV, Kuzmenkov YN, Minenko VP, Moschik KV, Nalivko AS, Robbins J, Shemiakina EV, Shinkarev S, Tochitskaya SI & Waclawiw MA (1998) Chernobyl-related thyroid cancer in children of Belarus: a case-control study. *Radiat. Res.* **150:** 349–356

Atchison M, Basu A, Zaprazna K & Papasani M (2011) Mechanisms of Yin Yang 1 in oncogenesis: the importance of indirect effects. *Crit Rev Oncog* **16:** 143–161

Baker SR & Bhatti WA (2006) The thyroid cancer epidemic: is it the dark side of the CT revolution? *Eur J Radiol* **60:** 67–69

Baloch ZW, Fleisher S, LiVolsi VA & Gupta PK (2002) Diagnosis of 'follicular neoplasm': a gray zone in thyroid fine-needle aspiration cytology. *Diagn. Cytopathol.* **26:** 41–44

Baloch ZW, LiVolsi VA, Asa SL, Rosai J, Merino MJ, Randolph G, Vielh P, DeMay RM, Sidawy MK & Frable WJ (2008) Diagnostic terminology and morphologic criteria for cytologic diagnosis of thyroid lesions: a synopsis of the National Cancer Institute Thyroid Fine-Needle Aspiration State of the Science Conference. *Diagn. Cytopathol.* **36:** 425–437

Basolo F, Pisaturo F, Pollina LE, Fontanini G, Elisei R, Molinaro E, Iacconi P, Miccoli P & Pacini F (2000) N-ras mutation in poorly differentiated thyroid carcinomas: correlation with bone metastases and inverse correlation to thyroglobulin expression. *Thyroid* **10:** 19–23

Basu A & Haldar S (1998) The Relationship Between Bcl2, Bax and P53: Consequences for Cell Cycle Progression and Cell Death. *Mol. Hum. Reprod.* **4:** 1099–1109

Becker K, Marchenko ND, Maurice M & Moll UM (2007) Hyperubiquitylation of wild-type p53 contributes to cytoplasmic sequestration in neuroblastoma. *Cell Death & Differentiation* **14:** 1350–1360

Bell S, Klein C, Müller L, Hansen S & Buchner J (2002) p53 contains large unstructured regions in its native state. *J. Mol. Biol.* **322:** 917–927

Blagosklonny MV, Giannakakou P, Wojtowicz M, Romanova LY, Ain KB, Bates SE & Fojo T (1998) Effects of p53-Expressing Adenovirus on the Chemosensitivity and Differentiation of Anaplastic Thyroid Cancer Cells. *JCEM* **83:** 2516–2522

Boehme KA, Kulikov R & Blattner C (2008) p53 stabilization in response to DNA damage requires Akt/PKB and DNA-PK. *PNAS* **105:** 7785–7790

Boelaert K, Smith VE, Stratford AL, Kogai T, Tannahill LA, Watkinson JC, Eggo MC, Franklyn JA & McCabe CJ (2007) PTTG and PBF repress the human sodium iodide symporter. *Oncogene* **26:** 4344–4356

Boelaert K, Tannahill LA, Bulmer JN, Kachilele S, Chan SY, Kim D, Gittoes NJL, Franklyn JA, Kilby MD & McCabe CJ (2003) A potential role for PTTG/securin in the developing human fetal brain. *FASEB J.* **17:** 1631–1639

Boice JD Jr (2012) Radiation epidemiology: a perspective on Fukushima. *J Radiol Prot* **32:** N33–40

Bond GL, Hu W & Levine AJ (2005) MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr Cancer Drug Targets* **5:** 3–8

Brady CA & Attardi LD (2010) p53 at a glance. *J Cell Sci* **123:** 2527–2532

Brenner-Gati L, Berg KA & Gershengorn MC (1988) Thyroid-stimulating hormone and insulin-like growth factor-1 synergize to elevate 1,2-diacylglycerol in rat thyroid cells. Stimulation of DNA synthesis via interaction between lipid and adenylyl cyclase signal transduction systems. *J Clin Invest* **82:** 1144–1148

Brooks CL & Gu W (2006) p53 Ubiquitination: Mdm2 and Beyond. *Molecular Cell* **21:** 307–315

Burgess JR & Tucker P (2006) Incidence trends for papillary thyroid carcinoma and their correlation with thyroid surgery and thyroid fine-needle aspirate cytology. *Thyroid* **16:** 47–53

Candau R, Scolnick DM, Darpino P, Ying CY, Halazonetis TD & Berger SL (1997) Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. *Oncogene* **15:** 807–816

Chakravarty D, Santos E, Ryder M, Knauf JA, Liao X-H, West BL, Bollag G, Kolesnick R, Thin TH, Rosen N, Zanzonico P, Larson SM, Refetoff S, Ghossein R & Fagin JA (2011) Small-molecule MAPK inhibitors restore radioiodine incorporation in mouse thyroid cancers with conditional BRAF activation. *J. Clin. Invest.* **121:** 4700–4711

Challeton C, Branea F, Schlumberger M, Gaillard N, Vathaire F de, Badie C, Antonini P & Parmentier C (1997) Characterization and radiosensitivity at high or low dose rate of four cell lines derived from human thyroid tumors. *International Journal of Radiation Oncology*Biology*Physics* **37:** 163–169

Charles R-P, Iezza G, Amendola E, Dankort D & McMahon M (2011) Mutationally activated BRAF(V600E) elicits papillary thyroid cancer in the adult mouse. *Cancer Res.* **71:** 3863–3871

Chen AY, Jemal A & Ward EM (2009) Increasing incidence of differentiated thyroid cancer in the United States, 1988-2005. *Cancer* **115:** 3801–3807

Chen J, Marechal V & Levine AJ (1993) Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* **13:** 4107–4114

Chien W & Pei L (2000) A novel binding factor facilitates nuclear translocation and transcriptional activation function of the pituitary tumor-transforming gene product. *J. Biol. Chem.* **275:** 19422–19427

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M & Green DR (2004) Direct Activation of Bax by p53 Mediates Mitochondrial Membrane Permeabilization and Apoptosis. *Science* **303:** 1010–1014

Cho Y, Gorina S, Jeffrey PD & Pavletich NP (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* **265:** 346–355

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159

Ciampi R & Nikiforov YE (2007) RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. *Endocrinology* **148:** 936–941

Cotter TG (2009) Apoptosis and cancer: the genesis of a research field. *Nature Reviews Cancer* **9:** 501–507

D'Avanzo A, Ituarte P, Treseler P, Kebebew E, Wu J, Wong M, Duh QY, Siperstein AE & Clark OH (2004) Prognostic scoring systems in patients with follicular thyroid cancer: a comparison of different staging systems in predicting the patient outcome. *Thyroid* **14:** 453–458

Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C & Eng C (1997) Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. *Cancer Res.* **57:** 4710–4713

Danovi D, Meulmeester E, Pasini D, Migliorini D, Capra M, Frenk R, Graaf P de, Francoz S, Gasparini P, Gobbi A, Helin K, Pelicci PG, Jochemsen AG & Marine J-C (2004) Amplification of Mdmx (or Mdm4) Directly Contributes to Tumor Formation by Inhibiting p53 Tumor Suppressor Activity. *Mol. Cell. Biol.* **24:** 5835–5843

Dauer LT, Zanzonico P, Tuttle RM, Quinn DM & Strauss HW (2011) The Japanese tsunami and resulting nuclear emergency at the Fukushima Daiichi power facility: technical, radiologic, and response perspectives. *J. Nucl. Med.* **52:** 1423–1432

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, *et al* (2002) Mutations of the BRAF gene in human cancer. *Nature* **417:** 949–954

DeLeo AB, Jay G, Appella E, Dubois GC, Law LW & Old LJ (1979) Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 2420–2424

Deleu S, Pirson I, Coulonval K, Drouin A, Taton M, Clermont F, Roger PP, Nakamura T, Dumont JE & Maenhaut C (1999) IGF-1 or insulin, and the TSH cyclic AMP cascade separately control dog and human thyroid cell growth and DNA synthesis, and complement each other in inducing mitogenesis. *Mol. Cell. Endocrinol.* **149:** 41–51

Dhillon AS & Kolch W (2004) Oncogenic B-Raf mutations: crystal clear at last. *Cancer Cell* **5:** 303–304

Dhomen N & Marais R (2007) New insight into BRAF mutations in cancer. *Curr. Opin. Genet. Dev.* **17:** 31–39

Eggo MC, King WJ, Black EG & Sheppard MC (1996) Functional human thyroid cells and their insulin-like growth factor-binding proteins: regulation by thyrotropin, cyclic 3',5' adenosine monophosphate, and growth factors. *J. Clin. Endocrinol. Metab.* **81:** 3056–3062

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW & Vogelstein B (1992) Definition of a consensus binding site for p53. *Nat. Genet.* **1:** 45–49

Eliyahu D, Goldfinger N, Pinhasi-Kimhi O, Shaulsky G, Skurnik Y, Arai N, Rotter V & Oren M (1988) Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene* **3:** 313–321

Evans RM, Barish GD & Wang Y-X (2004) PPARs and the complex journey to obesity. *Nat. Med.* **10:** 355–361

Fagin JA (2005) Genetics of papillary thyroid cancer initiation: implications for therapy. *Trans. Am. Clin. Climatol. Assoc.* **116:** 259–269; discussion 269–271

Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH & Koeffler HP (1993) High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. *J. Clin. Invest.* **91:** 179–184

Fang S, Jensen JP, Ludwig RL, Vousden KH & Weissman AM (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J. Biol. Chem.* **275:** 8945–8951

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M & Levine AJ (1988) Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* **8:** 531–539

Frasca F, Rustighi A, Malaguarnera R, Altamura S, Vigneri P, Del Sal G, Giancotti V, Pezzino V, Vigneri R & Manfioletti G (2006) HMGA1 Inhibits the Function of P53 Family Members in Thyroid Cancer Cells. *Cancer Res* **66:** 2980–2989

Frenzel A, Grespi F, Chmelewskij W & Villunger A (2009) Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis* **14:** 584–596

Fugazzola L, Pilotti S, Pinchera A, Vorontsova TV, Mondellini P, Bongarzone I, Greco A, Astakhova L, Butti MG, Demidchik EP, Pacini F & Pierotti MA (1995) Oncogenic Rearrangements of the RET Proto-Oncogene in Papillary Thyroid Carcinomas from Children Exposed to the Chernobyl Nuclear Accident. *Cancer Res* **55:** 5617–5620

Furukawa Y, Sutheesophon K, Wada T, Nishimura M, Saito Y, Ishii H & Furukawa Y (2005) Methylation silencing of the Apaf-1 gene in acute leukemia. *Mol. Cancer Res.* **3:** 325–334

Galy B, Créancier L, Zanibellato C, Prats AC & Prats H (2001) Tumour suppressor p53 inhibits human fibroblast growth factor 2 expression by a post-transcriptional mechanism. *Oncogene* **20:** 1669–1677

García B & Santisteban P (2002) PI3K is involved in the IGF-I inhibition of TSH-induced sodium/iodide symporter gene expression. *Mol. Endocrinol.* **16:** 342–352

García-Rostán G, Costa AM, Pereira-Castro I, Salvatore G, Hernandez R, Hermsem MJA, Herrero A, Fusco A, Cameselle-Teijeiro J & Santoro M (2005) Mutation of the PIK3CA gene in anaplastic thyroid cancer. *Cancer Res.* **65:** 10199–10207

Garcia-Rostan G, Tallini G, Herrero A, D'Aquila TG, Carcangiu ML & Rimm DL (1999) Frequent mutation and nuclear localization of beta-catenin in anaplastic thyroid carcinoma. *Cancer Res.* **59:** 1811–1815

Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, Wu R, Carcangiu ML, Costa J & Tallini G (2003) ras mutations are associated with aggressive

tumor phenotypes and poor prognosis in thyroid cancer. *J. Clin. Oncol.* **21:** 3226–3235

Gatza C, Moore L, Dumble M & Donehower LA (2007) Tumor suppressor dosage regulates stem cell dynamics during aging. *Cell Cycle* **6:** 52–55

Gilkes DM, Chen L & Chen J (2006) MDMX regulation of p53 response to ribosomal stress. *EMBO J* **25:** 5614–5625

Goretzki PE, Koob R, Koller C & Röher HD (1987) Thyrotropin (TSH) stimulates cell growth and DNA synthesis in monolayer cultures of human thyrocytes independent of the adenylate-cyclase system. *Acta Endocrinol Suppl (Copenh)* **281:** 273–280

Gottlieb TM, Leal JFM, Seger R, Taya Y & Oren M (2002) Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. *Oncogene* **21:** 1299–1303

Gregory Powell J, Wang X, Allard BL, Sahin M, Wang X-L, Hay ID, Hiddinga HJ, Deshpande SS, Kroll TG, Grebe SKG, Eberhardt NL & McIver B (2004) The PAX8/PPARgamma fusion oncoprotein transforms immortalized human thyrocytes through a mechanism probably involving wild-type PPARgamma inhibition. *Oncogene* **23:** 3634–3641

Gu Z, Flemington C, Chittenden T & Zambetti GP (2000) ei24, a p53 Response Gene Involved in Growth Suppression and Apoptosis. *Mol. Cell. Biol.* **20:** 233–241

Gudkov AV & Komarova EA (2003) The role of p53 in determining sensitivity to radiotherapy. *Nature Reviews Cancer* **3:** 117–129

H|[auml]|rtl B, Zeller T, Blanchette P, Kremmer E & Dobner T (2008) Adenovirus type 5 early region 1B 55-kDa oncoprotein can promote cell transformation by a mechanism independent from blocking p53-activated transcription. *Oncogene* **27:** 3673–3684

Halevy O, Rodel J, Peled A & Oren M (1991) Frequent p53 mutations in chemically induced murine fibrosarcoma. *Oncogene* **6:** 1593–1600

Hamatani K, Eguchi H, Ito R, Mukai M, Takahashi K, Taga M, Imai K, Cologne J, Soda M, Arihiro K, Fujihara M, Abe K, Hayashi T, Nakashima M, Sekine I, Yasui W, Hayashi Y & Nakachi K (2008) RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. *Cancer Res.* **68:** 7176–7182

Harms KL & Chen X (2006) The functional domains in p53 family proteins exhibit both common and distinct properties. *Cell Death Differ.* **13:** 890–897

Harvey M, Vogel H, Lee EY-HP, Bradley A & Donehower LA (1995) Mice Deficient in Both p53 and Rb Develop Tumors Primarily of Endocrine Origin. *Cancer Res* **55:** 1146–1151

Havre PA, Yuan J, Hedrick L, Cho KR & Glazer PM (1995) P53 Inactivation by HPV16 E6 Results in Increased Mutagenesis in Human Cells. *Cancer Res* **55:** 4420–4424

Heaney AP, Nelson V, Fernando M & Horwitz G (2001) Transforming events in thyroid tumorigenesis and their association with follicular lesions. *J. Clin. Endocrinol. Metab.* **86:** 5025–5032

Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M & Melmed S (2000) Expression of pituitary-tumour transforming gene in colorectal tumours. *Lancet* **355:** 716–719

Hess RD & Brandner G (1997) DNA-damage-inducible p53 activity in SV40-transformed cells. *Oncogene* **15:** 2501–2504

Honda R, Tanaka H & Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420:** 25–27

Horie S, Maeta H, Endo K, Ueta T, Takashima K & Terada T (2001) Overexpression of p53 protein and MDM2 in papillary carcinomas of the thyroid: Correlations with clinicopathologic features. *Pathol. Int.* **51:** 11–15

Hu B, Gilkes DM, Farooqi B, Sebti SM & Chen J (2006) MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. *J. Biol. Chem.* **281:** 33030–33035

Hu W, Feng Z, Teresky AK & Levine AJ (2007) p53 regulates maternal reproduction through LIF. *Nature* **450:** 721–724

Imaizumi M, Usa T, Tominaga T, Neriishi K, Akahoshi M, Nakashima E, Ashizawa K, Hida A, Soda M, Fujiwara S, Yamada M, Ejima E, Yokoyama N, Okubo M, Sugino K, Suzuki G, Maeda R, Nagataki S & Eguchi K (2006) Radiation dose-response relationships for thyroid nodules and autoimmune thyroid diseases in Hiroshima and Nagasaki atomic bomb survivors 55-58 years after radiation exposure. *JAMA* **295:** 1011–1022

Ionov Y, Yamamoto H, Krajewski S, Reed JC & Perucho M (2000) Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. *Proc. Natl. Acad. Sci. U.S.A.* **97:** 10872–10877

Ishikawa H, Heaney AP, Yu R, Horwitz GA & Melmed S (2001) Human Pituitary Tumor-Transforming Gene Induces Angiogenesis. *JCEM* **86:** 867–874

Ito T, Seyama T, Iwamoto KS, Hayashi T, Mizuno T, Tsuyama N, Dohi K, Nakamura N & Akiyama M (1993) In vitro irradiation is able to cause RET oncogene rearrangement. *Cancer Res.* **53:** 2940–2943

Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL & Zambetti GP (2003) Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4:** 321–328

Jeker LT, Hejazi M, Burek CL, Rose NR & Caturegli P (1999) Mouse thyroid primary culture. *Biochem. Biophys. Res. Commun.* **257:** 511–515

Jennings T, Bratslavsky G, Gerasimov G, Troshina K, Bronstein M, Dedov I, Alexandrova G & Figge J (1995) Nuclear accumulation of MDM2 protein in well-differentiated papillary thyroid carcinomas. *Exp. Mol. Pathol.* **62:** 199–206

Jin G, Cook S, Cui B, Chen WC, Keir ST, Killela P, Di C, Payne CA, Gregory SG, McLendon R, Bigner DD & Yan H (2010) HDMX Regulates P53 Activity and Confers Chemoresistance to 3-Bis(2-Chloroethyl)-1-Nitrosourea. *Neuro Oncol* **12:** 956–966

Johnson KR, Lehn DA, Elton TS, Barr PJ & Reeves R (1988) Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y). *J. Biol. Chem.* **263:** 18338–18342

Johnson TM, Hammond EM, Giaccia A & Attardi LD (2005) The p53QS transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. *Nat. Genet.* **37:** 145–152

Johnston LE, Tran Cao HS, Chang DC & Bouvet M (2012) Sociodemographic Predictors of Survival in Differentiated Thyroid Cancer: Results from the SEER Database. *ISRN Endocrinol* **2012:** Available at:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3431073/ [Accessed September 12, 2012]

Jones SN, Roe AE, Donehower LA & Bradley A (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378:** 206–208

Jonklaas J, Sarlis NJ, Litofsky D, Ain KB, Bigos ST, Brierley JD, Cooper DS, Haugen BR, Ladenson PW, Magner J, Robbins J, Ross DS, Skarulis M, Maxon HR & Sherman SI (2006) Outcomes of patients with differentiated thyroid carcinoma following initial therapy. *Thyroid* **16:** 1229–1242

Jung IL, Kang HJ, Kim KC & Kim IG (2010) PTEN/pAkt/p53 signaling pathway correlates with the radioresponse of non-small cell lung cancer. *Int. J. Mol. Med.* **25:** 517–523

Junttila MR & Evan GI (2009) p53 — a Jack of all trades but master of none. *Nature Reviews Cancer* **9:** 821–829

Kaeser MD, Pebernard S & Iggo RD (2004) Regulation of P53 Stability and Function in HCT116 Colon Cancer Cells. *J. Biol. Chem.* **279:** 7598–7605

Kamada R, Nomura T, Anderson CW & Sakaguchi K (2011) Cancer-associated p53 Tetramerization Domain Mutants QUANTITATIVE ANALYSIS REVEALS A LOW THRESHOLD FOR TUMOR SUPPRESSOR INACTIVATION. *J. Biol. Chem.* **286:** 252–258

Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF & Sherr CJ (1998) Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *PNAS* **95:** 8292–8297

Kanfi Y, Peshti V, Gozlan YM, Rathaus M, Gil R & Cohen HY (2008) Regulation of SIRT1 protein levels by nutrient availability. *FEBS Lett.* **582:** 2417–2423

Kawai H, Lopez-Pajares V, Kim MM, Wiederschain D & Yuan Z-M (2007) RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer Res.* **67:** 6026–6030

Kazakov VS, Demidchik EP & Astakhova LN (1992) Thyroid cancer after Chernobyl. *Nature* **359:** 21

Kim CS & Zhu X (2009) Lessons from mouse models of thyroid cancer. *Thyroid* **19:** 1317–1331

Kim D, Pemberton H, Stratford AL, Buelaert K, Watkinson JC, Lopes V, Franklyn JA & McCabe CJ (2005) Pituitary tumour transforming gene (PTTG) induces genetic instability in thyroid cells. *Oncogene* **24:** 4861–4866

Kim SK, Hwang TS, Yoo YB, Han HS, Kim D-L, Song K-H, Lim SD, Kim WS & Paik NS (2011) Surgical results of thyroid nodules according to a management guideline based on the BRAF(V600E) mutation status. *J. Clin. Endocrinol. Metab.* **96:** 658–664

Kimura T, Keymeulen AV, Golstein J, Fusco A, Dumont JE & Roger PP (2001) Regulation of Thyroid Cell Proliferation by TSH and Other Factors: A Critical Evaluation of in Vitro Models. *Endocrine Reviews* **22:** 631–656

Kinner A, Wu W, Staudt C & Iliakis G (2008) Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res.* **36:** 5678–5694

Klugbauer S, Lengfelder E, Demidchik EP & Rabes HM (1995) High prevalence of RET rearrangement in thyroid tumors of children from Belarus after the Chernobyl reactor accident. *Oncogene* **11:** 2459–2467

Kogai T, Taki K & Brent GA (2006) Enhancement of sodium/iodide symporter expression in thyroid and breast cancer. *Endocr. Relat. Cancer* **13:** 797–826

Kress M, May E, Cassingena R & May P (1979) Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *J. Virol.* **31:** 472–483

Kubbutat MHG, Jones SN & Vousden KH (1997) Regulation of p53 stability by Mdm2. *, Published online: 15 May 1997;* | *doi:10.1038/387299a0* **387:** 299–303

Kuo LJ & Yang L-X (2008) Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In Vivo* **22:** 305–309

Kurihara T, Ikeda S, Ishizaki Y, Fujimori M, Tokumoto N, Hirata Y, Ozaki S, Okajima M, Sugino K & Asahara T (2004) Immunohistochemical and sequencing analyses of the Wnt signaling components in Japanese anaplastic thyroid cancers. *Thyroid* **14:** 1020–1029

Kyo S, Inoue M, Nakazawa A, Ogawa H, Ueda G & Tanizawa O (1991) [Detection of HPV DNA in the uterine cervical lesions by polymerase chain reaction and in situ hybridization]. *Nippon Sanka Fujinka Gakkai Zasshi* **43:** 541–548

Lane DP & Crawford LV (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278:** 261–263

Lee JT & Gu W (2009) The multiple levels of regulation by p53 ubiquitination. *Cell Death & Differentiation* **17:** 86–92

Lehar SM, Nacht M, Jacks T, Vater CA, Chittenden T & Guild BC (1996) Identification and cloning of EI24, a gene induced by p53 in etoposide-treated cells. *Oncogene* **12:** 1181–1187

Levine AJ & Oren M (2009) The first 30 years of p53: growing ever more complex. *Nature Reviews Cancer* **9:** 749–758

Lewis AE, Fikaris AJ, Prendergast GV & Meinkoth JL (2004) Thyrotropin and serum regulate thyroid cell proliferation through differential effects on p27 expression and localization. *Mol. Endocrinol.* **18:** 2321–2332

Lewy GD, Sharma N, Seed RI, Smith VE, Boelaert K & McCabe CJ (2012) PTTG in Thyroid Cancer. *Journal of Endocrinological Investigation* Available at: http://www.ncbi.nlm.nih.gov/pubmed/22522436 [Accessed April 30, 2012]

Li H-F, Kim J-S & Waldman T (2009) Radiation-induced Akt activation modulates radioresistance in human glioblastoma cells. *Radiation Oncology* **4:** 43

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES & Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479–489

Liang SH & Clarke MF (1999) The nuclear import of p53 is determined by the presence of a basic domain and its relative position to the nuclear localization signal. *Oncogene* **18:** 2163–2166

Liao DJ & Dickson RB (2000) c-Myc in breast cancer. *Endocr. Relat. Cancer* **7:** 143–164

Linzer DI & Levine AJ (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17:** 43–52

Linzer DI, Maltzman W & Levine AJ (1979) The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen. *Virology* **98:** 308–318

Liu Y & Kulesz-Martin MF (2006) Sliding into home: facilitated p53 search for targets by the basic DNA binding domain. *Cell Death Differ.* **13:** 881–884

Lyle R, Béna F, Gagos S, Gehrig C, Lopez G, Schinzel A, Lespinasse J, Bottani A, Dahoun S, Taine L, Doco-Fenzy M, Cornillet-Lefèbvre P, Pelet A, Lyonnet S, Toutain A, Colleaux L, Horst J, Kennerknecht I, Wakamatsu N, Descartes M, *et al* (2009) Genotype-phenotype correlations in Down syndrome identified by array CGH in 30 cases of partial trisomy and partial monosomy chromosome 21. *Eur. J. Hum. Genet.* **17:** 454–466

MacCorkle RA & Tan T-H (2005) Mitogen-activated protein kinases in cell-cycle control. *Cell Biochem. Biophys.* **43:** 451–461

Mahaney BL, Meek K & Lees-Miller SP (2009) Repair of ionizing radiation-induced DNA double strand breaks by non-homologous end-joining. *Biochem J* **417:** 639–650

Malaguarnera R, Vella V, Vigneri R & Frasca F (2007a) P53 Family Proteins in Thyroid Cancer. *Endocr Relat Cancer* **14:** 43–60

Malaguarnera R, Vella V, Vigneri R & Frasca F (2007b) p53 family proteins in thyroid cancer. *Endocr Relat Cancer* **14:** 43–60

Manenti G, Pilotti S, Re FC, Della Porta G & Pierotti MA (1994) Selective activation of ras oncogenes in follicular and undifferentiated thyroid carcinomas. *Eur. J. Cancer* **30A:** 987–993

Marine J-CW, Dyer MA & Jochemsen AG (2007) MDMX: from bench to bedside. *J. Cell. Sci.* **120:** 371–378

Marmorstein LY, Ouchi T & Aaronson SA (1998a) The BRCA2 gene product functionally interacts with p53 and RAD51. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 13869–13874

Marmorstein LY, Ouchi T & Aaronson SA (1998b) The BRCA2 gene product functionally interacts with p53 and RAD51. *Proc Natl Acad Sci U S A* **95:** 13869–13874

Massimi P, Shai A, Lambert P & Banks L (2007) HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background. *Oncogene* **27:** 1800–1804

Mateu MG & Fersht AR (1998) Nine hydrophobic side chains are key determinants of the thermodynamic stability and oligomerization status of tumour suppressor p53 tetramerization domain. *EMBO J.* **17:** 2748–2758

Matoba S, Kang J-G, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F & Hwang PM (2006) p53 regulates mitochondrial respiration. *Science* **312:** 1650–1653

Mayo LD & Donner DB (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *PNAS* **98:** 11598–11603

Mazzaferri EL (1993) Management of a solitary thyroid nodule. *N. Engl. J. Med.* **328:** 553–559

McCabe CJ, Boelaert K, Tannahill LA, Heaney AP, Stratford AL, Khaira JS, Hussain S, Sheppard MC, Franklyn JA & Gittoes NJL (2002) Vascular endothelial growth factor, its receptor KDR/Flk-1, and pituitary tumor transforming gene in pituitary tumors. *J. Clin. Endocrinol. Metab.* **87:** 4238–4244

Medina DL, Toro MJ & Santisteban P (2000) Somatostatin interferes with thyrotropin-induced G1-S transition mediated by cAMP-dependent protein kinase and phosphatidylinositol 3-kinase. Involvement of RhoA and cyclin E x cyclin-dependent kinase 2 complexes. *J. Biol. Chem.* **275:** 15549–15556

Medina DL, Velasco JA & Santisteban P (1999) Somatostatin is expressed in FRTL-5 thyroid cells and prevents thyrotropin-mediated down-regulation of the cyclin-dependent kinase inhibitor p27kip1. *Endocrinology* **140:** 87–95

Meek DW (2004) The p53 response to DNA damage. *DNA Repair (Amst.)* **3:** 1049–1056

Meek DW & Anderson CW (2009) Posttranslational Modification of p53: Cooperative Integrators of Function. *Cold Spring Harb Perspect Biol* **1:** Available at: http://cshperspectives.cshlp.org/content/1/6/a000950 [Accessed September 9, 2012]

Meek DW & Hupp TR (2010) The regulation of MDM2 by multisite phosphorylation—Opportunities for molecular-based intervention to target tumours? *Seminars in Cancer Biology* **20:** 19–28

Mercer KE & Pritchard CA (2003) Raf proteins and cancer: B-Raf is identified as a mutational target. *Biochim. Biophys. Acta* **1653:** 25–40

Messina RL, Sanfilippo M, Vella V, Pandini G, Vigneri P, Nicolosi ML, Gianì F, Vigneri R & Frasca F (2012) Reactivation of p53 mutants by p53 reactivation and induction of massive apoptosis in thyroid cancer cells. *International Journal of Cancer* **130:** 2259–2270

Mietz JA, Unger T, Huibregtse JM & Howley PM (1992) The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J* **11:** 5013–5020

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P & Moll UM (2003) p53 Has a Direct Apoptogenic Role at the Mitochondria. *Molecular Cell* **11:** 577–590

Mishra A & Jana NR (2008) Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome. *Cell. Mol. Life Sci.* **65:** 656–666

Moll UM, LaQuaglia M, Bénard J & Riou G (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 4407–4411

Moll UM, Wolff S, Speidel D & Deppert W (2005) Transcription-independent pro-apoptotic functions of p53. *Curr. Opin. Cell Biol.* **17:** 631–636

Montes de Oca Luna R, Wagner DS & Lozano G (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378:** 203–206

Moro F, Ottaggio L, Bonatti S, Simili M, Miele M, Bozzo S & Abbondandolo A (1995) p53 expression in normal versus transformed mammalian cells. *Carcinogenesis* **16:** 2435–2440

Motoi N, Sakamoto A, Yamochi T, Horiuchi H, Motoi T & Machinami R (2000) Role of ras mutation in the progression of thyroid carcinoma of follicular epithelial origin. *Pathol. Res. Pract.* **196:** 1–7

Moynahan ME, Pierce AJ & Jasin M (2001) BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol. Cell* **7:** 263–272

Murray-Zmijewski F, Slee EA & Lu X (2008) A complex barcode underlies the heterogeneous response of p53 to stress. *Nature Reviews Molecular Cell Biology* **9:** 702–712

Nakano K & Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell* **7:** 683–694

Namba H, Rubin SA & Fagin JA (1990) Point mutations of ras oncogenes are an early event in thyroid tumorigenesis. *Mol. Endocrinol.* **4:** 1474–1479

Nandi D, Tahiliani P, Kumar A & Chandu D (2006) The ubiquitin-proteasome system. *J Biosci* **31:** 137–155

Nathanson KL, Wooster R, Weber BL & Nathanson KN (2001) Breast cancer genetics: what we know and what we need. *Nat. Med.* **7:** 552–556

Nedelcu AM & Tan C (2007) Early diversification and complex evolutionary history of the p53 tumor suppressor gene family. *Dev. Genes Evol.* **217:** 801–806

Nikiforov Y & Gnepp DR (1994) Pediatric thyroid cancer after the Chernobyl disaster. Pathomorphologic study of 84 cases (1991-1992) from the Republic of Belarus. *Cancer* **74:** 748–766

Nikiforov Y, Gnepp DR & Fagin JA (1996) Thyroid lesions in children and adolescents after the Chernobyl disaster: implications for the study of radiation tumorigenesis. *J. Clin. Endocrinol. Metab.* **81:** 9–14

Nikiforov YE & Nikiforova MN (2011) Molecular genetics and diagnosis of thyroid cancer. *Nature Reviews Endocrinology* **7:** 569–580

Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW 2nd, Tallini G, Kroll TG & Nikiforov YE (2003) RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. *J. Clin. Endocrinol. Metab.* **88:** 2318–2326

Nikiforova MN & Nikiforov YE (2009) Molecular diagnostics and predictors in thyroid cancer. *Thyroid* **19:** 1351–1361

Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N & Gotoh Y (2002) Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J. Biol. Chem.* **277:** 21843–21850

Okamoto K, Taya Y & Nakagama H (2009) Mdmx enhances p53 ubiquitination by altering the substrate preference of the Mdm2 ubiquitin ligase. *FEBS Letters* **583:** 2710–2714

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW & Vogelstein B (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *, Published online: 29 April 1993; | doi:10.1038/362857a0* **362:** 857–860

Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC & Hainaut P (2002) The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum. Mutat.* **19:** 607–614

Oltval ZN, Milliman CL & Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. *Cell* **74:** 609–619

Osman I, Drobnjak M, Fazzari M, Ferrara J, Scher HI & Cordon-Cardo C (1999) Inactivation of the p53 Pathway in Prostate Cancer: Impact on Tumor Progression. *Clin Cancer Res* **5:** 2082–2088

Pachnis V, Mankoo B & Costantini F (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119:** 1005–1017

Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M & Bonner WM (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr. Biol.* **10:** 886–895

Pei L & Melmed S (1997) Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Mol. Endocrinol.* **11:** 433–441

Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P & Olivier M (2007) Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum. Mutat.* **28:** 622–629

Pierantoni GM, Rinaldo C, Mottolese M, Di Benedetto A, Esposito F, Soddu S & Fusco A (2007) High-mobility group A1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2. *Journal of Clinical Investigation* **117:** 693–702

Pomerantz J, Schreiber-Agus N, Liégeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C & DePinho RA (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92:** 713–723

Puri R, Tousson A, Chen L & Kakar SS (2001) Molecular cloning of pituitary tumor transforming gene 1 from ovarian tumors and its expression in tumors. *Cancer Lett.* **163:** 131–139

Puzianowska-Kuznicka M, Krystyniak A, Madej A, Cheng S-Y & Nauman J (2002) Functionally impaired TR mutants are present in thyroid papillary cancer. *J. Clin. Endocrinol. Metab.* **87:** 1120–1128

Raghavan D, Boxerman J, Jeyapalan S & Rogg J (2012) Radiation necrosis of a high-grade glioma. *Med Health R I* **95:** 159–160

Ramsden JD, Yarram S, Mathews E, Watkinson JC & Eggo MC (2002) Thyroid Follicular Cells Secrete Plasminogen Activators and Can Form Angiostatin from Plasminogen. *J Endocrinol* **173:** 475–481

Read ML, Lewy GD, Fong JCW, Sharma N, Seed RI, Smith VE, Gentilin E, Warfield A, Eggo MC, Knauf JA, Leadbeater WE, Watkinson JC, Franklyn JA, Boelaert K & McCabe CJ (2011) Proto-oncogene PBF/PTTG1IP regulates thyroid cell growth and represses radioiodide treatment. *Cancer Res.* **71:** 6153–6164

Reeves R (2001) Molecular biology of HMGA proteins: hubs of nuclear function. *Gene* **277:** 63–81

Ricarte-Filho JC, Ryder M, Chitale DA, Rivera M, Heguy A, Ladanyi M, Janakiraman M, Solit D, Knauf JA, Tuttle RM, Ghossein RA & Fagin JA (2009) Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. *Cancer Res.* **69:** 4885–4893

Riemenschneider MJ, Büschges R, Wolter M, Reifenberger J, Boström J, Kraus JA, Schlegel U & Reifenberger G (1999) Amplification and Overexpression of the MDM4 (MDMX) Gene from 1q32 in a Subset of Malignant Gliomas without TP53 Mutation or MDM2 Amplification. *Cancer Res* **59:** 6091–6096

Risseeuw EP, Daskalchuk TE, Banks TW, Liu E, Cotelesage J, Hellmann H, Estelle M, Somers DE & Crosby WL (2003) Protein interaction analysis of SCF ubiquitin E3 ligase subunits from Arabidopsis. *The Plant Journal* **34:** 753–767

Robles AI, Bemmels NA, Foraker AB & Harris CC (2001) APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis. *Cancer Res.* **61:** 6660–6664

Rogakou EP, Pilch DR, Orr AH, Ivanova VS & Bonner WM (1998) DNA Double-Stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139. *J. Biol. Chem.* **273:** 5858–5868

Romei C, Fugazzola L, Puxeddu E, Frasca F, Viola D, Muzza M, Moretti S, Luisa Nicolosi M, Giani C, Cirello V, Avenia N, Rossi S, Vitti P, Pinchera A & Elisei R (2012) Modifications in the Papillary Thyroid Cancer Gene Profile Over the Last

15 Years. *The Journal of clinical endocrinology and metabolism* Available at: http://www.ncbi.nlm.nih.gov/pubmed/22745248 [Accessed August 31, 2012]

Rotter V (1983) p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* **80:** 2613–2617

Sadetzki S, Chetrit A, Lubina A, Stovall M & Novikov I (2006) Risk of Thyroid Cancer After Childhood Exposure to Ionizing Radiation for Tinea Capitis. *JCEM* **91:** 4798–4804

Sáez C, Martínez-Brocca MA, Castilla C, Soto A, Navarro E, Tortolero M, Pintor-Toro JA & Japón MA (2006) Prognostic Significance of Human Pituitary Tumor-Transforming Gene Immunohistochemical Expression in Differentiated Thyroid Cancer. *JCEM* **91:** 1404–1409

Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E & Anderson CW (2002) ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *J. Biol. Chem.* **277:** 12491–12494

Santarpia L, El-Naggar AK, Cote GJ, Myers JN & Sherman SI (2008) Phosphatidylinositol 3-kinase/akt and ras/raf-mitogen-activated protein kinase pathway mutations in anaplastic thyroid cancer. *J. Clin. Endocrinol. Metab.* **93:** 278–284

Sarnow P, Ho YS, Williams J & Levine AJ (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* **28:** 387–394

Scharnhorst V, Menke AL, Attema J, Haneveld JK, Riteco N, Van Steenbrugge GJ, Van der Eb AJ & Jochemsen AG (2000) EGR-1 enhances tumor growth and modulates the effect of the Wilms' tumor 1 gene products on tumorigenicity. *Oncogene* **19:** 791–800

Schulman BA & Harper JW (2009) Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Biol* **10:** 319–331

Scoumanne A, Harms KL & Chen X (2005) Structural basis for gene activation by p53 family members. *Cancer Biol. Ther.* **4:** 1178–1185

Sgarra R, Rustighi A, Tessari MA, Di Bernardo J, Altamura S, Fusco A, Manfioletti G & Giancotti V (2004) Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. *FEBS Lett.* **574:** 1–8

Shan B & Lee WH (1994) Deregulated Expression of E2F-1 Induces S-Phase Entry and Leads to Apoptosis. *Mol. Cell. Biol.* **14:** 8166–8173

Sharma N, Boelaert K & Watkinson JC (2010) Who should treat thyroid cancer? A UK surgical perspective. *Clin Oncol (R Coll Radiol)* **22:** 413–418

Sharma N, Timmers C, Trikha P, Saavedra HI, Obery A & Leone G (2006) Control of the P53-p21CIP1 Axis by E2f1, E2f2, and E2f3 Is Essential for G1/S Progression and Cellular Transformation. *J. Biol. Chem.* **281:** 36124–36131

Sharp DA, Kratowicz SA, Sank MJ & George DL (1999) Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J. Biol. Chem.* **274:** 38189–38196

Shen DH, Kloos RT, Mazzaferri EL & Jhian SM (2001) Sodium iodide symporter in health and disease. *Thyroid* **11:** 415–425

Sherr CJ (2006) Divorcing ARF and p53: an unsettled case. *Nature Reviews Cancer* **6:** 663–673

Shibata Y, Haruki N, Kuwabara Y, Nishiwaki T, Kato J, Shinoda N, Sato A, Kimura M, Koyama H, Toyama T, Ishiguro H, Kudo J, Terashita Y, Konishi S & Fujii Y (2002) Expression of PTTG (pituitary tumor transforming gene) in esophageal cancer. *Jpn. J. Clin. Oncol.* **32:** 233–237

Shvarts A, Bazuine M, Dekker P, Ramos YF, Steegenga WT, Merckx G, Van Ham RC, Van der Houven van Oordt W, Van der Eb AJ & Jochemsen AG (1997) Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. *Genomics* **43:** 34–42

Shvarts A, Steegenga WT, Riteco N, Van Laar T, Dekker P, Bazuine M, Van Ham RC, Van der Houven van Oordt W, Hateboer G, Van der Eb AJ & Jochemsen AG (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J.* **15:** 5349–5357

Sionov RV & Haupt Y (1999) The cellular response to p53: the decision between life and death. *Oncogene* **18:** 6145–6157

Smith VE, Franklyn JA & McCabe CJ (2011) Expression and function of the novel proto-oncogene PBF in thyroid cancer: a new target for augmenting radioiodine uptake. *J. Endocrinol.* **210:** 157–163

Smith VE, Read ML, Turnell AS, Sharma N, Lewy GD, Fong JCW, Seed RI, Kwan P, Ryan G, Mehanna H, Chan SY, Darras VM, Boelaert K, Franklyn JA & McCabe CJ (2012a) PTTG-Binding Factor (PBF) Is a Novel Regulator of the Thyroid Hormone Transporter MCT8. *Endocrinology* Available at: http://www.ncbi.nlm.nih.gov/pubmed/22535767 [Accessed April 30, 2012]

Smith VE, Read ML, Turnell AS, Sharma N, Lewy GD, Fong JCW, Seed RI, Kwan P, Ryan G, Mehanna H, Chan SY, Darras VM, Boelaert K, Franklyn JA & McCabe CJ (2012b) PTTG-Binding Factor (PBF) Is a Novel Regulator of the Thyroid Hormone Transporter MCT8. *Endocrinology* **153:** 3526–3536

Smith VE, Read ML, Turnell AS, Watkins RJ, Watkinson JC, Lewy GD, Fong JCW, James SR, Eggo MC, Boelaert K, Franklyn JA & McCabe CJ (2009) A novel mechanism of sodium iodide symporter repression in differentiated thyroid cancer. *J Cell Sci* **122:** 3393–3402

Soengas MS, Alarcón RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW & Lowe SW (1999) Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* **284:** 156–159

Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordón-Cardó C & Lowe SW (2001) Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* **409:** 207–211

Speidel D, Helmbold H & Deppert W (2006) Dissection of transcriptional and non-transcriptional p53 activities in the response to genotoxic stress. *Oncogene* **25:** 940–953

Spitzweg C, Harrington KJ, Pinke LA, Vile RG & Morris JC (2001) Clinical review 132: The sodium iodide symporter and its potential role in cancer therapy. *J. Clin. Endocrinol. Metab.* **86:** 3327–3335

Stratford AL, Boelaert K, Tannahill LA, Kim DS, Warfield A, Eggo MC, Gittoes NJL, Young LS, Franklyn JA & McCabe CJ (2005) Pituitary Tumor Transforming Gene Binding Factor: A Novel Transforming Gene in Thyroid Tumorigenesis. *JCEM* **90:** 4341–4349

Suarez HG, Du Villard JA, Severino M, Caillou B, Schlumberger M, Tubiana M, Parmentier C & Monier R (1990) Presence of mutations in all three ras genes in human thyroid tumors. *Oncogene* **5:** 565–570

Sui G, Affar EB, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR & Shi Y (2004) Yin Yang 1 Is a Negative Regulator of p53. *Cell* **117:** 859–872

Takeichi N, Ezaki H & Dohi K (1991) A review of forty-five years study of Hiroshima and Nagasaki atomic bomb survivors. Thyroid cancer: reports up to date and a review. *J. Radiat. Res.* **32 Suppl:** 180–188

Tanimura S, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A & Ohtsubo M (1999) MDM2 interacts with MDMX through their RING finger domains. *FEBS Lett.* **447:** 5–9

Thompson DE, Mabuchi K, Ron E, Soda M, Tokunaga M, Ochikubo S, Sugimoto S, Ikeda T, Terasaki M & Izumi S (1994) Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958-1987. *Radiat. Res.* **137:** S17–67

Toledo F & Wahl GM (2007) MDM2 and MDM4: p53 regulators as targets in anticancer therapy. *Int J Biochem Cell Biol* **39:** 1476–1482

Truant R, Antunovic J, Greenblatt J, Prives C & Cromlish JA (1995) Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. *J Virol* **69:** 1851–1859

Varley JM, Evans DG & Birch JM (1997) Li-Fraumeni syndrome--a molecular and clinical review. *Br. J. Cancer* **76:** 1–14

Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N & Liu EA (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303:** 844–848

Venkitaraman AR (2001) Functions of BRCA1 and BRCA2 in the Biological Response to DNA Damage. *J Cell Sci* **114:** 3591–3598

Viadiu H (2008) Molecular architecture of tumor suppressor p53. *Curr Top Med Chem* **8:** 1327–1334

Viniegra JG, Martínez N, Modirassari P, Hernández Losa J, Parada Cobo C, Sánchez-Arévalo Lobo VJ, Aceves Luquero CI, Alvarez-Vallina L, Ramón y Cajal S, Rojas JM & Sánchez-Prieto R (2005) Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. *J. Biol. Chem.* **280:** 4029–4036

Virolle T, Krones-Herzig A, Baron V, De Gregorio G, Adamson ED & Mercola D (2003) Egr1 promotes growth and survival of prostate cancer cells. Identification of novel Egr1 target genes. *J. Biol. Chem.* **278:** 11802–11810

Vogelstein B, Lane D & Levine AJ (2000) Surfing the p53 network. *Nature* **408:** 307–310

Vousden KH (2000) p53: Death Star. *Cell* **103:** 691–694

Vousden KH & Lane DP (2007) p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* **8:** 275–283

Vousden KH & Lu X (2002) Live or let die: the cell's response to p53. *Nat. Rev. Cancer* **2:** 594–604

Vousden KH & Prives C (2009) Blinded by the Light: The Growing Complexity of p53. *Cell* **137:** 413–431

Wakeford R (2011a) The silver anniversary of the Chernobyl accident. Where are we now? *J Radiol Prot* **31:** 1–7

Wakeford R (2011b) And now, Fukushima. *J Radiol Prot* **31:** 167–176

Waldman T, Kinzler KW & Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* **55:** 5187–5190

Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D & Marais R (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116:** 855–867

Wang J, Belcher JD, Marker PH, Wilcken DE, Vercellotti GM & Wang XL (2001) Cytomegalovirus inhibits p53 nuclear localization signal function. *J. Mol. Med.* **78:** 642–647

Wang T & Brown MJ (1999) mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal. Biochem.* **269:** 198–201

Watkins RJ, Read ML, Smith VE, Sharma N, Reynolds GM, Buckley L, Doig C, Campbell MJ, Lewy G, Eggo MC, Loubiere LS, Franklyn JA, Boelaert K & McCabe CJ (2010a) PTTG Binding Factor – a New Gene in Breast Cancer. *Cancer Res* **70:** 3739–3749

Watkins RJ, Read ML, Smith VE, Sharma N, Reynolds GM, Buckley L, Doig C, Campbell MJ, Lewy G, Eggo MC, Loubiere LS, Franklyn JA, Boelaert K & McCabe CJ (2010b) Pituitary tumor transforming gene binding factor: a new gene in breast cancer. *Cancer Res.* **70:** 3739–3749

Weibrecht I, Leuchowius K-J, Clausson C-M, Conze T, Jarvius M, Howell WM, Kamali-Moghaddam M & Söderberg O (2010) Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert Rev Proteomics* **7:** 401–409

Wiederschain D, Kawai H, Gu J, Shilatifard A & Yuan Z-M (2003) Molecular basis of p53 functional inactivation by the leukemic protein MLL-ELL. *Mol. Cell. Biol.* **23:** 4230–4246

Wolter KG, Hsu Y-T, Smith CL, Nechushtan A, Xi X-G & Youle RJ (1997) Movement of Bax from the Cytosol to Mitochondria During Apoptosis. *J Cell Biol* **139:** 1281–1292

Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, Collins N, Nguyen K, Seal S, Tran T, Averill D & Et A (1994) Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science* **265:** 2088–2090

Wu H, Pomeroy SL, Ferreira M, Teider N, Mariani J, Nakayama KI, Hatakeyama S, Tron VA, Saltibus LF, Spyracopoulos L & Leng RP (2011) UBE4B promotes Hdm2-mediated degradation of the tumor suppressor p53. *Nature Medicine* **17:** 347–355

Wu L & Levine AJ (1997) Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Mol Med* **3:** 441–451

Wyllie FS, Haughton MF, Rowson JM & Wynford-Thomas D (1999) Human thyroid cancer cells as a source of iso-genic, iso-phenotypic cell lines with or without functional p53. *Br J Cancer* **79:** 1111–1120

Xing M (2007) BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. *Endocr. Rev.* **28:** 742–762

Yaspo ML, Aaltonen J, Horelli-Kuitunen N, Peltonen L & Lehrach H (1998) Cloning of a novel human putative type Ia integral membrane protein mapping to 21q22.3. *Genomics* **49:** 133–136

Yasumura S (2011) Newsmaker interview: Seiji Yasumura. Fukushima begins 30-year odyssey in radiation health. Interview by Dennis Normile. *Science* **333:** 684–685

Yew PR & Berk AJ (1992) Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *, Published online: 07 May 1992;* | *doi:10.1038/357082a0* **357:** 82–85

Yew PR, Liu X & Berk AJ (1994) Adenovirus E1B Oncoprotein Tethers a Transcriptional Repression Domain to P53. *Genes Dev.* **8:** 190–202

Yuan J, Luo K, Zhang L, Cheville JC & Lou Z (2010a) USP10 regulates p53 localization and stability by deubiquitinating p53. *Cell* **140:** 384–396

Yuan J, Luo K, Zhang L, Cheville JC & Lou Z (2010b) USP10 Regulates p53 Localization and Stability by Deubiquitinating p53. *Cell* **140:** 384–396

Zaballos MA & Santisteban P (2012) FOXO1 Controls Thyroid Cell Proliferation in Response to TSH and IGF-I and Is Involved in Thyroid Tumorigenesis. *Molecular Endocrinology* Available at: http://mend.endojournals.org/content/early/2012/11/15/me.2012-1032 [Accessed December 18, 2012]

Zambetti GP (2007) The p53 mutation 'gradient effect' and its clinical implications. *J. Cell. Physiol.* **213:** 370–373

Zhang Q, Stovall DB, Inoue K & Sui G (2011) The oncogenic role of Yin Yang 1. *Crit Rev Oncog* **16:** 163–197

Zhao LY & Liao D (2003) Sequestration of p53 in the cytoplasm by adenovirus type 12 E1B 55-kilodalton oncoprotein is required for inhibition of p53-mediated apoptosis. *J. Virol.* **77:** 13171–13181

Zheng W-H, Kar S & Quirion R (2002) Insulin-like growth factor-1-induced phosphorylation of transcription factor FKHRL1 is mediated by phosphatidylinositol 3-kinase/Akt kinase and role of this pathway in insulin-like growth factor-1-induced survival of cultured hippocampal neurons. *Mol. Pharmacol.* **62:** 225–233

Zhou BP, Liao Y, Xia W, Zou Y, Spohn B & Hung MC (2001) HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat. Cell Biol.* **3:** 973–982

Zhu J, Zhou W, Jiang J & Chen X (1998) Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J. Biol. Chem.* **273:** 13030–13036

Zou H, McGarry TJ, Bernal T & Kirschner MW (1999) Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* **285:** 418–422

Chapter 10. **Bibliography**

10.1. **Publications relevant to thesis**

1. Robert I Seed and Martin L Read et al. The PTTG1-Binding Factor (PBF/PTTG1IP) acts as a novel regulator of p53 stability and function in human cancers. (Cancer Research under review).

2. Martin L. Read, Greg D. Lewy, Jim C.W. Fong, Neil Sharma, Robert I. Seed et al. Proto-oncogene PBF/PTTG1IP Regulates Thyroid Cell Growth and Represses Radioiodide Treatment. Cancer Res; 71(19); 6153–64.

3. Lewy GD, Sharma N, Seed RI, Smith VE, Boelaert K, McCabe CJ. PTTG in Thyroid Cancer. J Endocrinol Invest. [Epub ahead of print].

4. Smith VE, Read ML, Turnell AS, Sharma N, Lewy GD, Fong JC, Seed RI, Kwan P, Ryan G, Mehanna H, Chan SY, Darras VM, Boelaert K, Franklyn JA, McCabe CJ. PTTG-Binding Factor (PBF) Is a Novel Regulator of the Thyroid Hormone Transporter MCT8. Endocrinology. 2012 Apr 25. [Epub ahead of print].

10.2. **Other publications**

Natalie A Forrester, Rakesh N Patel, Thomas Speiseder, Peter Groitl, Garry G Sedgwick, Neil J Shimwell, Robert I Seed, Pól Ó Catnaigh, Christopher J McCabe, Grant S Stewart, Thomas Dobner, Roger J A Grand, Ashley Martin and Andrew S Turnell. Adenovirus E4orf3 targets Transcriptional Intermediary Factor 1γ 4 for proteasome-dependent degradation during infection. J. Virol. doi:10.1128/JVI.06583-11.