Nucleophosmin and p14ARF mediated regulation of p53

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Dedicated to the memory of my late parents and my late grand-mother

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Abstract

Tumour initiation and progression occur due to oncogenic mutations that also contribute to therapeutic resistance in many human tumours. Mutations activating the "PI3K/AKT" signalling pathway and inactivating the "*TP53"* tumour suppressor gene are common mechanisms that cancer cells require to proliferate and escape pre-programmed cell death. p53 mutant (p53mut) tumours not only fail to respond to DNA damaging therapy, but are also described to promote therapeutic resistance by dominant negative suppression of p53 dependent promoter activity. Our work identifies the crucial interaction between the PI3K/AKT pathway and p53 mutations that regulate treatment sensitivity in tumours.

Using a combination of *in vitro* and *in vivo* techniques we demonstrate that AKT inhibition promotes reduced cellular levels of p53mut via a novel Nucleophosmin 1 (NPM) mediated regulation of the tumour suppressor p14ARF and promotes re-engagement of cell cycle arrest, senescence and increased sensitivity to ionising radiation in both *in vivo* and *in vitro* systems. We show that the PI3K/AKT pathway plays an important role in the regulation of p53mut and inhibitors of this pathway can re-sensitise treatment resistant tumours. This has helped us to simultaneously highlight the cohort of patients where the greatest efficacy may be achieved in clinical practise.

We further show that the AKT mediated regulation of NPM that we describe in solid tumours is relevant in Acute Myeloid Leukaemia (AML) with mutated NPM, albeit showing physiologically different effects. This further highlights the necessity for rational treatment planning with the newer targeted agents that inhibit specific signalling pathways in AML patients.

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Lastly to all my patients, thank you for understanding. Everything I do is for you.

Abbreviations

4EBP1 - eukaryotic translation initiation factor 4E Binding Partner 1

γ-H2AX – Phosphorylated Histone 2A variant X

ADP- Adenosine Diphosphate

AF – AlexaFluor

ALK – Anaplastic Lymphoma Kinase

APAF-1 – Apoptotic protease-activating factor-1

APS – Ammonium PerSulphate

ATCC – American Type Culture Collection

ATM – Ataxia Telengeictasia Mutated

ARF – Alternate Reading Frame

ARFBP1 - Alternate Reading Frame Binding Partner 1

ATR- ATM and Rad3 Related

BSA – Bovine Serum Albumin

CDC – Cell Division Cycle

CDK – Cyclin Dependent Kinase

CHK – Checkpoint Kinase

Co-IP – Co Immunoprecipitation

Crm1- Chromosome Region Maintenance 1 protein homolog

DAPI - 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle's Medium

DNA – DeoxyriboNucleic Acid

DNA-PK - DNA Dependent Protein Kinase

DRAM- Damage Regulated Autophagy Modulator

DTT - Dithiothreitol

EDTA - ethylenediaminetetraacetic acid

EGFR – Epidermal Growth Factor Receptor

eIF4A - eukaryotic translation initiation factor 4E

FBS – Fetal Bovine Serum

FAM- 6-Carboxy Fluorescein

FLT3- Fms Like Tyrosine Kinase 3

FLT3-ITD- FLT3 Internal Tandem Duplication

G_1 – Gap 1 phase

G_2 – Gap 2 phase

GAP – GTPase Activating Protein

GDP - Guanosine diphosphate

GEF – Guanine Exchange Factor

GFP – Green Fluorescent Protein

GRB2- Growth Factor Receptor Bound protein 2

GSK3 – Glycogen Synthase Kinase

GTP – Guanosine triphosphate

H2AX – Histone 2A variant X

HCC – Hepatocellular Carcinoma

HDM2 – Human MDM2

IF – ImmunoFluorescent

IGF – Insulin-like Growth Factor

Ig – Immunoglobulin

IHC - Immunohitsochemistry

IP – Immunoprecipitation

IR – Ionising Radiation

KeV – Kiloelectron Volt

LB – Luria Bertani

MAPK- Mitogen Activated Protein Kinase

MEFs – Mouse Embryo Fibroblasts

MOPS - 3-(N-morpholino) propanesulfonic acid

MRN - Mre11, Rad50 and Nbs1 complex

mRNA – messenger RiboNucleic Acid

MTA – Materials Transfer Agreement

MCS – Multiple Cloning Site

MDM2 – Murine Double Minute

mTOR – mammalian Target of Rapamycin

MULE – Mcl Ubiquitin Ligase E3

NF1 - NeuroFibromatosis type 1

NPM – NucleoPhosMin

NPM-ALK – Nucleophosmin-Anaplastic Lymphoma Kinase

NSCLC – Non Small Cell Lung Cancer

PTEN- Phosphatase and Tensin Homolog

PARP- Poly ADP-Ribose Polymerase

PBS – Phosphate buffered Saline

PCR – Polymerase Chain Reaction

PFA – Para-FormAldehyde

PI – Propidium Iodide

PIP2 - Phosphatidylinositol (4, 5)-bisphosphate

PIP3 - Phosphatidylinositol (3, 4, 5)-triphosphate

PI3K – PhosphatidyInositol-3-Kinase

PKA – Protein Kinase A

PKB – Protein Kinase B

PKC – Protein Kinase C

PRAS40 - Proline-Rich AKT Substrate of 40KDa

P/S – Penicillin / Streptomycin

PTEN – Phosphatase and Tensin homologue deleted on chromosome 10

PVDF – PolyVinyl DiFluoride

pRT-PCR- Quantitative Real Time Polymerase Chain Reaction

Raptor – Rapamycin sensitive adaptor protein of mTOR

Rb- Retinoblastoma gene

RBD – RAS Binding Domain

Rictor – Rapamycin insensitive companion of mTOR

RNA – RiboNucleic Acid

ROS – Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

SCC – Squamous Cell Carcinoma

SD – Standard Deviation

SDS-PAGE – Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

ssDNA – single stranded DeoxyriboNucleic Acid

TAE – Tris Acetate EDTA

TBS-T – Tris Buffered Saline – Tween 20

TEMED - *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine

TNF- Tumour Necrosis Factor

UV – Ultra Violet

Contents

Chapter 1

Introduction

Chapter 1 – Introduction

1.1– Cancer

Cancers are genetic diseases where specific mutations result in cells losing their proliferation regulation capacities. They may be broadly classified into solid tumours and haematological malignancies. Cancers are treated with various therapeutic modalities which include surgery, radiation, chemotherapy and combination therapies. The response to treatment and cure from disease varies between different cancers as well as patients, and is dependent on various factors.

Common characteristics and molecular pathways that have been referred to as the hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) have been studied in great detail over the last decade and this has played a major role in the understanding of how tumours grow and respond to treatment. Early studies identified that development and progression of cancer was dependent on sustained proliferation signals, mechanisms to evade the growth suppressors as well as cell death and the extra cellular components of increased invasion and nutrient supply by way of angiogenesis (Hanahan and Weinberg, 2000). While they are certainly the core requirements for cancers unlike benign tumours, it has since been understood that they require other characteristics for the evolution from a normal and functional tissue or cell to a malignant tumour (Hanahan and Weinberg, 2011). The emerging view is that of the acquisition of the core tumour promoting hallmarks listed above secondary to enabling genomic alterations which lead to heritable changes that can be passed on from one cell to its progeny, eventually giving rise to a clone of cancer cells. A number of

mutations and genomic alterations have been identified that can play a role in regulating this caretaker mechanism (Hanahan and Weinberg, 2011; Negrini et al., 2010). In this context, the Tp53 is a central regulator where it influences cell cycle and cell death mechanisms and will be described in detail in the proceeding chapters. Along with Tp53 a number of caretaker genes that play a role in maintenance of the genome integrity have been identified that are deregulated prior to transformative changes in the cells (Jackson and Bartek, 2009; Negrini et al., 2010). Another interesting new addition to the wealth of knowledge about the development of cancer is that of the requirement for tumour-promoting inflammation in the tumour micro-environment. Various studies have identified characteristics of inflammation associated with malignancies including distinct immune cells, all of which may play a role in anti-tumour effect. But quite interestingly, a paradoxical pro-tumour enabling effect of local inflammation has garnered a lot of attention and is implicated in the contribution to the core hallmarks of cancer including survival and pro-angiogenic effects, local invasion and metastasis (DeNardo et al., 2010; Grivennikov et al., 2010; Hanahan and Weinberg, 2011). While these “Enabling Characteristics” (Hanahan and Weinberg, 2011) are understood to play a vital role in cancer development, two further core hallmarks are the deregulated metabolism characterised by cancer cells and the ability of the cancer cells to evade the immune system. In the former, the uncontrolled cell proliferation of cancers is associated with adjustments in the metabolic pathways in the cancer cells. Due to the variable oxygen availability to the cancer cells, they reprogram their energy production to an aerobic glycolysis state by upregulating glucose transporters (Jones and Thompson, 2009). Glycolytic changes have also been associated with oncogenes like MYC and RAS

as well as mutant Tp53 (DeBerardinis et al., 2008; Vousden and Lane, 2007). Interestingly, this reliance on the glycolytic pathway is accentuated by hypoxia (in a HIF1 and HIF2 mediated manner) which is characteristic of tumours (Semenza, 2010), while different clones of cells in a tumour have been reported to possess different, but symbiotic metabolic pathways that maintain the malignant tumour mass (Feron, 2009). Another emerging hallmark that has been identified as being essential for the development of tumours is the micro-environment in which the tumour cells proliferate. A number of cell types and extra cellular components have been identified that interact with the cancer cells and the cancer stem cells to finally produce the tumour 'stroma'. A quick overview of the stromal constituents would include a prominent contribution from the tumour vasculature associated endothelial cells (Chung et al., 2010) as well as the closely associated lymphatic endothelial cells. In close association with the tumour endothelial cells are the supporting pericytes, all of which together constitute the tumour vasculature. Other well studied stromal constituents include cancer associated fibroblasts (Cirri and Chiarugi, 2011) as well as the bone marrow derived stromal cells that have been characterised in the stroma of solid tumours. As mentioned above in the case of local inflammation associated with cancers, the immune inflammatory cells are a significant component of the tumours stroma and include macrophages, mast cells, neutrophils and lymphocytes, all of which promote growth by producing growth factors (Grivennikov et al., 2010).

The use of ionising radiation as well as DNA damaging drugs are some of the common and most effective modes of cancer treatment. Unfortunately, resistance to therapy is a clinical problem caused by both intrinsic (signalling) and extrinsic factors (micro-environmental factors). The normal cellular response to DNA

damaging treatments like radiation is the activation of cell cycle checkpoints, induction of DNA repair and, if extreme, apoptosis via the p53 family of transcription factors. For these reasons p53 is viewed as the ‘guardian of genome’ (Lane, 1992) that is not only important for the response to therapeutic intervention but is a fundamental suppressor of tumour initiation. A loss of function or inactivation of p53 is a prerequisite for development of many cancers (Freed-Pastor and Prives, 2012; Levine and Oren, 2009). The loss of p53 activity is also associated with resistance to DNA damaging therapies (Brown and Wouters, 1999) commonly used in cancer treatment and is increasingly seen to have detrimental effects on patient outcome (Olivier et al., 2006; Robles and Harris, 2010) via suppression of alternative apoptotic and cell death pathways. The phosphatidylinositol 3 kinase (PI3K) /AKT signalling cascade is a mediator of essential activities required by tumour cells, such as growth and survival (Davies, 2011; Osaki et al., 2004). Mutations, amplification and deletions of the upstream regulators of AKT are among the most frequent somatic events in cancer. As is the case with p53 mutations, deregulation of AKT is a major event enhancing both oncogenesis and resistance to treatment in many human malignancies (Osaki et al., 2004).

1.2– Regulatory mechanisms in mammalian cells

1.2.1– DNA repair and the cell cycle

The repair of DNA damage that occurs as a result of genotoxic stress is essential for maintaining genomic integrity and is exploited in cancer therapies including radiation. These repair mechanisms are quite often deregulated in cancer cells and forms the basis for therapeutic intervention, where normal cells can overcome the treatment associated DNA damage while the cancer cells with multiple defects in their genome have impaired damage response, which subsequently forces them into cell cycle arrest or cell death. The choice of the repair pathway selected by the cellular machinery depends on the stage of the cell cycle (Table 1.2.1.1) which in turn is selected and controlled through cell-cycle regulators like cyclin-dependent kinases (CDKs). Following DNA damage, a complex repair process is initiated called the DNA Damage Response (DDR) which is mediated through a network of cellular pathways that senses, signals and repairs DNA lesions and which is regulated by the various check-point proteins. Cell cycle checkpoints, regulated by the checkpoint proteins, act as surveillance and signalling pathways that co-ordinate the DNA damage repair and cell-cycle progression. Severe damage as in the case of therapeutic radiation that cannot be repaired will result in blocking the cell at one of the checkpoints and initiation of the death pathways. The most important components of the DDR machinery include the kinases Ataxia Telangiectasia Mutated (ATM), ATM-Rad3-related (ATR) and DNA Dependent Protein Kinase (DNA-PK). DNA double strand breaks (DSBs) as in the case of radiation, usually initiate the ATM and DNA-PK response while single stranded DNA and stalled replication forks initiate the ATR response (Bartek and Lukas,

2007). Activation of these kinases results in their recruitment to the DNA damage sites along with other repair proteins.

	G1	S	G2-M
DSBs or single-strand breaks	Non Homologous End Joining	Homologous Recombination-fork restart	Homologous Recombination mediated repair
Mismatches	Mismatch repair		
Bulky lesion	Base/Nucleotide Excision Repair	Damage bypass	

Table 1.2.1.1: DNA repair pathways functioning in different cell cycle stages. Adapted from (Branzei and Foiani, 2008)

Cells in the G1 phase of cell cycle have multiple mechanisms to repair DNA damage sustained as a result of exposure to ROS, Ionizing Radiation (IR) or UV and this is initiated and completed before the onset of replication (Table 1.2.1.1). Nucleotide Excision Repair (NER/BER) repairs pyrimidine dimers that are formed on exposure to UV (Sancar et al., 2004) and can also support other repair mechanisms including the Homologous Recombination (HR) repair pathway (Nojima et al., 2005). IR causes DSBs which are repaired by the HR and Non Homologous End Joining (NHEJ) (Sonoda et al., 2006) pathways, with the former using information from the identical sister chromatid to repair the defect, while the latter uses the Ku70/Ku80 complex to bind to the broken ends in order to recruit DNA-PKs and ligases to ligate the ends together. The NHEJ pathway is the predominant DSB repair pathway in the G1 phase of cell cycle. The cell-cycle phase is an important determinant of the DSB repair mechanism that is chosen to repair the defect (whether NHEJ or HR) as has been shown in defective repair mutant studies (Branzei and Foiani, 2008).

The S phase of cell cycle where critical DNA synthesis takes place is prone to nucleotide mis-incorporation, accumulation of nicks and gaps, fork collapse at DNA breaks and aberrant transition at collapsed forks (Branzei and Foiani, 2005). The course of synthesis of new DNA is associated with topological modifications that can disrupt progression of the S phase. These are generally resolved by the topoisomerases that help in completion of the cell cycle, condensation of the chromosomes and progression into the G2 and M phase. Base-base mismatches are repaired by the mismatch repair pathway in the S phase as well as the BER which was originally encountered in the G1 phase (Jiricny, 2006; Sancar et al., 2004). DSBs can also occur in the S phase as a result of replication fork collapse. These DSBs are repaired by HR utilising the RAD52 group of genes (Sung and Klein, 2006) and facilitated by the CDK activity. This helps prevent NHEJ, although reports suggest the possibility of a competition between the Ku mediated NHEJ and the HR pathways (Kim et al., 2005). Recent studies also show the pro-HR role that PARP and CtIP proteins play in S phase (Hochegger et al., 2006; Sartori et al., 2007).

1.2.2– Cell death mechanisms and senescence

Mammalian cells undergo various forms of cell death to maintain tissue integrity and homeostasis. Normal cells have complex programmes in place that limit their numbers to the adequate range. This is commonly deregulated in cancers where the absence or failure of the intrinsic cell death mechanisms leads to aberrant proliferation of cells. The best recognised forms of cell death include Apoptosis, Necrosis and Autophagy (Fig.1.2.2.1) (Galluzzi et al., 2007; Kroemer et al., 2009). Apoptosis is associated with cleavage of cytoskeletal proteins by proteases and subsequent collapse of the subcellular components as well as chromatin

condensation, nuclear fragmentation and formation of plasma membrane blebs (Adams and Cory, 2007; Hotchkiss et al., 2009). Necrosis on the other hand is associated with early loss of integrity of plasma membrane, an influx of extracellular ions and fluids and a subsequent swelling of the organelles and the cell itself (Golstein and Kroemer, 2007). Autophagy is associated with the cells digesting its organelles to generate energy and metabolites until sustained nutrient deprivation results in the digestion of all available substrates and the cell dies (Hotchkiss et al., 2009; Klionsky, 2007; Kroemer and Jaattela, 2005). The cell death mechanisms are highly regulated and are under the control of a number of genetic processes functioning under the influence of cellular stress response elements.

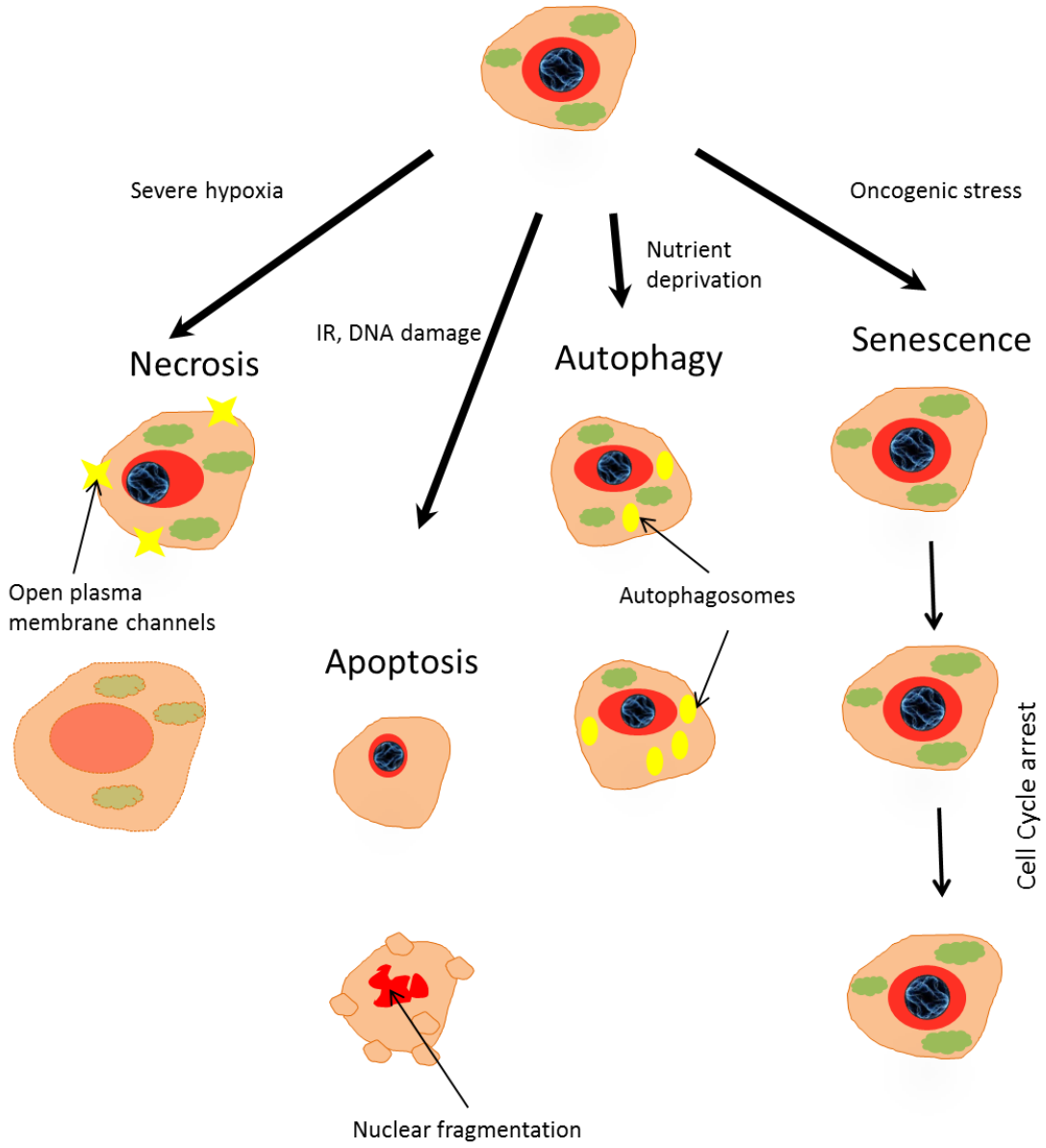


Fig. 1.2.2.1- Cell death mechanisms and senescence in cells

Apoptosis: The apoptotic pathway is activated in response to severe stress that cannot be repaired by the cellular machinery. While a number of intricate interactions play a role in initiating the pathway, a brief description would involve the extrinsic and intrinsic pathways as well as the important role played by p53 in mediating the apoptotic response. The p53 component will be discussed in proceeding sections. The extrinsic death receptor mediated apoptotic pathway is activated by death ligands like FAS, TNF and TRAIL (Green, 2005) which

activates the death receptors that recruit the FAS-associated death domain (FADD) which in turn recruits Caspase 8 that activates the executioner Caspase 3 leading to apoptosis. The intrinsic pathway on the other hand centres on the mitochondria. Following exposure to stress including oncogenic stress, the proapototic BH3 class of proteins are activated and inhibit the BCL2 and BCL-XL antiapoptotic proteins which under normal conditions inhibit BAX and BAK (Chipuk and Green, 2008). Active BAX and BAK induce permeabilization of the mitochondria, release of cytochrome c and an Apoptotic Protease Activating Factor (APAF1) mediated activation of Caspase 9 which leads to cleavage of caspase 3 and activation of apoptosis. The apoptotic machinery is quite often deregulated in disease conditions with cancers being the most common, where more than 50% of neoplasms have a defect in apoptosis.

Necrosis: Necrosis or oncosis is mediated by reactive oxygen species (ROS), calcium ions, poly-ADP-Ribose polymerase (PARP) calpains and cathepsins (Hotchkiss et al., 2009). Unlike in apoptosis where PARP is cleaved and inactivated, necrosis is associated with retained PARP activity that results in ATP shortage and a shift of the cells towards necrosis rather than apoptosis. The increased permeability of the plasma membrane as mentioned earlier, results in activation of proteases that degrade critical proteins and lead to cell death (Golstein and Kroemer, 2007). Although initially described to be a non-programmed cell death, necrosis has since been associated with regulatory mechanisms, which include TNF mediated control as well as the intracellular protease inhibitor Serpin mediated inhibition of necrosis (Laster et al., 1988; Luke et al., 2007), pointing to the possibility of as yet unknown mechanisms of regulation.

Autophagy: Unlike necrosis, autophagy is considered a form of programmed cell death that can control cell proliferation. Autophagy is associated with cells recycling their non-essential or damaged organelles and macromolecular components in response to sub-lethal stress like nutrient deprivation, in the process having a role in tumour suppression, deletion of toxic mis-folded proteins and antigen presentation (Klionsky, 2007; Kroemer and Jaattela, 2005). In spite of it tumour suppressive properties, autophagy has been controversial, as it has been described to mediate cellular resistance to therapy especially in the context of cancer. Many oncogenes including PI3K/AKT are known to suppress autophagy whereas tumour suppressors like PTEN and TSC2 promote autophagy (Maiuri et al., 2009). Autophagy can promote resistance to cell-death especially in response to DNA-damaging agents by initiating the same adaptive response. Not surprisingly, the focus of many therapeutic approaches has been to use autophagy inhibitors in combination with other agents to target cancers.

Senescence: Cellular senescence is an essentially irreversible arrest of cell proliferation that is initiated following cell stress. Senescence is mediated by two main pathways- the p53/p21 and the $p16^{INK4a}$/pRb tumour suppressor pathways (Schmitt et al., 2002a). Along with cell growth and proliferation arrest, the cells undergoing senescence show chromatin organisation and gene expression including expression of cytokines, chemokines, proteases and growth factors, which are together called the Senescence Associated Secretory Phenotype (SASP) (Campisi, 2013; Campisi and d'Adda di Fagagna, 2007). A number of factors have been shown to induce the senescence response in cells. These include telomere shortening which is characteristically seen in ageing cells, where repeated cell divisions result in shorter telomeres that evoke the DNA Damage

Response (DDR) and subsequent p53 mediated cell cycle arrest or cell death (di Fagagna et al., 2003; Fumagalli et al., 2012). Other factors that can induce senescence are DNA damage (including double strand breaks) causing genomic instability and epigenomic damage. Two vital initiators of senescence of specific interest in the context of this manuscript are the Mitogen signalling activated senescence and the tumour suppressor mediated activation of senescence. Oncogene induced mitogenic signalling results in the activation of the p53/p21 and p16/pRb tumour suppressor pathways that initiates senescence. This has characteristically been seen in the case of cells with the oncogenically active H-RAS (H-RAS^{V12}) that results in activation of the mitogen-activated protein kinase (MAPK) signalling pathway (Serrano et al., 1997), over-expression of growth factor receptors like ERBB2 (Trost et al., 2005) or loss of tumour suppressors like PTEN that regulate the signalling cascade (Alimonti et al., 2010). These mitogenic signalling cascades lead to activation of the DDR and subsequent activation of the p53/p21 or the p16/pRb pathways (Bartkova et al., 2006). Other DNA damage response elements like ATR activation and the p38 MAPK signalling can induce a similar DDR response and senescence.

The p53/p21 and the p16/pRb pathways are the principle effectors of the senescence response. The vital role of p53 and p21 mediated cell cycle regulation is discussed in the following section. As with p53, the Retinoblastoma (Rb) tumour suppressor plays a major role in cell cycle regulation and is in turn regulated by the cyclin dependent kinase inhibitor p16^{INK4a} and results in cell cycle arrest and senescence depending on the magnitude of the cellular damage/stress (Chau and Wang, 2003).

1.3– p53 tumour suppressor and cancer

The transcription factor TP53 is an important mediator of cell death and replicative senescence responses to oncogenic stress. It belongs to a family of transcription factors that include p63 and p73, which together form a network controlling cell proliferation, differentiation, and death (Collavin et al., 2010). A number of biochemical activities have been attributed to p53. However the most important of these is its role as a transcription factor. The ability of p53 to inhibit cell proliferation by either promoting cell cycle arrest or apoptosis has been attributed in a large part to this transcriptional activity. The activation of p53 can occur in response to oncogenic insults, genotoxic damage, hypoxia and other cell stress (fig 1.3.2). p53 via its DNA binding domain binds to specific DNA sequences and induces transcription of genes that promote cell cycle arrest, or if the signals are strong enough, activate the apoptotic machinery. The other p53 family members' p63 and p73 encode proteins that have many functions similar to that of p53 (Kaghad et al., 1997; Yang et al., 1998). Both p63 and p73 are differentially spliced to give rise to mRNAs that are translated to different proteins in normal cells. These include N-terminal variants that encode either a full length (Transactivation proficient) or an N-terminal truncated ΔN variant of the α, β and γ variants of both p73 and p63. Along with p53, they show homology at both genomic and protein levels, with all three having a Trans Activation Domain, a DNA binding domain and an Oligomerisation domain. Unlike p53, both p63 and p73 possess long C termini, which although not essential for dimerization, can interact with other proteins. Most importantly, both proteins have significant homology in the DNA binding domain with that of p53, suggesting that the three proteins can bind to the same DNA sequence and trans-activate the same

promoters (Harms et al., 2004; Levrero et al., 2000). In spite of the said sequence homologies which can promote transcription of the established p53 target genes, there is indeed differences in the functions of the p63 and p73 isoforms. Promoters that respond to p53 like those controlling the cell cycle and checkpoints like p21, GADD45, PCNA, Cyclin G as well as those that promote apoptosis like BAX, BCL-XL and IGFBP3 can be activated by p63 and p73. Yet the response of the different gene promoters to the different p53 family members differs (Allocati et al., 2012; Harms et al., 2004). p73, which has been well characterised in the last 15 years, can promote p21 mediated cell-cycle arrest in response to stress (Das et al., 2003; Jost et al., 1997). Interestingly, it can also have pro-apoptotic activity which is in many ways similar to that of p53. In response to certain DNA damage inducers, like Cisplatin, p73 can induce apoptosis in an MLH1 and ATM-cAbl dependent manner. But this is dependent on specific cellular stimuli and is a possible explanation for the differential activity of the p53 family members.

Regulation of p53 family: Regulation of the p53 family involves both activation and inhibition of the proteins. The activity of these proteins is mainly regulated by post translational modifications. Many proteins regulators play a major role in regulating this stabilisation. The Ataxia Telangiectasia Mutated (ATM) DNA dependent protein kinase (DNA-PK), ATR, Chk1 and Chk2 phosphorylate and stabilise p53 in response to IR and UV. Similarly, acetylation of p53 and p73 by p300/CBP can activate them. On the other hand interaction with proteins like Murine Double Minute 2 (MDM2), High Mobility Group 1 (HMG-1) and Insulin-like Growth Factor type 1 Receptor (IGF-1R) can negatively regulate the protein levels of the p53 family. Interestingly, information about the transcription of the p53 family, especially p53 is scarce and is restricted to a few reports like the

transcriptional regulation of p53 by proteins like C/EPBβ, a member of the CAAT/ enhancer-binding protein (C/EPB) family of transcription factors(Boggs and Reisman, 2007).

The p53 Protein: The p53 protein is a 393 amino acid protein with a complex domain structure, whose activity is regulated by its protein structure (Joerger and Fersht, 2007) as well as by post translational modifications (Bode and Dong, 2004). The N-terminal region of the p53 protein from residues 1-42 contains the transactivation domain which plays a major role in interaction with a number of regulatory proteins including MDM2 (Marine et al., 2006), components of the transcription initiation complexes and the acetyltransferases p300 and CBP (Grossman, 2001). The transactivation domain is followed by a proline rich region (amino acid 63-92) that contains the SH3-domain binding motif that plays a regulatory role (Walker and Levine, 1996). The central or core domain comprising of residues 101-292 binds double stranded DNA and contains p53 promoter recognition motifs that recognise the promoters of downstream targets. Four core domains bind the DNA response elements in a co-operative manner to give a 4:1 complex (Weinberg et al., 2005). The C-terminal region is comprised of the residues 307-356 which forms the tetramerisation domain and the extreme C-terminus contains the self-regulatory acetylation (Friedler et al., 2005; Gu et al., 1997)as well as the non-specific DNA binding sites.

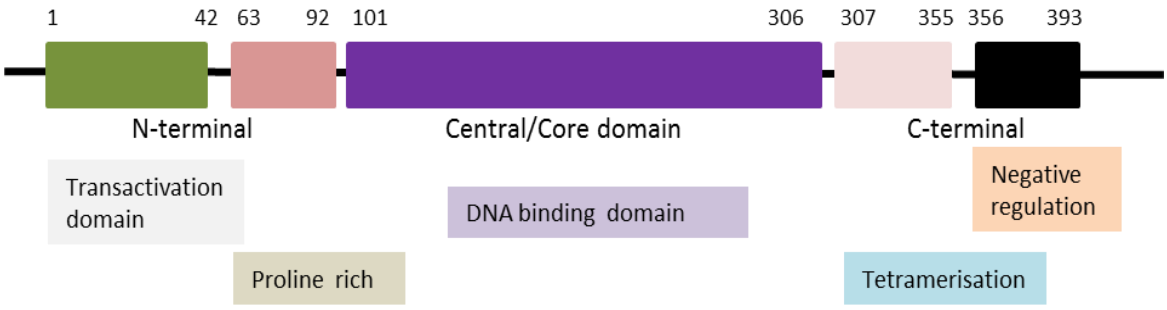


Fig.1.3.1: p53 protein structure

p53 and apoptosis: The control of apoptosis by p53 is an extensively studied area in the years since its discovery. p53 was initially identified to play an important role in apoptosis by cells (Yonish-Rouach et al., 1991) which was followed by the discovery that apoptosis following DNA damage by radiation required p53 activity (Lowe et al., 1993). These studies together with other studies where it was seen that loss of apoptosis in p53 null mice resulted in increased tumour progression (Symonds et al., 1994), identified the important role that p53 played in controlling malignant transformation in cells. The apoptotic program consists of the extrinsic and intrinsic pathways, and p53 has been shown to control key points in both. The extrinsic pathway consists of cell surface receptors, associated regulatory proteins and the inhibitory ligands which are all required for procaspase activation and initiation of apoptosis (Peter and Krammer, 2003). The intrinsic pathway on the other hand centres on the mitochondria which key apoptogenic factors (Kroemer and Reed, 2000) and is controlled to a large extend by the pro and anti-death members of the Bcl2 family of proteins (Gross et al., 1999a; Gross et al., 1999b; Hemann and Lowe, 2006). p53 apoptotic activity has been linked to its transactivation activity and is thought to be essential for apoptosis regulation in normal cells.

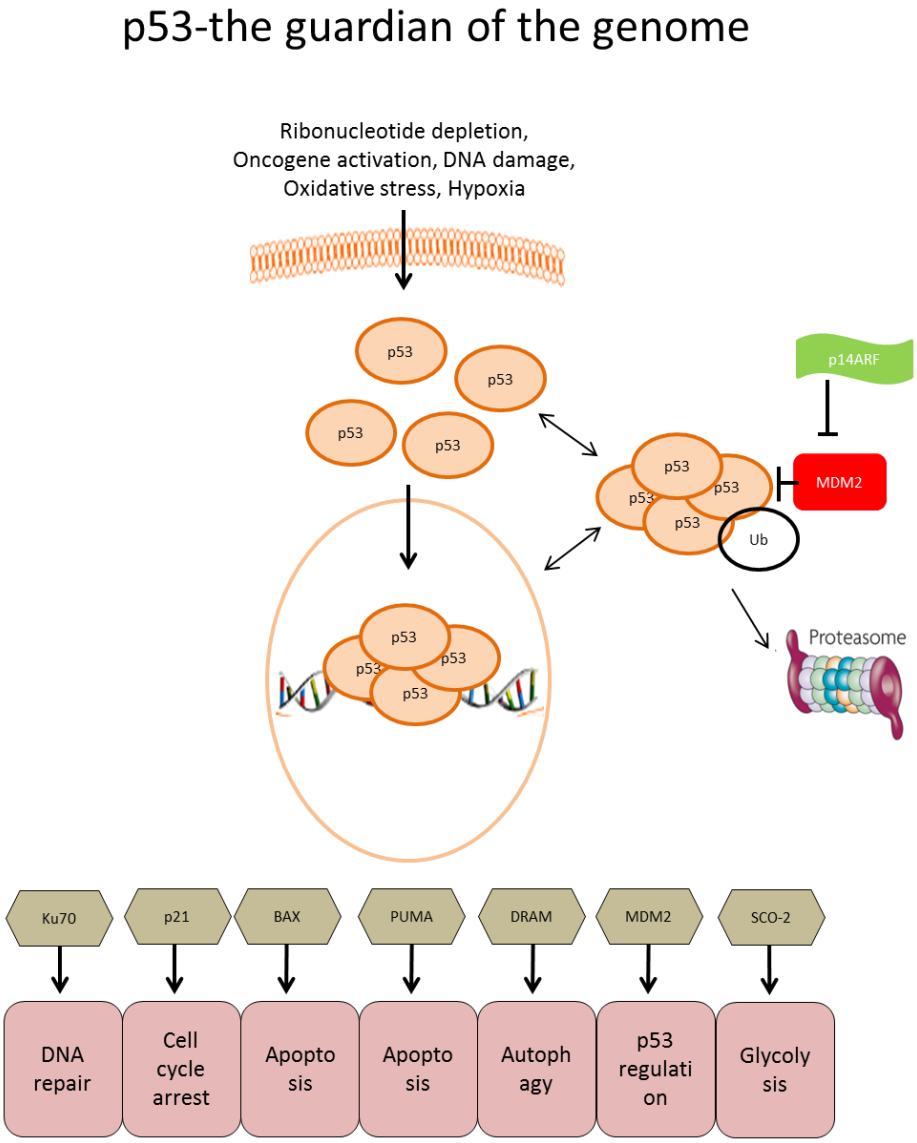


Fig. 1.3.1: Representative targets of p53 mediated tumour suppressive effects and p53 protein regulation

The Bcl2 family of pro-apoptotic genes are controlled by p53 and include many members including *Bax*, *Puma*, *Noxa* and *Bid* (Fridman and Lowe, 2003; Riley et al., 2008). All of these genes have p53 response elements in their promoter regions and increased transcription of these genes following severe cell stress like DNA damage and oncogenic signalling results in the activation of the mitochondria mediated apoptotic pathway. This has been established in both *in vitro* and *in vivo* settings as loss or deficiency of any of the p53 targets can result in varying levels

of apoptotic defects (Yin et al., 1997; Yu and Zhang, 2005; Zhang et al., 2000). A loss of p53 has a similar attenuation of the apoptotic mechanism confirming the effector pathways. The role of p53 is not restricted to transcriptional regulation of the apoptotic factors. It also acts as a transcriptional regulator of the apoptotic effector pathway where it transactivates APAF-1(Moroni et al., 2001; Soengas et al., 1999) which can initiate the caspase cascade and can also up-regulate the expression of Caspase 6, both of which can potentiate the cell death. The influence of p53 on the intrinsic pathway does not end with the transcriptional regulation of the components of the pathway. Wild type p53 can interact with anti and pro-apoptotic members of the Bcl2 family at the mitochondria and regulate the mitochondrial permeabilisation. p53 interacts with Bcl-xL and Bcl2 and neutralizes their inhibitory effects on proapoptotic Bax and Bak and thereby promotes mitochondrial membrane permeabilisation. This non-transcriptional effect is believed to occur only during p53-dependant cell death (Fridman and Lowe, 2003; Vaseva and Moll, 2009) and though important, comes secondary to its role in the transcriptional activation of the cell death mechanism. p53 also has a poorly understood role in the transcriptional control of the extrinsic apoptotic pathway where it has been shown to encode the DR5 death receptor loci and the Fas ligand TNFSF6 (Maecker et al., 2000; Wu et al., 1997). Bid, a member of the intrinsic pathway, can also play a role in this mechanism by interacting with the extrinsic pathway, thereby adding another p53 mediated/regulated apoptotic effect (Sax et al., 2002).

p53 regulates cell cycle and inhibits cells growth and proliferation: p53 plays a significant role in the regulation of the cell cycle in normal cells. While much of this may be attributed to the regulation of the cyclin dependent kinase inhibitor

p21, this effect can also be due to its interaction with the cell cycle regulator Polo Like Kinases (PLK). PLK1 acts as a sensor for DNA integrity/damage especially in the various cell cycle stages, wherein via ATM and ATR, it mediates the DNA damage response that closely requires functional p53 activity (Xie et al., 2005). Plk1 negatively regulates the activity as well as stability of p53 (Ando et al., 2004), whereas the other PLK family member Plk2 is a direct transcriptional target of p53 (Burns et al., 2003), and Plk3 mediates phosphorylation of p53 at Ser-20 and its subsequent stabilisation (Xie et al., 2001). Interestingly the requirement of p53 was examined in a panel of isogenic cell-lines with wild type and non-functional p53 and it showed that cells without a functional Wild Type p53 became sensitive to PLK1 inhibition and resulted in cell-cycle arrest (Sur et al., 2009). This finding was all the more substantial as cells carrying a non-functional mutant p53 also showed a similar effect and showed that prior activation of the p53 pathway with the MDM2 inhibitor Nutlin protects against the neutrophil depletion that is induced by mitotic inhibitors that block the activity of PLK1. Using this drug combination could thus possibly alleviate the side effects of chemotherapy with anti-mitotic agents without reducing its ability to kill p53 mutant tumour cells. Recently, PLK1 has also been shown to regulate p21 expression in a p53 dependent manner leading to senescence (Kim et al., 2013) while p53 dependent epigenetic changes of the PLK family members following tumour micro-environmental stress regulates their activity and consequently, various stages of the cell cycle (Ward and Hudson, 2014). Many models of p53 function have shown that programmed cell death is the primary mechanism by which wild type p53 prevents transformation and eliminates malignant cells (Vousden and Prives, 2009). However, p53 has additional mechanisms by which it can prevent cancer development most

significant of which is its ability to inhibit cell growth and proliferation. In non-transformed cells, p53 exerts this influence by activating the expression of the cyclin-dependent kinase (CDK) inhibitor p21(Abbas and Dutta, 2009a; Bunz et al., 1998) as well as other targets like 14-3-3 and GADD45 (Barak et al., 1993; Zhan et al., 1999).

p53 in adult and embryonic stem cells: The p53-p21 mediated cell cycle arrest has been shown to be essential in adult cells while early pluripotent embryonic cells have G1/S checkpoint and DNA repair that are p53 independent (Aladjem et al., 1998; Prost et al., 1998) and is instead dependent on the p53 analogue p73. Surprisingly though, the exposure of embryos to exogenous DNA damage, like γ-radiation, indicates a requirement for functionally active p53 to maintain embryogenesis where it regulates the repair and elimination of cells possessing a less committed state. $p53^{-/-}$ embryos on the other hand do not show this early effect, but rather a global activation of apoptosis in the committed and differentiated cells that appear at later stages. Adult $p53^{-/-}$ mice are also deficient for the G1/S checkpoint as well as apoptotic response to DNA damage (Bouffler et al., 1995; Donehower et al., 1992). Indeed the complex role that p53 plays in regulating cells both at embryonic and later stages have promoted the idea that the cancer suppressive effects of p53 are an evolutionary co-option of p53 activity that was initially evolved to protect the germ-line (Donzelli et al., 2008; Vousden and Prives, 2009). To this end, it has now been proposed that in most normal cells, acute stress is not required for activation of p53, but rather, basal levels of p53 or low levels of stress can activate the p53 pathway. This regulatory mechanism has been shown to control stem cell renewal as well as the potential

for developing induced pluripotent stem cells from fibroblasts (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009).

p53 regulates p21 dependent senescence: Another interesting finding of p53 in normal cells is that whilst p53 mediated G1/S cell cycle arrest can give time for minor DNA damage to be corrected, cells that are exposed to significant stress in the form of DNA damage by oncogene activation or in response to telomere dysfunction, are blocked permanently in the irreversible cell cycle arrest otherwise known as senescence (Campisi and d'Adda di Fagagna, 2007). This is another important mechanism for preventing malignant transformation and is also seen in response to wild-type p53 activation in transformed cancer cells. The cytostatic effects of senescence seen in *in vitro* studies are complemented by immune system mediated tumour clearance in the *in vivo* system, making it almost as important as apoptosis in controlling tumour cells (Xue et al., 2007). One of the key mediators of p53 induced senescence is the cell cycle regulator p21 (Schumm et al., 2006). p21 belongs to the Cip/Kip family of CDKs which includes p21, p27 and p57 (Abbas and Dutta, 2009b). p21 exerts its functions primarily through its inhibition of CDK2 activity (by inhibiting the cyclin-CDK complexes) which is required not only for the phosphorylation of the Retinoblastoma (Rb) protein with the consequent release and activation of E2F-dependent gene expression, but also for the firing of replication origins and for the activity of proteins directly involved in DNA synthesis (Brugarolas et al., 1995; Deng et al., 1995; Zhu et al., 2005). The expression of p21 is very sensitive to wild type p53 levels and can result in a G1/S cell cycle arrest following stress. Interestingly, the loss of p21 does not promote tumour development, in a manner quite possibly similar to the activity of pro-apoptotic PUMA, where loss of PUMA alone, does not promote

tumour development (Mandke et al., 2012). The existing understanding thus is that apoptosis and senescence support each other in ensuring the prevention of malignant transformation in normal cells. Not surprisingly, this brings up the question of what determines the outcome of p53 activation. The decision of damage repair or cell death/arrest is dependent on the cell type as well as the nature of the stress signal (Vousden and Prives, 2009). Evidence also suggests that this decision is also dependant on the extent of the damage or the duration of stress with repairable damage eliciting the survival response and more severe damage promoting the anti-proliferative cell death mechanisms (Stott et al., 1998). The p53 mediated regulation of p21 and subsequent cell cycle modulation primarily involves the G1/S phases of the cell cycle. But it has also been shown by various authors that p53 may also be playing a role in other stages of the cell cycle including G2 and the pre-meiotic stages of the cell cycle (Weisz et al., 2004). p21, Cyclin G as well as the 14-3-3σ are all proteins described to have G2 regulatory effects and are also known to be p53 transcriptional targets. DNA damage repair response following radiation induced damage is another transcriptional role exhibited by p53. In an ATM dependent manner, p53 has been shown to stabilise the levels of Ku70 in the nucleus, which promotes the Ku70/Ku80 complex formation at the DNA damage site followed by activation of the repair pathways (Brown et al., 2000).

p53 and metabolic pathways: p53 can also regulate metabolism which in turn may help its function as a tumour suppressor. Starvation and other metabolic stress can induce the activity of p53 which promotes AMP-activated protein kinase and other metabolism regulating proteins which can negatively regulate the mammalian target of Rapamycin (mTOR) (Petitjean et al., 2007). The mTOR

regulation together with the ability of p53 to induce lysosomal proteins like DRAM (damage-regulated autophagy modulator) (Lang et al., 2004) can result in other cellular control mechanisms like autophagy (Muller and Vousden, 2013). In addition to the afore-mentioned regulation of metabolism, p53 has been shown to regulate glucose uptake, glycolysis and mitochondrial respiration (Vousden and Prives, 2009; Zalcenstein et al., 2006). The p53 pathway components while being essential for regulating cell growth and survival can also occasionally be utilised by cancer cells to promote survival and is an interesting area about which very little is known.

p53 mediated regulation of MicroRNAs: The transcriptional activity of p53 is not just restricted to the regulation of proteins. p53 has been shown to regulate the expression of a number of MicroRNAs. The miR-34 locus is regulated by p53 and expression of this miRNA has been demonstrated to promote cell cycle arrest, cell death and senescence (Bommer et al., 2007; Chang et al., 2007; Tazawa et al., 2007). Other microRNAs have also been reported to be transcriptional targets of p53 including miR-107, miR-145, miR-192 and miR-215 (Feng et al., 2011) and will be an interesting area that is likely to be investigated further in the near future. Though p53 has mainly been known for its transcriptional activity, it can also act as a transcriptional repressor of genes that promote growth and survival (Brady et al., 2011; Riley et al., 2008). This is another potentially important role that p53 plays to prevent malignant transformation and is yet to be completely understood.

Post-translational modifications of p53: p53 itself undergoes numerous post-translational modifications including acetylation and phosphorylation and these are known to regulate its function as a transcriptional regulator (Di Agostino et al., 2006) These include phosphorylation of sites like Serine (S) 46 by the homeo-

domain interacting protein kinase 2 (HIPK2), AMPK, Protein Kinase C delta and p38 Mitogen Activated Protein Kinase (p38-MAPK), all of which regulates the transcriptional activity. Various other p53 sites are phosphorylated by a number of kinases and play important roles in the response to cell stress. Many Lysine residues in the C-terminus of p53 including K120 are acetylated by acetyl transferases like Tip60 (Liu et al., 2011), while deacetylases like Sirtuin 1 can reverse this effect (Cheng et al., 2003). Various other post-translational modifications like ubiquitination, methylation, sumoylation and neddylation also have the potential to regulate p53 transcriptional activity. A number of other p53 regulation events have also been identified and play varying roles in regulating cellular response to stress. Many non-covalent modifiers of p53 have been identified that can exert an effect on the interplay between the protein and its binding partners. One of the best studied and reported is that of the p53 regulatory protein and E3 ubiquitin ligase MDM2 and its homolog MDMX. In addition to their E3 ligase properties, these proteins can affect p53 activity by displacing p300 and reducing p53 acetylation (Ito et al., 2001) or by recruiting Histone Deacetylases (HDAC1) (Ito et al., 2002) and KAP1 (Wang et al., 2005a) which can regulate acetylation of histones in the vicinity of p53 binding sites. MDM2 can also associate with p53 at the p21 promoter (Vousden and Prives, 2009), while ectopically expressed MDM2 can bind to other p53 target promoters and regulate their expression. The p53 transactivation domains TAD I (residues 20-40) and TAD II (residues 40-60) can recruit components of the multi-subunit transcriptional activator STAGA to activate transcription of target genes like p21, PUMA and GADD45 (Gamper and Roeder, 2008). The transactivation domains TAD I and TAD II may regulate transcriptional activity of p53 by being the binding sites for

specific inhibitors like RITA (Ding et al., 2005; Enge et al., 2009; Vassilev et al., 2004). Arginine methyl transferases like CARM1 and PRMT1 facilitate p53 mediated transcription from DNA via the histone acetyl transferase p300 (An et al., 2004), while the ASPP family member iASPP binds specifically to the P72 SNP of p53 making it less pro-apoptotic than the similar R72 p53 SNP (Bergamaschi et al., 2006). Another interesting regulator of p53 is the p52 subunit of NFκB which can inhibit p21 expression, while at the same time promoting PUMA, DR5 and GADD45 expression (Schumm et al., 2006) . These are but a representative list of a number of proteins that have been known to interact with p53 and regulate its function.

MDM2, MDMX and p53- The potent tumour suppressor functions of p53 require that the activity of this protein is under tight control to prevent unnecessary induction of apoptosis or cellular senescence. In non- transformed cells, p53 is targeted for proteasomal degradation by the E3 ubiquitin ligase MDM2 (Murine Double Minute 2) (Momand et al., 1992; Wade et al., 2013a). Another MDM family member that lacks E3 ligase activity, but is essential for regulating p53 levels is MDMX (Bottger et al., 1999). The MDM2 family is not the only E3 ligase that is known to ubiquitinate p53. Other E3 ligases including CHIP (Esser et al., 2005) and COP1 (Dornan et al., 2004) can also have similar properties. While both the MDM proteins can regulate p53 transactivation by engaging its N terminal transactivation domain, they differ in other functions as well as sensitivity to p53 activation. MDM2 is more sensitive to p53 activation when compared to MDMX. MDM2 homo-oligomers regulate p53 by binding and ubiquitylating p53 which leads to its proteasomal degradation(Fang et al., 2000). This keeps the p53 levels low in normal cells. In contrast, MDMX does not possess ubiquitin ligase activity,

but instead hetero oligomerises with MDM2 to increase its activity in controlling p53 (Linares et al., 2003). The aromatic residues present on both MDM2 and MDMX is further required for the E2 ubiquitin conjugating enzyme recruitment and subsequent p53 ubiquitination (Uldrijan et al., 2007). The regulation of MDM2/MDMX is essential for regulating p53, and the fine balance that exists in cells that will decide their fate. Gene amplification can result in increased MDM2/MDMX transcription, although many tumours show high levels of the proteins even without gene amplifications, suggesting post translational regulation (Wade et al., 2013b). Proliferating cells are more sensitive to MDM regulation especially depletion (Francoz et al., 2006) and not surprisingly, many mitogenic signals have been found to regulate MDM2 activity. The over-expression of the RAS signalling pathway can lead to up-regulation of MDM2/MDMX which is dependent on the ETS transcription factors (Ries et al., 2000). Like-wise, the MYC oncogene activation can result in an MDM2/MDMX transcription dependent regulation of p53 (Alt et al., 2003), although the exact mechanism is not yet clearly identified. While many more mitogenic signals may similarly affect MDM2/MDMX activity, what is well known is that p53 itself is the most potent regulator of MDM2 transcription (Barak et al., 1993). Although this was initially believed to be a MDM2 specific function, reports also show that p53 may have a regulatory role in MDMX as well (Phillips et al., 2010). This feed-back mechanism is essential for maintaining normal cellular homeostasis and to prevent abnormal initiation of the cellular death mechanisms.

The regulation of the MDM proteins does not stop just at the transcriptional level. Many post-translational modifications also occur that can play a role in regulating their activity. One such event that has been reported is the role of AKT in

phosphorylating serine (S) residues on MDM2 and MDMX, thereby increasing the ubiquitinating activity on p53. AKT has been shown to phosphorylate MDM2 at S166 and S186 (Mayo and Donner, 2001) while MDMX has been shown to be phosphorylated on S367 (Lopez-Pajares et al., 2008). These phosphorylations stabilise the MDM proteins and promote the ubiquitination of p53. Interestingly, the studies showing these post-translational modifications have invariably been done with exogenous expression of MDM2 and MDMX, as well as in ARF incompetent cells. In this context, more work looking at the dynamics of the endogenous proteins will be required in order to confirm the findings in the physiological setting. AKT is not the only kinase that has been known to produce post-translational modifications on the MDM proteins. Casein Kinase 1α (CK1α), DNA-dependant protein kinase (DNA-PK) and ABL have all been shown to phosphorylate the MDM proteins and thereby regulate the p53 activity (Chen et al., 2005b; Mayo et al., 1997; Meek and Hupp, 2010; Zuckerman et al., 2009). Likewise, the ATM and CHK kinases can also phosphorylate MDM2 following DNA damage (Chen et al., 2005a; Meek and Hupp, 2010) leading to dissociation of the oligomers and stabilisation of p53. As mentioned earlier, p53 plays an important role in regulating the transcription of miRNAs. p53 mediated transcription of miR-192, miR-195 and miR-215 can down-regulate MDM2 expression (Pichiorri et al., 2010) while miR-34 can regulate MDMX (Mandke et al., 2012), although *in vivo* studies do not support that claim (Concepcion et al., 2012).

The p53 regulating/ubiquitinating activity of MDM2 is most robustly antagonized by p14ARF (p19ARF in mouse – hereafter ARF), a product of the INK4A/ARF locus (Sherr, 2006; Stott et al., 1998). In the presence of hyperactive oncogenic signals, p53 responds in a manner that has been unequivocally demonstrated to require

ARF (Christophorou et al., 2006; Efeyan et al., 2006; Kamijo et al., 1997). A prerequisite for benign neoplasms to develop into tumours is the inactivation of p53, which most frequently is achieved through mutations in p53 itself, loss of INK4A/ARF or deregulation of ARF protein by promoter hyper-methylation (Sharpless, 2005; Vousden and Prives, 2009).

Functional p53 regulatory mechanisms are essential for normal regulation of ageing and tumour suppression: As mentioned previously, multiple stress signals converge on p53 and regulate its activity. Upon exposure to stress cues, p53 attains full transcriptional activity and activates a number of targets. The outcome of this is dependent on the type and intensity of the stress as well as the cellular context (Vousden and Lane, 2007). The same mechanisms that regulate p53 in its tumour suppressive role, are essential for another normal cellular function-namely that of cellular and tissue ageing (Finkel et al., 2007). Interestingly, it has been unambiguously shown that the normal regulatory mechanism of p53 (primarily via MDM2 and ARF) is essential for the normal cellular function of p53 and plays a vital role in the ageing process. Enhanced p53 activity in the presence of normal regulation is associated with cancer protection without any effect on the ageing process. This is characteristically seen in mice that have extra copies of the p53 gene (also referred to as super-p53) (Garcia-Cao et al., 2002), extra gene copies of ARF (super-ARF) (Matheu et al., 2004) and decreased MDM2 activity (Mendrysa et al., 2006). Furthermore compound super p53/ARF mice were associated with delayed ageing in the same normal regulatory context (Matheu et al., 2007). On the contrary, losses of the normal regulatory mechanisms were associated with a completely different picture. Mouse models with N-terminal p53 truncations (resulting in impaired MDM2 interaction) were

associated with enhanced tumour suppression, but at the expense of accelerated ageing (Maier et al., 2004; Tyner et al., 2002). In a similar manner, constitutive activation of p53 as a result of permanent damage as in case of mice deficient for BRCA1 results in embryonic lethality, which can be rescued by deleting p53 function (Cao et al., 2003; Gudmundsdottir and Ashworth, 2006). All of this points to the vital role that the p53 regulatory mechanisms play in tumour prevention and normal cellular ageing.

1.4– p53 mutations in cancer

The functional inactivation of the p53 pathway, either through mutation of p53 or the mis-regulation of upstream regulatory elements is a universal feature of human cancer. Indeed somatic mutations of p53 are found in nearly half of all human cancers (Brosh and Rotter, 2009; Freed-Pastor and Prives, 2012). Recently, mutant p53 (p53mut) has also been demonstrated to respond to many of the same stimuli that promotes wild type p53 stabilization indicating that wild type and mutant p53 share similar regulatory mechanisms (Suh et al., 2011). An interesting characteristic of tumours carrying missense mutations in p53 is that more than 75% express p53mut protein within tumour tissue, which contributes to the many gain of function phenotypes attributed to p53mut (Brosh and Rotter, 2009; Petitjean et al., 2007). Furthermore, many genetic mutations found in cancer including RAS mutation, c-MYC activation, p16INK4A loss and PML deletion have also been demonstrated to stabilize p53mut (Haupt et al., 2013; Terzian et al., 2008). In normal tissue, mutations of p53 are in themselves not sufficient to promote p53mut accumulation. Likewise, tumours originating from p53mut mice do not accumulate p53mut to the same degree, suggesting that there may be

other mechanisms which contribute to p53mut stability (Lang et al., 2004; Olive et al., 2004; Terzian et al., 2008). Growing evidence indicates that p53mut tumour cells must acquire additional modifications that will allow them to overcome the same regulatory mechanisms which inhibit wild type p53 accumulation (Brosh and Rotter, 2009; Freed-Pastor and Prives, 2012; Li et al., 2011a; Li et al., 2011b; Terzian et al., 2008). Although MDM2 has been demonstrated to restrict the stabilization of p53mut (Terzian et al., 2008) the molecular determinants and pathways that promote p53mut stabilization remain to be fully determined.

p53mut as an oncogene: p53mut protein has been shown previously to possess oncogene like properties (Sigal and Rotter, 2000), which was further strengthened by the work done on genetically engineered mice, where p53 null or heterozygous mice expressing a mutant p53 allele was seen to have increased incidence of tumours of different types (Lang et al., 2004; Olive et al., 2004). The oncogenic function of the mutant protein also includes increased metastasis, genome instability, stem cell expansion, invasion, survival and tissue remodelling (Muller and Vousden, 2013). Most missense or point mutations that are commonly seen in p53 are localised to its DNA binding region (including the most common ‘hotspot’ mutations R175, G245, R248, R249, R273 and R282) (Fig. 1.4.1) suggesting that the transcriptional function of the p53mut is key to its pro-oncogenic properties (Freed-Pastor and Prives, 2012). These mutations which occur at the DNA binding domain can be broadly classified into structural mutants that change the conformation of the p53 protein and contact mutants that change the amino acids essential for binding to specific promoters. The structural mutations can cause changes in the structural core of the p53 protein which in turn can influence its conformation and subsequent stability. In fact the wild type p53 itself is inherently

unstable and the mutations in the DNA binding and core domains can alter the stability of the mutant protein (Bullock et al., 1997).

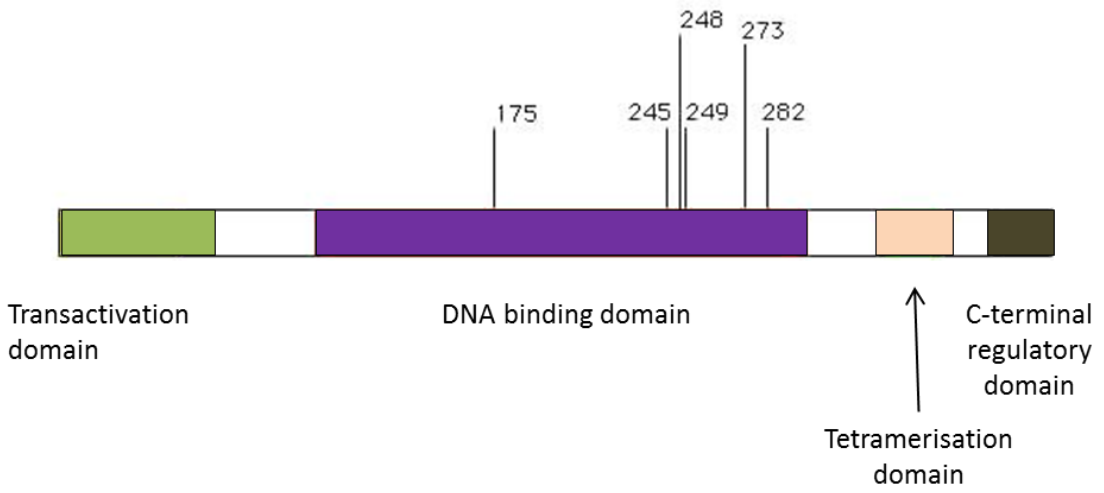


Fig. 1.4.1: p53mut DNA binding domain associated 'hotspot' mutations

p53 "hot-spot" mutations: DNA contact mutations inactivate p53 by replacing residues that form essential contacts with the DNA response elements like R248, R273 and R280. This results in impaired transactivation of the DNA response elements without causing significant structural changes to the p53 protein (Joerger et al., 2006; Joerger and Fersht, 2007).On the other hand, structural mutants like R282, G245 and R249 result in conformational changes of the L3 loop of the tertiary structure of p53 that leads to impaired binding to the DNA and affinity loss to the DNA promoter site (Joerger and Fersht, 2007). The most common hotspot mutant- the R175 mutant, is a structural variant which impairs the zinc binding region of the p53 protein core which is essential for protein loop formation, especially when replaced by a bulky reside like Histidine.

p53mut Gain of Function: Mutations of p53 have been described to give gain-of-function (GOF) properties to the protein, where it is not just the loss of p53 function but rather the selective maintenance of specific p53mut protein variants

that has a positive role for tumorigenesis. Quite interestingly, previous authors have shown that the above mentioned DNA binding domain hotspot mutants are all associated with varying degrees of gain-of function properties (Table 1.4.1) (Hanel et al., 2013; Lang et al., 2004; Olive et al., 2004). But the GOF properties are not uniform between the different mutant variants and can be described to occur via various distinct downstream interactions and activity (Hanel et al., 2013; Mello and Attardi, 2013).

Mutation	Frequency	Class of mutation	GOF	Reference
R175H	4.6%	Structural	Yes	(Olive et al., 2004)
R248Q	3.5%	Contact	Yes	(Hanel et al., 2013)
R273H	3.1%	Contact	Yes	(Lang et al., 2004)
R248W	2.8%	Contact	Yes	(Song et al., 2007) (Xu et al., 2014)
G245S	2.8%	Structural	Yes	(Hanel et al., 2013)
R273C	2.7%	Contact	Yes	(Tepper et al., 2005)
R282W	2.4%	Contact	Yes	(Xu et al., 2014)
R249S	1.8%	Structural	? Yes	(Lee and Sabapathy, 2008)
G245D	0.68%	Structural	?	(Zhang et al., 2012)

Table 1.4.1: Table showing the most common mutations at the “hotspots” and their GOF activity

Many p53mut forms can exert dominant-negative effects on the remaining wild type p53 allele in the cell and excessive production of the mutant protein can abrogate the function of the wild type protein (Brosh and Rotter, 2009). While this dominant-negative effect can occur due to formation of wild type/mutant complexes resulting in impaired function, it can also be followed by loss of

heterozygosity of the wild-type allele, which would suggest a selective advantage for losing the wild type allele. Interestingly, the effect on the wild type p53 is not the only gain-of-function exhibited by the mutant protein.

Role of p53mut as an oncogenic transcription regulator: The tumour associated p53mut retains its N-terminal transactivation domain, albeit one which quite possibly recognises other DNA binding regions that is different from the normal response elements (Ludwig et al., 1996). The aberrant p53 function may allow it to function as the transcription regulator for oncogenic proteins, as seen with its association with gene promoters encoding CD95, EGR1 and MSP (Weisz et al., 2004; Zalcenstein et al., 2003; Zalcenstein et al., 2006). This will raise the question of how the aberrant p53 protein achieves specificity for DNA binding. Various authors have tried to answer this and at least two areas of interest have been identified. One line of thought has been that of a consensus p53mut specific DNA response element, although surprisingly it has been quite difficult to characterize (Dell'Orso et al., 2011; Donzelli et al., 2008). p53mut has also been shown to preferentially bind nuclear matrix attachment regions that anchor chromatin fibers to the nuclear matrix, generating chromatin domains that may enhance or repress transcription, although it has since been shown that this is solely dependent on the stereo-specific configuration of the DNA and not on DNA sequence(Gohler et al., 2005; Will et al., 1998). Another explanation about how promoter specific activation can be mediated by p53mut, is that of other Single Stranded DNA Binding proteins which may recruit p53mut through protein-protein interaction. This is exemplified by the Sp1 protein whose interaction with p53mut amplifies its transcriptional activity (Chicas et al., 2000) as well as the interaction of p53mut with NF-Y (Di Agostino et al., 2006). The former is responsible for

promoting transcription of pro tumorigenic factors like VEGF. The latter interaction deregulates cell cycle checkpoint following DNA damage (Di Agostino et al., 2006) and results in TopBP1 and p300 recruitment to target promoters (Liu et al., 2011). Interestingly this is in conjunction with other family members of the p53 family, namely p63 and p73. This gives way to another gain of function property of p53mut, which is also probably the best characterized. The transcription inhibitory interaction of p53mut with p63 and p73 (Gaiddon et al., 2001; Strano et al., 2002) impairs their binding to DNA and subsequent activity. p53mut inhibits the activity of the full length isoforms of both p63 and p73, but has a different activating effect on an N-terminus lacking (ΔN) isoform of p63(Neilsen et al., 2011). Thus p53mut may have both activating and repressing functions on other transcription factors. TopBP1 promotes p53mut and p63 interaction (Liu et al., 2011) whereas interaction of the C-terminus of p53mut with Ankyrin Repeat Domain 11 (ANKRD11) disrupts the p53mut-p63 interaction (Noll et al., 2012). On the other hand TGF-β treatment results in promotion of SMAD2 mediated complex formation and inhibition of p63 (Adorno et al., 2009). Thus the p53mut mediated repression of the activity of other transcription factors is indeed very complex and quite possibly may also depend on the cell type and cell context. Recently p53mut has also been shown to form protein aggregates that resemble amyloid aggregates which in turn has been shown to form complexes with other transcription factors, thereby inhibiting their activity (Xu et al., 2011). p53mut has also been shown to have an effect on other proteins by directly regulating their activity due to its protein-protein interaction. This includes the interaction with MRE11, which impairs the MRE11-RAD50-NSB1 phosphorylation of ATM and subsequent damage repair by HR (Muller and Vousden, 2013; Song et al., 2007).

Other proteins affected by p53mut interaction include BTG2, an H-Ras inactivating protein, whose activity is impaired and Topoisomerase 1 (Top1) whose activity is enhanced by p53mut binding (Restle et al., 2008; Solomon et al., 2012). These are but a snap shot of the various p53mut protein-protein interactions that have been shown to have significant effects on cancer progression and response to treatment.

p53mut interaction with p63 and p73: The information about the effect of p53mut interaction with the p63/p73 transcription factors and loss of their anti-tumour activity has been based on *in vivo* work done on p63 and p73 heterozygous mice that show increased incidence of tumours (Flores et al., 2005; Su et al., 2010; Tomasini et al., 2008) in a manner that recapitulated the tumour incidence in p53mut mice. The loss of the full length versions of p63/p73 show increased incidence of tumours which are more aggressive and metastatic. On the other hand loss of the ΔN forms of p63 and p73 show no increase in tumorigenesis, while the same in cells are associated with both pro-survival and anti-apoptotic effects (Lee et al., 2006; Wilhelm et al., 2010). This gives rise to the view that p53mut inhibits the anti-tumorigenic activity of full length p63 and p73 while promoting the anti-apoptotic effects of ΔN p63 and p73 (Muller and Vousden, 2013). p53mut also modulates the expression of p63 target genes, which have been associated with invasion and metastasis. These include SHARP1(Adorno et al., 2009), Cyclin G2 and Dicer (Girardini et al., 2011; Martello et al., 2010; Su et al., 2010) which have been shown to be p53mut regulated both in *in vitro* as well as *in vivo* systems, although the exact mechanism is still not clear. Likewise, the p53mut-p63 interaction has also been shown to enhance recycling and signalling of cell surface receptors. This occurs via the G protein and

Rab family member RAB11 effector, RAB Coupling Protein (RCP) which mediates recycling of growth factor receptors like the Epidermal Growth Factor Receptor and Hepatocyte Growth Factor Receptor (Muller et al., 2009). While a similar role of p73 in invasion and metastasis has not been shown, its role in enhancing apoptosis and senescence is well characterised (Lowe et al., 2004; Melino et al., 2003). In this context, p53mut can interact with p73 and prevent the apoptotic and senescence response, especially in response to treatment (Di Como et al., 1999a; Dulloo and Sabapathy, 2005; Strano and Blandino, 2003).

p53mut and its regulation of miRNAs: The regulation of miRNAs by p53 has been mentioned before, and not surprisingly, p53mut has been shown to alter the stability of their target transcripts. These include miR-130b, miR-155 and miR-205, which regulate ZEB1 and ZNF652 (Dong et al., 2013; Neilsen et al., 2013; Tucci et al., 2012). A number of other p53mut interactions have been studied and are believed to play different roles in the p53mut gain-of-function activity. What is also interesting to note is the requirement of the p53mut DNA binding domain for the interaction with p63 and p73 (Gaiddon et al., 2001; Strano et al., 2002), as well as the requirement of the C-terminal of the mutant protein for the interaction with ANKRD11, PLK2, ETS2 and VDR for regulating the gain-of-function activity (Do et al., 2012; Muller and Vousden, 2013; Noll et al., 2012; Valenti et al., 2011). This variation in the p53mut interacting sites is also characterised by the fact that the different hot spot mutations result in different gain of function outcomes. The different mutant forms (broadly the DNA binding and structural proteins) show different abilities to bind other transcription factors and proteins although eventually converging on the same pathway to elicit pro-tumorigenic properties in most instances (Muller and Vousden, 2013).

Regulation of p53mut protein: An important factor when discussing about p53mut is the regulation of the protein itself in the cells. A key fact that is all the more important in the context of this manuscript is that many of the mechanisms that regulate wild-type p53 have been shown to regulate p53mut as well. The MDM2 mediated regulation of wild-type p53 that was discussed earlier has also been shown to regulate the protein levels of p53mut as the mutant protein by itself is not intrinsically resistant to MDM2 mediated ubiquitination and degradation (Suh et al., 2011; Terzian et al., 2008). Like-wise, the oncogenic and other cell stress mechanisms that normally promote the wild-type p53 stabilization also promote the stability of p53mut protein (Terzian et al., 2008). These include the commonly deregulated signalling pathways like PI3K/AKT and the RAS pathways, which could explain many of the *in vivo* findings where p53mut and PI3K/AKT pathway mutations have been known to co-exist and promote tumour progression (Blanco-Aparicio et al., 2010; Hanel et al., 2013). This further indicates that targeting the pathways that promote the p53mut stability could reverse the gain of function related resistance to therapy. Much of the work on destabilizing the p53mut protein was done by using histone deacetylases that target heat shock proteins to rescue MDM2 dependent degradation of mutant p53 (Li et al., 2011a) as well as the small molecule RETRA (Reactivation of Transcriptional Reporter Activity) that prevents the association of p53mut with p73 (Kravchenko et al., 2008). While these initial works are interesting, on the other hand, this also strengthens the argument about the rationale for selecting patients based on their p53mut status, as the very same therapeutic agents that maybe used to decrease the p53mut stability may be deleterious to a p53 wild-type tumour (Hamilton et al., 2014; Muller and Vousden, 2013).

1.5– p14^{ARF} signalling in cancer

p14ARF (Alternate Reading Frame) is a product of the INK4A locus at 9p21 along with the Cyclin Dependant Kinase (CDK) and Retinoblastoma tumour suppressor regulator p16^{INK4A}. Separate splicing events result in protein sequences that are completely unrelated and have separate functions (Duro et al., 1995; Mao et al., 1995). ARF is a key tumour suppressor responsible for the protection of cells against oncogenic activation by invoking p53 (Kamijo et al., 1997; Sherr, 2006). ARF is sensitive to active oncogenic signalling and stabilises p53 activity by inhibiting the p53 ubiquitinating proteins MDM2 and Mule/Huwe1. This prevents the subsequent proteasomal degradation of p53 and a consequential increase in p53 activity. The central dogma surrounding tumour initiation dictates that neoplasms must bypass the senescence barrier, through loss of INK4A/ARF locus or inactivating mutations of p53. While ARF loss and p53mut may be mutually exclusive, mounting evidence points to transcriptional, translational and post-translational regulation of ARF as independent and clinically relevant modes of escaping senescence (Burd et al., 2010; Chen et al., 2010a; Chen et al., 2010b; Gonzalez et al., 2006; Maeda et al., 2005). A primary regulation of ARF protein is achieved through modulating association with the nucleolar protein Nucleophosmin, which sequesters ARF into the nucleolus (Enomoto et al., 2006; Gjerset, 2006; Gjerset and Bandyopadhyay, 2006; Itahana et al., 2003; Korgaonkar et al., 2005; Kuo et al., 2004).

ARF as an "oncogene" in p53^{mut} tumours- Oncogene induced senescence occurs upon the mobilization of ARF and the subsequent promotion of a sustained p53 response. Inactivation of p53 is a prerequisite for tumour cells to further develop and reports suggest that acquisition of p53 mutations result in tumours

that are more therapeutically recalcitrant compared to loss or regulatory inactivation. The origin of these effects are attributed in part to suppression of alternative p53 related tumour suppressors p63, p73 or a remaining wild type p53, either by forming non-functional heterodimers and/or active repression of p53 dependent promoters (Bergamaschi et al., 2003; Bruno et al., 2010; Di Como et al., 1999b; Goh et al., 2011; Lang et al., 2004; Li and Prives, 2007; Olive et al., 2004). In tumours that retain *INK4A/ARF*, the oncogenic insult persists and continues to drive $p53^{mut}$ expression, further exacerbating dominant negative suppression and a failure to respond to normal cues such as DNA damage (Abraham and O'Neill, 2014). In this context ARF acts like an oncogene rather than a tumour suppressor and can potentially be a therapeutic target that can regulate the p53mut levels.

p53 independent functions of ARF: ARF has been shown to possess anti-tumour functions independent of its MDM-p53 regulation. Triple knock-out of ARF, p53 and MDM2 led to wide-spread tumours in mice when compared to double knock-out of p53 and MDM2, suggesting a p53 independent role of ARF in tumour suppression (Weber et al., 2000). Interaction of ARF with various transcription factors like E2F1, Myc, NFκB, and many of the nucleolar proteins like Nucleolin and Nucleophosmin as well as components of the ribosomal biogenesis machinery, have been found to produce many p53 independent tumour-suppressive functions (Ozenne et al., 2010; Sherr et al., 2005). Induction of ARF has been found to regulate ribosome biogenesis by interacting with the 45/47S and 32S subunits (Sugimoto et al., 2003). Similarly, p19ARF has been shown to regulate platelet-derived growth factor (PDGF) in a p53 independent manner (Silva et al., 2005). ARF has also been implicated in post translational modification

of various targets like HDM2 and Werner's Helicase by sumoylation (Sherr, 2006; Tago et al., 2005). The p53 independent functions of ARF are an evolving area of research with more information likely to emerge in the coming years.

1.6 – The RAS - PI3K – AKT pathway

RAS- The RAS proteins are members of a GTP binding superfamily of proteins that play crucial roles in signalling pathways that regulate cellular proliferation. This is also one of the commonest signalling pathways that is de-regulated in tumour cells and which regulates their growth, invasiveness and ability to promote angiogenesis (Oft et al., 1996; Rak et al., 1995; Shaw and Cantley, 2006; Shields et al., 2000). Three members of the RAS family have been identified and include KRAS, HRAS and NRAS. The RAS family members commonly undergo activating mutations (Downward, 2003; Lowy and Willumsen, 1993) that confer them pro-tumorigenic properties. All the RAS family members are widely expressed, but KRAS itself is the most essential for normal cellular function and so not surprisingly, is the most ubiquitous and most commonly mutated (85%) of the RAS family members. Post-translational modifications that localise the RAS proteins to the inner side of the plasma membrane are essential for their activity in normal cells (Hancock et al., 1989; Hancock et al., 1990). This also makes them attractive therapeutic targets during drug development. Since the RAS proteins are GTP binding proteins, their activity depends on whether they have been bound to GTP (active) or GDP (inactive). The ratio between the GTP and GDP binding decides the activation and subsequent downstream signalling (Campbell et al., 1998). The exchange between GDP and GTP and the hydrolysis of GTP to GDP is catalysed

by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) respectively and this involves a number of proteins acting together. GEFs including SOS1 and SOS2 are essential for activating RAS following upstream receptor tyrosine kinase (RTK) signalling. Activation of the upstream components like the Epidermal Growth Factor Receptor (EGFR), results in its binding to the SH2 domain of the adapter protein Growth Factor Receptor Bound protein 2 (GRB2), following which GRB2 is bound to the SOS proteins via its SH3 domain. This recruitment occurs at the plasma membrane, where RAS is also localised, and the close proximity between the two proteins result in the increased exchange of GTP for GDP as GTP is the predominant guanine nucleotide in the cytosol, and the resultant activation of RAS (Daub et al., 1996; Downward, 2003; Gale et al., 1993). The activation of RAS can also involve other proteins like the SHC adapter protein (Rozakisadcock et al., 1992) as well as several other non-receptor tyrosine kinases which can anchor GRB2 to promote RAS GTP loading (Cullen and Lockyer, 2002; Ebinu et al., 1998). The activation of RAS is reversed by GTPase activating proteins (Donovan et al., 2002) which are essential for regulating the mitogenic signalling cascade that proceeds from RAS. This brings us to the question of how the RAS mediated downstream growth and survival signalling, proceeds. It is important, since the constitutive activation of RAS is a common feature in cancer cells which promotes proliferation and survival. Activated RAS binds and activates downstream effector proteins, which can in turn activate distinct signalling pathways.

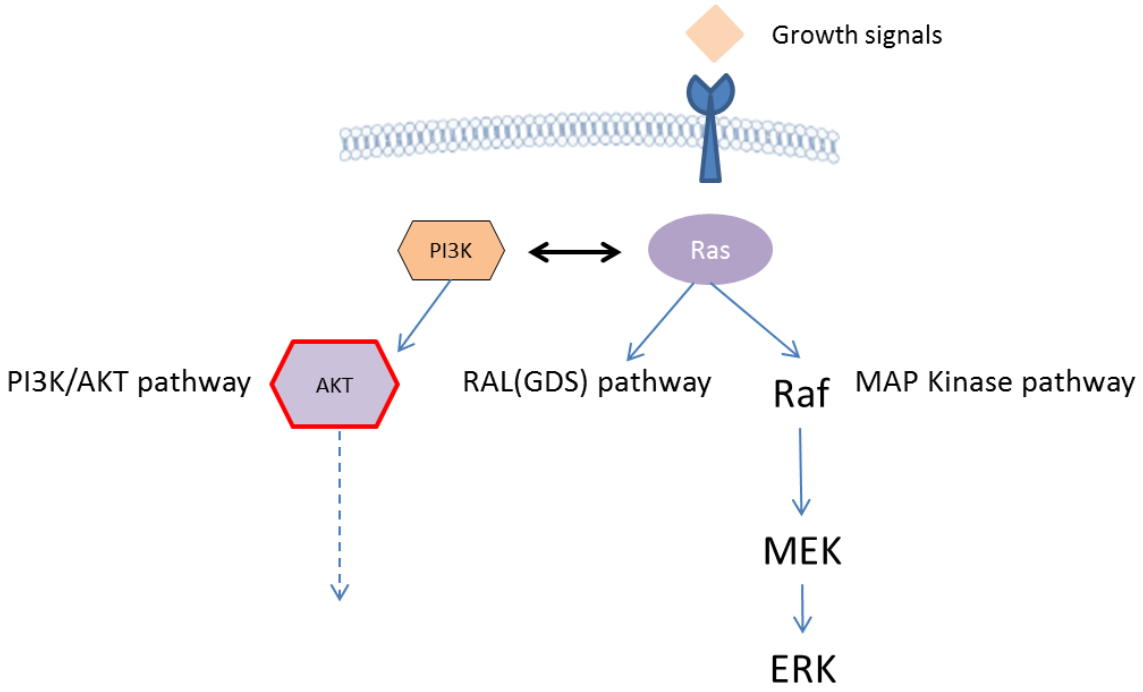


Fig. 1.6.1: The principle RAS effector pathways

MAPK pathway regulation by Ras: The pathway most commonly associated with RAS activation is the RAF effector and its downstream components, together commonly referred to as the RAF/MAPK pathway (Chang et al., 2003; Leevers et al., 1994). The GTP-bound RAS activates the three closely related RAF proteins c-RAF1, BRAF and ARAF. This occurs at the plasma membrane and is subsequently followed by the RAF mediated phosphorylation and activation of the mitogen activated protein kinase kinase 1 and 2 (MEK 1 and 2). These kinases phosphorylate and activate the mitogen-activated protein kinases (MAPKs) ERK 1 and ERK 2. The ERKs can directly phosphorylate many downstream transcription factors including Ets-1, c-Jun and c-Myc (McCubrey et al., 2007). The ETS family in turn, regulates expression of FOS and the JUN-FOS heterodimers formed by the above mentioned activation cascade and plays a key role in regulation of transcription factors that are responsible for cell cycle regulation, including Cyclins

(Woods et al., 1997). ERK also phosphorylates and activates the p90 Ribosomal S6 kinase which leads to activation of the transcription factor CREB (Steelman et al., 2004) as also an *i*nhibitor *κ*B *k*inase (IKK) mediated activation of the NFκB transcription factor (Zhao and Lee, 1999). The above highlights the multitude of cell cycle regulatory components that are influenced by the RAF/MEK/ERK pathway and signifies the important role that RAS activation play in normal and transformed cells.

PI3K/AKT pathway regulation by RAS: In addition to the MAPK pathway, RAS also activates other pathways, the most important of which is the PI3K/AKT pathway. RAS interacts with the catalytic subunit of type I Phosphatidyl Inositol 3-Kinases (PI3Ks) (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994), leading to its phosphorylation and subsequent activation of the pathway, the details of which will be discussed later in this section. Another effector pathway that has been shown to be regulated by RAS is the RAL proteins- RAL guanine nucleotide dissociation simulator (RALGDS), RALGDS-like gene (RGL/RSB2) and RGL2/RLF. The RALGDS pathway along with the PI3K/AKT pathway inhibits the Forkhead transcription factors. The Forkhead transcription factors have been shown to promote cell cycle arrest and apoptosis (Accili and Arden, 2004) and their inhibition by the mitogenic signalling through the RAS/RAL and PI3K/AKT pathways, results in the promotion of proliferation and transformation of cells. Thus aberrant RAS signalling can affect a number of essential growth and survival pathways, and is quite often utilised by transformed cells, by way of activating mutations of the pathway members, especially KRAS. The crosstalk between the MAPK and PI3K/AKT pathways that is mediated by RAS, makes it an important therapeutic target in many cancers.

PI3K/AKT- The PI3K family is a large group of Serine /Threonine kinases including phosphatidylinositol and other related kinases. They are grouped into 3 classes (I-III) according to their substrate preference and sequence homology (Cantley, 2002; Engelman et al., 2006). They possess distinct signalling roles in cells in regulating growth and survival. The Class I PI3Ks are further sub-divided into two- 1A and 1B, but possessing different regulatory sub-units. Class 1A integrates signals from G-Protein Coupled Receptors (GPCRs) and Receptor Tyrosine Kinases (RTKs), while the class 1B are exclusively associated with GPCRs. The class 1A will be discussed here further since it falls within the context of the manuscript. The Class II PI3Ks play a role in membrane trafficking and internalisation of receptors (Gaidarov et al., 2001) and they are also activated by RTKs, Cytokine receptors and integrins. Unfortunately, not much is known about further downstream activity of this class of the PI3Ks. The Class III PI3Ks (also known as vps34 in yeast) is known to regulate the Mammalian Target of Rapamycin (mTOR) (Nobukuni et al., 2005) and may play a role similar to that of Class 1 Kinases.

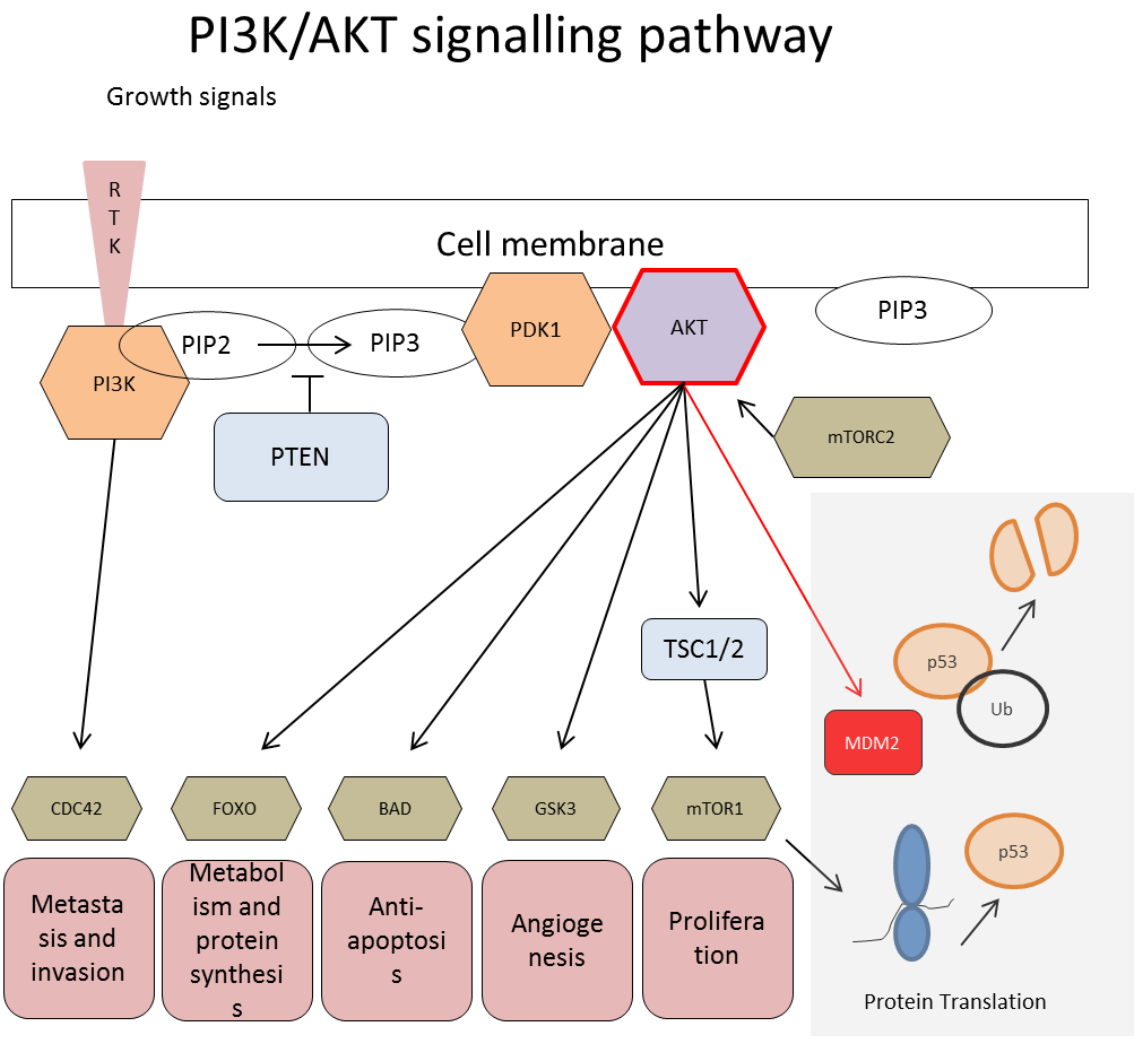


Fig. 1.6.2: The PI3K/AKT signalling pathway in cancer

The Class 1A PI3Ks are heterodimers of regulatory p85 subunits and catalytic p110 subunits. p85 binds and integrates signals from various signalling proteins which includes protein kinase C (PKC), SHP1, Rac, Rho, hormonal receptors, mutated Ras and Src, providing an integration point for activation of p110 and subsequent downstream signalling (Fruman et al., 1998; Hennessy et al., 2005). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to phosphatidylinositol-3,4,5-tri phosphate (PtdIns(3,4,5)P3)/ PIP3 which binds lipid binding domains of downstream targets to target them to the cell membrane for

subsequent activation. The primary downstream targets that are thus phosphorylated include the kinases Phosphoinositide Dependent Kinase (PDK) and AKT. The haplo-insufficient tumour suppressor Phosphatase and Tensin Homologue (PTEN) regulates the pathway by dephosphorylating PtdIns(3,4,5)P3 and preventing activation of downstream kinases (Song et al., 2012). AKT, which is the human homologue of the viral oncogene v-AKT is recruited to the membrane by the 3'-Phosphoinositides (PIP3), which interacts with the PH domain of AKT, as does PIP3 mediated recruitment of PDK1. The PIP3 bound PDK1 phosphorylates AKT at the activation loop Threonine 308 which in turn promotes auto-phosphorylation of S473 on AKT. This activation site can also be phosphorylated by the mammalian Target of Rapamycin Complex 2 (mTORC2) and dephosphorylated by the phosphatase PHLPP (Bayascas and Alessi, 2005; Gao et al., 2005). Phosphorylated AKT activates its various down-stream targets to produce the pro-growth and survival effects. Due to the vital growth and survival signalling that occurs through the PI3K/AKT pathway, it is essential for normal cellular function. Not surprisingly, this pathway is commonly de-regulated in cancers by activating mutations of the kinases and/or loss of the tumour suppressor PTEN (Hennessy et al., 2005; Osaki et al., 2004). Furthermore, while alteration of upstream pathway components, such as activating mutations in RAS, PI3K or loss of PTEN, can be the primary oncogenic events leading to therapeutic failure, it is the activation of AKT that is proposed to modulate cell death responses to therapeutic agents and mediate resistance to treatment (Hafsi et al., 2012).

AKT isoforms and substrate recognition: AKT has three isoforms- AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ. These have extensive homology to the protein

kinases A, G and C within their kinase domains and so are classed as members of the AGC kinase family. The substrate recognition motif of AKT was identified by *in vitro* studies and was defined as R-X-R-X-X-S/T-B where X represents any amino acids, B a bulky hydrophobic residue and the arginine (R) residue at -3 and -5 position from the Serine/Threonine residue (Alessi et al., 1996). But AKT is a relatively promiscuous kinase in the *in vitro* setting and studies have shown that it can phosphorylate most R-X-R-X-X-S/T as well as certain R-X-X-S/T sites (Maddika et al., 2008). A number of AKT substrates have been identified and while most of them conform to the consensus AKT recognition sites, approximately 25% do not (Manning and Cantley, 2007).

Regulation of Cell Survival: AKT has a critical role in regulating cell survival downstream of growth factor signalling and cell stress. A primary role has been the blocking of pro-apoptotic proteins in the cells. AKT has been shown to phosphorylate and inhibit the pro-apoptotic Bcl-2 homology domain 3 (BH3)- only protein BAD at S136, creating a binding site for 14-3-3 proteins that triggers release of BAD from its target genes (Datta et al., 1997; Datta et al., 2000). AKT also inhibits other pro-apoptotic proteins through effects on transcription factors including FOXO and p53. AKT phosphorylates FOXO at T24, S256 and S319 and blocks FOXO mediated transcription of pro-apoptotic genes like BIM (Dijkers et al., 2002; Tran et al., 2003). Unphosphorylated FOXO can also induce the expression of the pro-apoptotic Fas ligand (Brunet et al., 1999). As mentioned earlier, AKT has also been shown to phosphorylate MDM2 at S166 and S186 and promote its translocation to the nucleus and subsequent ubiquitination of p53 (Mayo and Donner, 2001; Zhou et al., 2001b). Thus AKT has been shown to have important pro-survival signal transduction activities. Other less understood AKT mediated

anti-apoptotic effects include the phosphorylation and inactivation of the AKT substrate GSK3 (Cross et al., 1995), which results in the stabilisation of the GSK3 target and anti-apoptotic Bcl-2 family member MCL-1 (Maurer et al., 2006). Another target of AKT is caspase-9 which is phosphorylated at S196 which results in its losing the protease activity to cleave other pro-apoptotic caspases (Cardone et al., 1998).

Regulation of Growth and Proliferation: Promoting cell growth has been a highly conserved role of AKT and one of its predominant downstream targets that regulates this role is the mTOR complex 1 (mTORC1). AKT regulates mTORC1 by inhibiting the upstream regulator of mTORC1, the Tuberous Sclerosis (TSC) TSC1/TSC2 complex (Gao and Pan, 2001), where TSC2 is directly phosphorylated by AKT at S939 and T1462 (Inoki et al., 2002; Manning et al., 2002; Wullschleger et al., 2006). TSC2 and TSC1 forms a complex that acts as a GTPase-activating protein (GAP) for the RAS related G- protein Rheb which in turn strongly activates mTORC1 when it is GTP bound. Proline-Rich AKT Substrate of 40KDa (PRAS40), a regulator of the mTORC1 is another substrate phosphorylated by AKT. AKT phosphorylates PRAS-40 at T246 and activates the mTORC1 signalling. Thus AKT regulates two parallel mTORC1 activating mechanism, further highlighting its importance in cell growth.

The PI3K/AKT pathway has other roles as well, and along with the MAPK pathway, it is also known to regulate cell proliferation. AKT phosphorylates $p27^{Kip1}$ at T157 (Shin et al., 2002; Viglietto et al., 2002) and $p21^{Cip1/Waf1}$ at T145 (Zhou et al., 2001a), which promotes their cytosolic sequestration and thereby preventing their cyclin-dependant cell-cycle regulation activity at the nucleus. Other cell proliferation regulatory roles include the AKT mediated phosphorylation of GSK3

which regulates cyclin D and cyclin E as well as the transcription factors c-Jun and c-Myc, all of which play regulatory roles in G1/S transition of the cell cycle (Diehl et al., 1998; Wei et al., 2005; Yeh et al., 2004). The AKT mediated mTORC1 regulation is also believed to play a role in causing a G1 cell cycle arrest (Skeen et al., 2006), while AKT mediated phosphorylation of S280 on the checkpoint kinase 1 (Chk1) and its inactivation (King et al., 2004) promotes progression through mitosis even in the presence of DNA damage as the CHK1 protein is unable to interact with the DNA damage sensing proteins ATM and ATR (Puc et al., 2005).

AKT plays important roles in other cellular functions as well. It has been shown to regulate angiogenesis in physiological conditions where AKT activates endothelial nitric oxide synthase that releases Nitric Oxide and thereby stimulates vasodilation, vascular remodelling and angiogenesis (Dimmeler et al., 1999; Morbidelli et al., 2003). Interestingly, AKT signalling also leads to increased Hypoxia Inducible Factor α (HIF1α and HIF2α) by a mTORC1 mediated increase in translation (Semenza, 2003).

The cell growth, survival and proliferation regulation have been very well characterised by many studies. An equally important role of AKT is in the regulation of metabolism. AKT2 has been shown to stimulate glucose uptake in cells like adipocytes that have Glucose Transporter 4 (GLT4) containing vesicles and are sensitive to insulin (Calera et al., 1998). This has been found to occur through a direct AKT target Rab-GAP AS160 (Eguez et al., 2005), where phosphorylation at different sites on the AS160 inhibits its GAP activity and instead allows a Rab-family GTPase to become GTP loaded and stimulate the GLUT4 containing vesicle translocation in the adipocytes. Unlike in the adipocytes, GLUT1 is the main glucose transporter in most cells and its regulation is

dependent on the changes in transcription levels of the protein. AKT mediated regulation of the mTORC1 complex, which was detailed previously, contributes to a HIFα dependent transcription of the GLUT1 gene and subsequent translation of the mRNA (Taha et al., 1999; Zelzer et al., 1998). AKT can also regulate the metabolism of glucose and lipids within the cells. Glucose-6-phosphate, the active form of glucose in the cells, is converted to glycogen or catabolised during glycolysis to supply energy for cellular processes. AKT has also been shown to regulate both the glycogen synthesis as well as glycolysis in cells. The phosphorylation of GSK3 as mentioned earlier, inhibits its activity on its substrate glycogen synthase and this result in increased activity and glycogen synthesis. Along with the glycogen synthesis activity, AKT also promotes glycolysis via a HIFα mechanism (Elstrom et al., 2004), while the inhibition of FOXO1 as mentioned previously also promotes glucose homeostasis (Zhang et al., 2006) especially in liver tissue. The GSK3 effects referred previously is not restricted to glycolysis or glycogen synthesis. It has been shown to promote the Sterol Regulatory Element-Binding Proteins (SREBPs) involved in cholesterol and fatty acid biosynthesis (Sundqvist et al., 2005). Thus AKT has a plethora of regulatory effects in normal cells, all of which are also utilised by malignant cells to promote their survival.

A poorly understood area of AKT has been its effect on migration and invasion. Some studies have reported the decreased migratory potential of cells in the presence of AKT, especially AKT1, and loss of AKT1 has been shown to increase migration in an ERK1/ERK2 dependant manner, where inhibition of AKT1 up regulates the MAPK pathway members (Irie et al., 2005). This point also brings us to the important role of PI3K/AKT cross-talk in cells, which acts as vital feed-back

loops as well as non-redundancy mechanisms utilised by cells to promote growth and survival. One of the best studied has been the cross-talk between the PI3K/AKT pathway and the MAPK pathway. The PI3K/AKT pathway via mTORC1 and S6K-1 can inhibit the MAPK pathway (Carracedo et al., 2008) and has been seen in different cellular/tissue settings (Wang et al., 2013). AKT has also been shown to block ERK signalling through inhibition of RAF1 (Zimmermann and Moelling, 1999) as well as the MAPKKK5 which is an upstream regulator in the MAPK related JNK-p38 pathway (Kim et al., 2001). A number of other pathway cross-talks involving the PI3K/AKT pathway exist, which is beyond the scope of this brief description of the signalling pathway.

1.7– AKT activation in Cancer

AKT has been shown to have a central role in the signalling pathway as we have seen and not surprisingly, the inappropriate activation of the PI3K/AKT signalling pathway has been implicated in tumorigenesis. Deregulation of several elements of this pathway including upstream signalling proteins as well as downstream effectors of AKT have been shown to play important roles in this regard.

AKT mutations: AKT itself has been shown to exhibit alterations that results in its activation in cancer cells. Alterations including activating mutations, amplifications and overexpression in all the three isoforms of AKT have been observed in different cancers. Activating mutations in AKT1 though relatively rarer than mutations of the other members of the family has been reported in breast, colorectal and ovarian cancers (Carpten et al., 2007; Kim et al., 2008). The E17K activating mutation in the PH domain of AKT1 has been the characterised

mutation in this context and has also been reported in lesser frequency in squamous cell cancers of the lung (Malanga et al., 2008). Similar E17K activating mutations in AKT3 have been reported, albeit as a rare occurrence in melanomas (Davies et al., 2008).

Amplification and overexpression of AKT2 has been reported in ovarian carcinomas (Bellacosa et al., 1995; Cheng et al., 1992) as well as the observation that the amplifications were more frequent in the undifferentiated tumours suggesting increased aggressiveness. The overexpression also makes the tumour cells overly responsive to growth factors. Amplifications of the native location of AKT2 on the chromosome in ovarian cancers have also been reported (Bellacosa et al., 1995; Thompson et al., 1996). In addition to ovarian cancers, AKT2 amplifications have also been reported in non-Hodgkin's lymphoma, pancreatic cancers and hepatocellular cancers (Arranz et al., 1996; Cheng et al., 1996; Xu et al., 2004). Unlike AKT2, amplifications in AKT1 and AKT3 have been reported infrequently. Increased AKT3 mRNA levels without gene amplification has been reported in estrogen receptor negative breast cancers (Nakatani et al., 1999) as well as increased AKT1 protein levels in breast cancers (Stal et al., 2003) that are not associated with amplification, but rather with possible activating mutations as mentioned earlier.

Activating mutations of PI3K/AKT pathway components: The best studied and most widely reported mechanism of PI3K/AKT activation in cancers are by receptor tyrosine kinases and by mutations in specific components of the upstream signalling pathway (Altomare and Testa, 2005; Engelman, 2009). By extension, these mechanisms modulate the AKT effect in cancer cells.

The activation of Class 1A PI3Ks is mediated by RTK and is essential for its oncogenic activity. The p85 regulatory subunit plays an important role in this regard where the Src homolgy 2 (SH2) domains present on it binds to the phosphotyrosine residues of activated RTKs (Songyang et al., 1993). Cancers exhibiting oncogene addiction to an RTK have increased PI3K activity that is controlled by the RTK including EGFR/ERBB2 (Pinkas-Kramarski et al., 1996). This is further highlighted by the fact that inhibitors targeting the RTKs require the down-regulation of PI3K for effective therapeutic activity (Bianco et al., 2003; Yakes et al., 2002). Multiple RTKs have also been reported to be activated in many cancers (Stommel et al., 2007) and this means that a single RTK inhibitor is ineffective in such cancers. As mentioned previously, the direct RAS mediated activation of PI3K is another mechanism that plays a role in promoting the PI3K/AKT mediated effects in cancer cells and is likely to play an important role in cancers commonly associated with activating RAS mutations.

Genetic abnormalities in the upstream components of the PI3K/AKT pathway can activate AKT. Loss of the PTEN tumour suppressor and a consequent loss of the phosphatidylinositol-3,4,5-triphosphate (PIP_3)3' Phosphatase leads to accumulation of the active PI3K (Li et al., 1997; Maehama and Dixon, 1998). This leads to increased incidence of tumours (in the heterozygous setting) as well as more aggressive disease (homozygous loss of PTEN) (Podsypanina et al., 1999; Wang et al., 2003). Somatic activating mutations of the p110α, the catalytic subunit encoded by PIK3CA (Samuels et al., 2004) occur in about 30% of epithelial cancers and results in maintenance of the PI3K/AKT activity even in the absence of growth factor signalling. Mutations in the PIK3R1 gene that

encodes p85α regulatory subunit of PI3K also results in aberrant activation of PI3K (Huang et al., 2007).

Downstream of AKT, many of its targets are affected by the aberrant activation of the upstream components of the pathway. The FOXO transcription factors mentioned previously are up-regulated in the presence of aberrant AKT signalling, resulting in tumour suppressive effects. In contrast, the eukaryotic initiation factor of translation eLF4E acts like an oncoprotein and co-operates with other proteins like c-myc to promote tumorigenesis (Ruggero et al., 2004; Sun et al., 2005). The aberrant constitutive activity of AKT in response to mutations or upstream signals also results in increased mTORC1 activation and subsequent tumour cell growth signalling, details of which have been discussed in previous sections.

The PI3K/AKT and mTOR pathway has been associated with certain hereditary cancer syndromes as well. These include various phacomatoses which are characterised by scattered hamartomatous or adenomatous lesions that encode tumour suppressor proteins which deregulate signalling pathways like the PI3K/AKT/mTOR which in turn subsequently promote abnormal cellular proliferation (Tucker et al., 2000). Germ-line mutation of the PTEN gene that leads to activation of the PI3K/AKT pathway occurs in Cowden disease, which is associated with a high risk of breast, thyroid and endometrial carcinomas (Liaw et al., 1997). Similarly, Tuberous Sclerosis Complex is a disorder associated with the germ-line mutations in the TSC1 and TSC2 genes which leads to an activation of the mTOR pathway that is independent of the AKT activation status and which gives rise to hamartomas that can progress to cancer (Zhang et al., 2003). The TSC proteins can be inactivated by other mechanisms as well. Mutations in the Tuberin phosphorylating protein AMP-activated protein kinase (AMPK kinase)

which is encoded by a gene that is inactivated in Peutz-Jeghers syndrome, leads to activation of mTOR signalling and subsequent increase in the incidence of gastro-intestinal hamartomatous polyps that are associated with a high incidence of malignant transformation (Shaw et al., 2004). The Neurofibromatosis 1 (NF1) gene encoded protein neurofibromin is known to function as a RAS-GAP. Deregulation of NF1, as is seen in neurofibromatosis is associated with increased Ras activity and consequent PI3K/AKT activation leading to increased incidence of benign as well as malignant tumours including Peripheral Nerve Sheath Tumours (Johannessen et al., 2005). Von-Hippel-Lindau syndrome is another example of a hamartomatous disease where the PI3K/AKT pathway plays a role. The VHL protein is involved in the regulation of the HIF-1α transcription factor downstream of mTOR. Loss of VHL leads to increased stability of the HIF-1α transcription factor, which in-turn results in increased transcription of its pro-tumorigenic targets (Krieg et al., 2000).

1.8- PI3K - AKT pathway inhibitors

The importance of PI3K/AKT in cancer has led to the development of a number of inhibitors targeting various components of the pathway. Many of these drugs are in various stages of clinical trials in both solid tumours and haematological malignancies. The PI3K/AKT inhibitors can be broadly divided into PI3K specific inhibitors, mTOR inhibitors, PI3K-mTOR dual inhibitors and AKT inhibitors.

PI3K inhibitors: The PI3K specific inhibitors are isoform specific or pan Class 1A PI3K inhibitors. Pan Class 1A PI3K inhibitors target the p110 catalytic subunit irrespective of the isoform and thereby inhibit the PI3K/AKT pathway. They include

BKM120 and XL147 among others and are potent inhibitors that can affect the activity of the downstream components including AKT and mTOR. Isoform specific PI3K inhibitors on the other hand specifically target the p110 isoforms and may be particularly effective in different cancers. p110α specific inhibitors like INK1117 (So et al., 2013) specifically target the alpha subunit and can effectively inhibit the PI3K/AKT pathway. p110α specific inhibition may also help to block angiogenesis (Graupera et al., 2008) and have been reported to be effective in ERBB2 amplification positive breast cancers (Torbett et al., 2008). Similarly, the p110β specific inhibitors may be effective in PTEN deficient cancers (Jia et al., 2008; Wee et al., 2008) while p110δ specific inhibitor CAL101 (GS-1101) has been recently shown to be effective for recurrent Non-Hodgkins lymphoma and Chronic Lymphocytic Leukaemia (Flinn et al., 2009a; Flinn et al., 2009b). The PI3K specific inhibitors while being very effective as inhibitors of the PI3K/AKT pathway have the advantage of decreased toxicity when compared to dual PI3K-AKT inhibitors and can potentially be used with other inhibitors like MAPK pathway inhibitors. A disadvantage of these agents is that they may not be effective in the presence of activating mutations in the downstream components of the pathway such as mutations or amplifications of AKT or activating mutations in the mTOR components. Tumours that exploit more than one component of the pathway will also not be sensitive to such treatments and in all such cases, the dual PI3K/AKT-mTOR pathway inhibitors maybe more effective as single or combination agents.

mTOR inhibitors: mTOR catalytic site inhibitors inhibit both mTORC1 and mTORC2 and consequently also inhibit AKT-S473 phosphorylation through the inhibition of mTORC2. While this may inhibit AKT feed-back loops into mTOR, studies have shown that these inhibitors do not inhibit AKT-T308 phosphorylation

as well as activation of many AKT substrates (Feldman et al., 2009; Jacinto et al., 2006). The activity of many of these agents is thus likely to be the effect on mTORC1 rather than any effect on other components of the PI3K/AKT pathway.

Dual PI3K-mTOR inhibitors: Dual PI3K-mTOR pathway inhibitors take advantage of the similarity between the p110 subunits of both PI3K and mTOR (Garcia-Echeverria and Sellers, 2008). Most of them target the α, β and δ isoforms of PTEN as well as mTORC1 and 2 (Engelman, 2009). These pan PI3K-mTOR inhibitors are effective in cancers where multiple components of the pathway are deregulated, including PIK3CA and PIK3R1 mutations as well as PTEN loss and RTK dependent activation. PI-103 (Fan et al., 2006) is one such dual pathway inhibitor which shows potent PI3K/AKT pathway inhibition as well as mTOR inhibition. Other dual inhibitors being trialled in early phase clinical trial include BEZ235, BGT226, XL765 and SF1126. Unfortunately, the increased spectrum of activity of this class of inhibitors comes at the price of increasing toxicity which may become intolerable to patients, further precluding their use in combination therapy with other agents.

PI-103 binds to the p110 catalytic domain and prevents the accumulation of PIP3 at the plasma membrane, which subsequently prevents the activation of AKT. PI-103 also inhibits the mTOR1 and 2 complexes and thereby inhibits a feed-back mechanism that can activate AKT. Thus it is an ideal agent to study the PI3K/AKT pathway inhibition *in vitro* and *in vivo*. The drug itself is not under clinical trials due to its pharmacokinetic property of rapid metabolism (Raynaud et al., 2007).

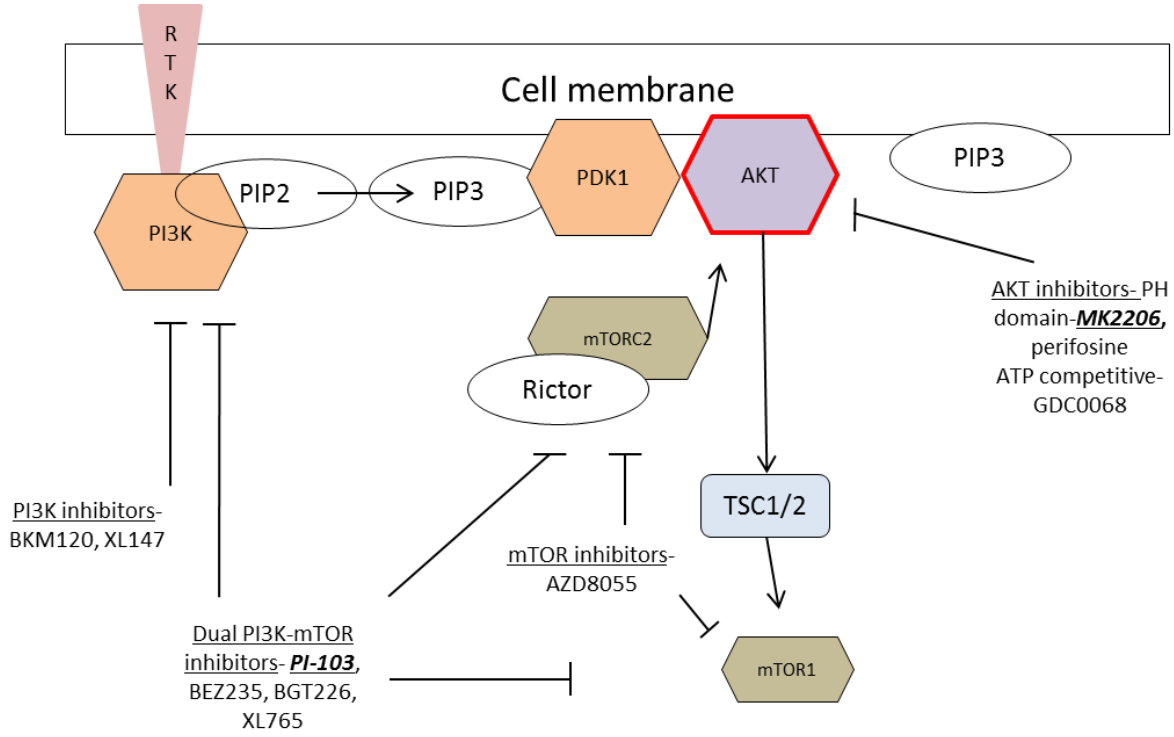


Fig. 1.8.1: PI3K/AKT pathway inhibitors

AKT inhibitors: AKT specific inhibitors are the fourth category of inhibitors that is gaining wide-spread use as potent PI3K/AKT pathway inhibitors. A number of ATP mimetics and non-catalytic site inhibitors are under development (Pal et al., 2010) and unlike the other classes of PI3K/AKT inhibitors, these agents are expected to be active in AKT amplified or mutated tumour cells as well. The allosteric AKT inhibitors block the recruitment of AKT to the membrane by preventing the interaction between the PH domain of AKT and the phosphoinositides, while the ATP mimetics block the AKT catalytic site activity. What is interesting in the context of the two different categories of the AKT inhibitors is the pharmacodynamic analyses of each. The allosteric inhibitors can be monitored by looking at the AKT phosphorylation (T308 or S473), while in contrast, the ATP mimetics do not block the AKT phosphorylation as a result of the negative feed-back activation of PI3K, and instead will have to be monitored by assessing the

phosphorylation of one of the downstream substrates (mentioned previously) like PRAS-40 or GSK3.

MK-2206 is an orally active highly potent allosteric AKT inhibitor, equally potent toward purified recombinant human Akt1 (IC_{50}, 5 nmol/L) and Akt2 enzyme (IC_{50}, 12 nmol/L) and approximately 5-fold less potent against human Akt3 (IC_{50}, 65 nmol/L) (Hirai et al., 2010; Yan, 2009; Yap et al., 2011). MK-2206 has shown promising pre-clinical and clinical results as an AKT inhibitor and a number of early phase trials are progressing with combination therapy using either targeted agents or cytotoxic drugs.

A number of AKT inhibitors are currently undergoing pre-clinical or early phase clinical trials and are providing early clinical data on the subset of patients who are most likely to benefit from them. Breast cancers with activated PI3K/AKT pathway have been found to be sensitive to the inhibitors, although surprisingly they have been seen to promote tumour growth stasis and growth delay rather than tumour shrinkage (Serra et al., 2008; She et al., 2008a). This is also exhibited by breast cancers with ERBB2 mutations, highlighting the important of PI3K/AKT signalling in such tumours. Not surprisingly many studies also reveal the absence of significant anti-tumour effects of these drugs as mono-therapy agents (Hirai et al., 2010) while instead promoting increased sensitivity in combination with other agents. All these points stresses the need for increasing our understanding of the molecular mechanisms of the cancers that can help identify the subset of patients who are most likely to benefit from the treatments.

1.9– NPM signalling in cancer

Nucleophosmin (NPM) is a phosphoprotein belonging to the nucleoplasmin family of chaperones (Frehlick et al., 2007) which includes NPM1, NPM2 and NPM3. NPM is primarily localised in the nucleolus, although a portion acts as a chaperone protein, shuttling between the nucleus and the cytoplasm in response to various import and export signals (Colombo et al., 2005b; Colombo et al., 2006). NPM has been found to interact with a number of proteins and plays an important role in many cellular functions, including response to stress, DNA repair, ribosome biogenesis and modulation of chromatin condensation/decondensation events (Colombo et al., 2011; Li and Hann, 2009). In the cell, NPM is closely associated with a number of proteins including p14/ARF (Bertwistle et al., 2004; Korgaonkar et al., 2005; Kuo et al., 2004) F-box and WD repeat domain containing 7 (Fbw7) (Bonetti et al., 2008) and SUMO1/sentrin/SMT3 specific peptidases 3 and 5 (SENP3-5) (Yun et al., 2008) amongst others. NPM interaction with p14/ARF inhibits its functional activity (Colombo et al., 2006). With a decrease in the levels of active p14/ARF, there is an increase in free active MDM2, which subsequently targets p53 for degradation. NPM mutations that shuttle p14/ARF to the cytoplasm, thereby promoting MDM2 dependent p53 degradation, have been identified in haematological malignancies(Colombo et al., 2011).

The NPM protein: NPM exists in two splice forms designated B23.1 and B23.2, with B23.1 found in the nucleolus, nucleoplasm and cytoplasm and the shorter B23.2 mainly restricted to the nucleoplasm and cytoplasm (Chang and Olson, 1990; Wang et al., 1993). The two variants also have different expression levels in tissues, with B23.1 the predominant variant in most tissues. The NPM B23.1 variant (hereafter referred to as NPM in the context of this manuscript) has a

modular structure containing distinct sequence motifs. Various functional domains have been identified and starting from the N-terminal end, the protein contains a hydrophobic oligomerisation domain (fig. 1.9.1) followed by an acidic histone binding region and a C-terminal nucleic acid binding region that is comprised of a basic region followed by an aromatic stretch which contains tryptophan residues at 288 and 290 which is essential for nucleolar localisation (Nishimura et al., 2002). In addition the protein also contains two nuclear export signal (NES) regions between amino acid residues (aa) 42-49 and 94-102 and two nuclear localisation signal (NLS) regions between aa152-157 and 190-197 (Hingorani et al., 2000; Wang et al., 2005b).

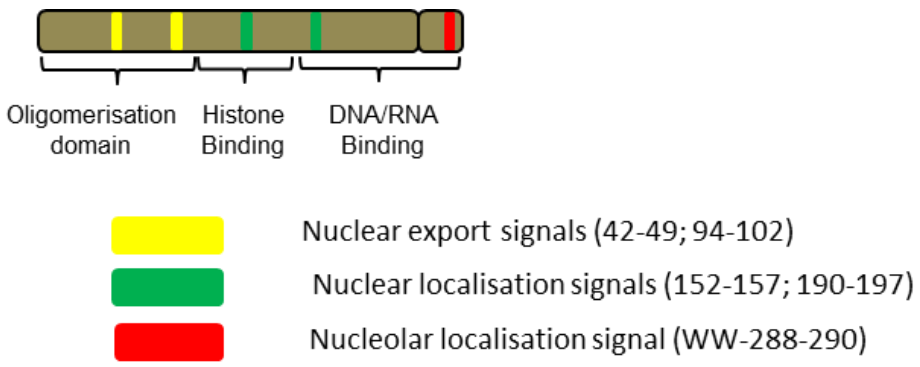


Fig. 1.9.1: NPM1 protein structure showing the sub-cellular localisation signal regions

The sub-cellular localisation (Liu et al., 2007) of NPM has been reported to be dependent on the tertiary structure of NPM. The NPM protein is predominantly found as an oligomer in cells. The monomeric protein is folded to form an 8 stranded β pleated structure that coalesce to form NPM pentamers (fig.1.9.2) (Dutta et al., 2001; Enomoto et al., 2006; Lee et al., 2007).

Fig. 1.9.2: Structure of NPM pentameric ring formation

Two NPM core pentamers associate head to head to form NPM decamers. The formation of oligomers results in the exposure of the C-terminal nucleolar localisation signal (NLS) residues and promotes trafficking of the oligomers to the nucleolus. Interestingly the ARF binding site on the NPM decamer is located on the molecular surface and is not involved in the inter-subunit interaction (Lee et al., 2007). This promotes increased ARF binding to the oligomer and subsequent translocation to the nucleolus. This regulation is of particular relevance to this study and will be discussed in detail later.

Functions of NPM: Through the functional domains, NPM interacts with many partner proteins and this produces the characteristic functions associated with the protein. They include nucleolar factors like nucleolin and fibrillarin, transcription factors like interferon regulatory factor and NFκB, histones, proteins associated with cell proliferation/mitosis and arguably in the context of cancers, one of their most important function of response to oncogenic stress via ARF and p53 (Grisendi et al., 2006). Because of this diversity of interactions, NPM has been

described to be both oncogenic and tumour suppressive. The NPM protein has been shown to be overexpressed in a number of cancers including gastric, colon, ovarian and prostate, while advanced stage bladder cancers have been associated with increased NPM mRNA levels (Grisendi et al., 2006; Nozawa et al., 1996; Shields et al., 1997; Subong et al., 1999; Tanaka et al., 1992; Tsui et al., 2004). Unlike in solid tumours, NPM is also a target of genetic alterations in haematological cancers, where they have been associated with chromosomal translocation leading to activated proteins as well as NPM mutations characteristically seen in AML. In the former case, NPM has been seen to form oncogenic fusion proteins together with loss of a functional allele of the gene. These include NPM-RARα (Retinoic Acid Receptor α) (Redner et al., 1996), NPM-ALK (Anaplastic Lymphoma Kinase) (Morris et al., 1994) and NPM-MLF1 (Myeloid Leukaemia Factor 1) (Yoneda-Kato et al., 1996). NPM mutations resulting in the formation of cytoplasmic NPM variants (NPMc) (Falini et al., 2005) have been characterised in Acute Myeloid Leukaemia (AML) and is also seen to be associated with other mutations conferring increased tumorigenic potential for the malignant cells. Some of the most important roles of NPM in cancer cells have been its association with cell growth and inhibition of apoptosis. Not surprisingly, the expression of the NPM protein in cancer cells is associated with the aberrant mitogenic signalling characteristic of transformed cells (Dergunova et al., 2002). NPM is a transcriptional target of oncogenic MYC (Zeller et al., 2001) and conversely has been associated with decreased levels in cells undergoing apoptosis (Wu et al., 1999). The over-expression of NPM in cells also makes them resistant to drugs (Hsu and Yung, 2000), while absence of the protein is associated with decreased proliferation and increased apoptosis (Grisendi et al.,

2005). The characteristic structural features and chaperone properties of NPM mentioned above also helps the protein in its other major function in the cell- that of ribosomal processing and assembly (Olson et al., 1986). The chaperone property of NPM plays a role in transport of maturing pre-ribosomal particles and down-regulation of NPM has been shown to impair the processing of pre-rRNA (Itahana et al., 2003). In cancer cells, this regulatory role may be utilised by aberrant NPM expression, which promotes increased ribosomal activity (Ruggero and Pandolfi, 2003). This is characteristically seen in MYC over-expressing cells, which show increased protein synthesis.

NPM expression is also associated with inhibition of the cell death mechanisms where it has been shown to interfere with the apoptotic activity of the Interferon Regulatory Factor (IRF1) (Kondo et al., 1997) as well as inhibition of the apoptotic response to hypoxia in a HIF1α and p53 dependent manner (Li et al., 2004). Various other anti-apoptotic mechanisms mediated by NPM have been described including the effect on the RNA- dependent protein kinase PKR, as well as the interaction with PIP3 that inhibits the DNA fragmentation activity of the caspase activated DNase (DNA fragmentation factor 40KDa) (Ahn et al., 2005). NPM also exhibits anti-apoptotic activity through the functional inhibition of p53 where its interaction with the S15 phosphorylation site of p53 and blocks p21 induction, which is also characteristically seen in the presence of hypoxia (Li et al., 2005). Interestingly, $NPM^{-/-}$ mouse embryos show high levels of apoptosis (Grisendi et al., 2005) which was seen to be p53 dependent (Colombo et al., 2005a). On the other hand, increased expression of NPM as is characteristically seen in normal cells exposed to different stress stimuli including DNA damage is associated with translocation of the NPM from the nucleolus to the nucleoplasm and the cytoplasm

and a subsequent delay in cell cycle that allows repair of the DNA damage (Chan, 1992).

NPM in the context of ARF and p53: One of the most important functions of NPM that has been described in the context of haematological malignancies and in the context of maintenance of genomic stability has been its role in regulation of ARF. As mentioned earlier, ARF is vital for the MDM2 mediated regulation of p53. NPM associates with ARF to form high molecular weight complexes which are localised within the nucleolus and stabilises ARF turnover (Kuo et al., 2004). This stabilisation has been characteristically found to be both proteasome dependent and independent. $NPM^{-/-}$, $p53^{-/-}$ fibroblast cells have been shown to have increased turnover of ARF, and interestingly these cells are also associated with increased proliferation and potential for transformation (Colombo et al., 2005a; Grisendi et al., 2005). Thus NPM has been shown to have an important role in the regulation of ARF stability and localisation which in turn can regulate cell proliferation through both p53 dependent and p53 independent mechanisms. ARF can promote p53 mediated cell cycle arrest and apoptosis in response to cell stress signals by way of its regulatory effect on MDM2 (Sherr, 1998). It can also antagonise cell proliferation by its inhibitory effect on ribosome biogenesis, where it inhibits processing of 47S, 45S and 32S precursors (Sugimoto et al., 2003). This data points out that ARF has a dual effect on controlling cell proliferation, which could be regulated by its association with NPM. The above hypothesis is further supported by the fact that NPM has a role in ribosomal processing and this in turn may facilitate the ribosome specific ARF activity (Bertwistle et al., 2004; Okuwaki et al., 2002). The interaction of ARF with NPM has also been reported to impede its trafficking from the nucleus to the cytoplasm (Brady et al., 2004b) while the

oncogenic stress induced increase in ARF and its subsequent association with NPM may lead to increased ARF interaction with the ribosomal machinery and a decrease in rRNA processing. Both the ARF-NPM interactions are expected to promote proliferation inhibitory effects in the cell. The ARF association with NPM and localisation to the nucleus also has another very important regulatory effect. The localisation of ARF to the nucleolus by NPM sequesters it away from the nucleoplasmic pool and this in turn reduces the ARF mediated inhibitory effect on MDM2, subsequently destabilising the p53 protein levels (Korgaonkar et al., 2005). It has also been reported that MDM2 competes with NPM for ARF binding (Brady et al., 2004a), raising the hypothesis that following cellular stress, NPM/ARF complexes traffics from the nucleolus to the nucleoplasm (and cytoplasm), where MDM2 competes with NPM for ARF binding and in turn gets deactivated, promoting p53 stability. The dynamics of the NPM-ARF interaction and regulation of p53 is further complicated by the reports of NPM-MDM2 interaction, where following cell stress, NPM has been shown to bind to MDM2 and stabilise p53 (Kurki et al., 2004). Taken together, an interesting question raised by many studies is whether ARF which is bound to NPM can still interact with and regulate the activity of MDM2 (Grisendi et al., 2006).

The regulation of p53 is controlled by a number of factors that have been discussed previously. The direct interactions with p53 by NPM, as well as the indirect interactions with the regulatory components, are expected to play an important role in the NPM mediated p53 regulation. The points discussed previously highlights the role that NPM plays in stabilizing p53 and promoting DNA repair, but is at odds with other data showing stabilisation of p53 in $NPM^{-/-}$ cells (Colombo et al., 2005b; Grisendi et al., 2005). Furthermore, NPM has been shown

to induce cellular senescence in a p53 dependent manner, leading to the suspicion of direct NPM interaction and stabilisation of p53 (Colombo et al., 2002). Thus the NPM mediated regulation of p53 and the tumour suppressive role of NPM is a highly complex arena which quite possibly depends on the cell type and cell context. An examination of NPM mediated maintenance of genomic stability further shows that NPM has been implicated in the control of chromosomal ploidy and DNA repair. Following DNA damage, the up-regulation of NPM protein levels is associated with increased DNA repair (Wu et al., 2002) and it is also implicated with regulating the ATM and ATR activity in DNA damage repair (Colombo et al., 2005a; Velimezi et al., 2013).

Post-translational modifications play a major role in the cell cycle regulating and genomic stability maintenance functions of NPM. Phosphorylations are the most common of post translational modifications and many kinases have been reported to phosphorylate NPM. These include Cdk2-cyclinE complex which phosphorylates T199 of NPM and plays a role in regulating cell cycle. T 199 NPM is also found to accumulate at sites of DNA damage. Several other kinases have been reported to phosphorylate NPM including S4 by Polo-like Kinase 1 (PLK1) and NimA-like protein Kinase (Nek2A). Casein Kinase 2 phosphorylates S125 as does ATR which modulates NPM chaperone activity and DNA repair in the cells (Colombo et al., 2011; Grisendi et al., 2006). Other post translational modifications seen in NPM include acetylation (p300 mediated acetylation and SIRT1 mediated de-acetylation), sumoylation which controls NPM interaction with the retinoblastoma protein, ubiquitination by the BRCA1-BARD1 ubiquitin ligase which leads to NPM localisation to centrosomes (Sato et al., 2004) and poly-(ADP-ribosyl)-ation (Colombo et al., 2011; Kraus, 2008).

NPM and Acute Myeloid Leukaemia- Acute Myeloid Leukaemia (AML) is a haematological malignancy mainly affecting the elderly and correspond to about 6.3% of all cases of leukemias in the United Kingdom (https://www.hmrn.org/statistics/incidence). Malignant cells carrying NPM mutations are seen in about 35% of adult AML cases and have been thought to be a founder mutation that is highly stable throughout the course of the disease (Falini et al., 2008b; Falini et al., 2005; Falini et al., 2007; Falini et al., 2009; Schnittger et al., 2009). These mutations are closely associated with normal cytogenetics and are mostly restricted to the AML-M3 and AML-M4 sub-types. They are associated with favourable prognosis in the absence of FMS-Like Tyrosine Kinase-Internal Tandem Duplication (FLT3-ITD) mutations. A number of mutations in NPM have been identified, but the most prevalent is the Type A mutation of exon 12 of the NPM gene that results in duplication of a TCTG tetranucleotide at position 956 to 959 of the NPM reference sequence (Falini et al., 2007). This results in a loss of either 290 or both 290 and 288 tryptophan residues that causes the helical structure of NPM C-terminal domain to unfold (Grummitt et al., 2008) and the generation of a new nuclear export sequence (NES) motif. This reinforces the Exportin 1 or Chromosome Region Maintenance 1 protein homolog (Crm1) - dependent nuclear export of NPM that is characteristic of the AML associated NPM mutant (NPMc). The NPMc forms heterodimers with wild-type NPM and the strong nuclear export signals on the NPMc results in the translocation of the heterodimers to the cytoplasm (Falini et al., 2006). This is associated with a concomitant translocation of the NPM chaperoned proteins including ARF, as well as a disruption of the normal nucleolar structure. Inhibitors of the Crm1 transporter like Leptomycin B reverts the NPMc back into the nucleus,

confirming the role that nuclear export plays in the sub-cellular localization of the mutant protein (Falini et al., 2006). FLT3-ITDs are secondary activating mutations commonly seen in AML and are associated with poor prognosis in patients when present along with NPMc, where they have been strongly associated with normal karyotype (Dohner et al., 2005). These RTK mutations are associated with activation of the downstream MAPK pathway (Dosil et al., 1993), the PI3K/AKT pathway (Gilliland and Griffin, 2002; Takahashi, 2011) and the STAT5 pathway (Mizuki et al., 2003). The molecular significance of the co-existence of the two mutations has not yet been clearly established and continues to be a focus of interest among many groups. Interestingly, it was reported recently that FLT3-ITD and NPMc mutations co-operate to induce acute leukemia in a mouse model (Mallardo et al., 2013). While the exact molecular mechanism is unknown, this further raises the stakes in identifying the relationship between the two mutations. Identification of the molecular mechanisms that play a role in the NPMc activity as well as the mechanistic reasons behind the interaction of FLT3 and NPMc will thus go a long way in the development of treatment strategies for the subset of patients with the mutations.

Aims

The aims of this DPhil project was to test whether AKT mediated phosphorylation of NPM was responsible for regulating oligomerisation and sub-cellular localisation of NPM, p14/ARF nucleolar retention and $p53^{mut}$ stability in human tumours. In addition we wanted to derive mechanistically relevant biomarkers and test whether reversal of this activity upon PI3K-AKT pathway inhibition could be exploited for therapeutic gain in the setting of $p53^{mut}$ tumours. We further wanted to examine if the same regulatory mechanism played a role in AML cells carrying NPM mutations and whether we could target this mechanism for therapeutic benefit.

Chapter 2

Material and Methods

Chapter 2 – Materials and Methods

2.1 – Buffer compositions

PBS – 137 mM Sodium chloride, 2.7 mM potassium chloride, 10.1 mM sodium phosphate, 1.8 mM potassium phosphate, adjusted to pH 7.4 with HCl

Cryogenic storage media – 90 % FBS, 10 % DMSO

Crystal violet staining solution - 0.5 % (w/v) crystal violet; 10 % methanol; 20 % ethanol, in dH_2O

Laemmli lysis buffer – 50 mM Tris pH 6.8, 2% (w/v) SDS, 10 % (v/v) glycerol

NP40 lysis buffer – 150 mM sodium chloride (NaCl), 1% (v/v) Nonident 40 (NP-40), 1 mM sodium orthovanadate (Na_3VO_4), 50 mM NaF, 5 mM sodium pyrophosphate ($Na_4P_2O_7$).

Laemmli loading buffer - 5% (w/v) SDS, 0.16 M Tris HCl pH 6.8, 12.5 % (v/v) β-mercaptoethanol, 30 % (v/v) glycerol, trace bromophenol blue

SDS running buffer - 3.03 g Tris, 14.4 g glycine and 1 g SDS in 1L dH_2O

Stripping Buffer - 62.5 mM Tris pH 6.8; 2 % w/v SDS; 0.7 % β-Mercaptoethanol

Western blot transfer buffer – 20 % (v/v) methanol, 0.25 M Tris, 1.92 M glycine

Antigen retrieval buffer **–** 10 mM Tri-sodium citrate, 0.05 % Tween pH 6.0

2.2 – Tissue Culture and cell freezing / thawing

HT-1080-SG1 and SG2 tumour cell lines were kindly provided by Eric Stanbridge (Stanford, CA), AKT knockout MEF's were kindly provided by Dr. Birnbaum (University of Pennsylvania PA). *NPM*$^{-/-}$, *p53*$^{-/-}$ and *p53* $^{-/-}$ MEFs were kindly provided by Pier Paolo Pandolfi (Harvard, Boston MA). PSN1 cells were kindly provided by Thomas Brunner (University of Oxford) and subsequently genotyped

at the DDC laboratories, London, to confirm cell identity. The mutation data reflects the information on the COSMIC Cell Line Project database http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/. OCI-AML3 cell-lines were obtained from Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). All other cell lines were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM containing 4.5 g/l glucose (Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 mg/ml). OCI-AML3 cells were cultured in MEM-Alpha (Invitrogen) supplemented with 20% heat inactivated fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 mg/ml). All cultures were maintained at 37°C in water saturated 5 % CO_2 with the exception of MRC5 cells which were cultured at 37°C in 3 % O_2/5 % CO_2.

KPC mice derived cell lines (work undertaken at the Owen Sansom lab, Beatson Institute, Glasgow)**:** *Pdx1-cre*, *KrasG12D*, *Trp53flox*, *Trp53R172H* and *ARF-/-* mice have been described previously (Ding et al., 2005; Gamper and Roeder, 2008; Kamijo et al., 1997; Olive et al., 2004; Wang et al., 2005a) . Mice were kept in conventional animal facilities and experiments were carried out in compliance with UK Home Office guidelines. Genotyping was performed by Transnetyx (Cordova, TN, USA). Animals were monitored until showing symptoms of late stage pancreatic cancer and then sacrificed as per institutional guidelines. Tumour and metastatic burden was assessed by gross pathology and histology.

Tumour tissue for preparation of PDAC cell lines was harvested in DMEM. Tumours were disaggregated by fine mincing with scalpels, and plated in growth media (Dulbecco's modified Eagle medium containing 10% fetal bovine serum and

2mmol/L L-glutamine). Cells were allowed to adhere, washed, grown to confluence and then passaged as normal.

2.3 – Drug treatments

The following pharmaceutical agents were used in the experiments:

PI-103-Merck

CAS 301305-73-7- Sellekchem

CC1-779- Sigma Aldrich

Cycloheximide (CHX)- Sigma Aldrich

LY2228820- Sellekchem

MG132- Sigma Aldrich

MK2206.2HCl and MK2206.HCl-ChemieTek and Sellekchem

NSC348884- Sigma Aldrich

Nutlin 3a- Sigma Aldrich

2.4 – siRNA and plasmid expression system transfection

75000 cells were plated in 10cm dishes and when they were approximately 50-70% confluent, the cells were transfected with siRNAs the day after they have been plated in antibiotic-free medium (DMEM supplemented with 10% FBS and 1% glutamine). Different tranfection reagents were used according to the cell-line

studied. A) DharmaFect 1 (Thermo Scientific). The siRNA:DharmaFect ratio was indicated by the Thermo Scientific protocol for RNA interference (12.5µl siRNA: 10µl DharmaFect). B) Lipofectamine 2000. The siRNA: Lipofectamine 2000 was used at the ratio 12.5µl siRNA: 10µl Lipofectamine 2000, as advised by the manufacturer. OPTI-MEM serum-reduced medium (Invitrogen) was used for the dilution of the transfection reagent and the siRNAs. 6h after the transfection, the anti-biotic free media was changed into complete media (containing P/S). The cells were harvested/further treated 48h after transfection and the knockdowns were assessed by western blotting.

OCI-AML3 cells were transfected by nucleofection using Cell-line Kit T (Amaxa Biosystems) and nucleofector program X-001 according to manufacurer's instructions. In short 1x 10^6 cell suspension in MEF Nucleofector Solution 1 was mixed with 50nM SiRNA and transferred to a manufacturer supplied cuvette before placing in the Nucleofector and running programme X-001 as instructed by the manufacturer. The cells were also transfected with Lipofectamine 3000 (Life technologies) at the concentrations advised by the manufacturer. OPTI-MEM serum-reduced medium (Invitrogen) was used for the dilution of the transfection reagent and the siRNAs. The antibiotic free media was not changed and the transfections assessed 48 hours later by Immunofluorescence after cyto-spinning the cells onto glass slides.

2.5- Retroviral Transfection and molecular biology:

6 x 10^6 293 T cells were plated on 10 cm dishes the day before transfection. The cells were transfected the next day according to standard calcium phosphate

transfection protocols (10µg of retroviral genome plasmid, 10µg packaging plasmid, 100µl 2.5M $CaCl_2$, sterile water, Hanks Balanced Salt Solution (HBSS) buffer). Media was changed the next day. 200 x 10^3 MEFs were plated on 10 cm dishes, in order to have actively dividing cells at time of transfection. The next day retro-virus containing media from the 293T cells was collected, filtered, diluted (1:2-1:3) with growth media and polybrene (hexadimethrine bromide) added (8µg/ml). The media on the recipient cells were replaced with the virus containing media. This was repeated 3 to 4 times. Selection agent (Puromycin) was added to the recipient cells the next day, after replacing with fresh growth media.

The following plasmids were purchased from Addgene(Hamilton et al., 2014): pBABE puro-myr-FLAGAKT1 (Addgene plasmid 15294), (Boehm et al., 2007), pBABE PuroL myr-HA-AKT2 (Addgene plasmid 9018), pBABE-puro-K-Ras V12 (Addgene plasmid 9052) and pcDNA3 MDM2 S166D S186D (Addgene plasmid 16236). The image clone (IMAGE 6411700, accession number BC054755) encoding mouse Npm was purchased from Source Bioscience. Human NPM was PCR amplified according to standard protocols using the primers sense-aatgaattcatggaagattcgatggacatggacatgagc and antisense-aatctcgagaagagacttcctccactgccagagatcttg and cloned into the C-terminal FLAG tagging vector PCMV 4 (Aligent), between the EcorI and XhoI restriction enzyme sites. Human NPM was PCR amplified using the primers NPM_pbabe_FWD aataatggatccatggaagattcgatggacatgg and NPM_pbabe_REV aataatgaattcttaaagagacttcctccactgcc and cloned into the retroviral vector pBABE Puro between the BamH and EcoI restriction sites. Primers used for mutation of Ser48 to Ala; Hu_NPM_S48A_sense gttatctttaagaacggtcgctttaggggctggtgcaaag & Hu_NPM_S48A_antisense ctttgcaccagcccctaaagcgaccgttcttaaagataac. Primers

used for the mutation of Ser48 to Glu Hu_NPM_S48E_sense ccagttatctttaagaacggtcgagttaggggctggtgcaaaggatg and Hu_NPM_S48E_antisense catcctttgcaccagcccctaactcgaccgttcttaaagataactgg. Primers used for mutation of siRNA (ACAAGAAUCCUUCAAGAAA) recognition site sense- catcaacaccaagatcaaaaggacaagagagctttaagaaacaggaaaaaactcctaaaacac & antisense gtgttttaggagtttttttcctgtttcttaaagctctcttgtccttttgatcttggtgttgatg. Mouse Npm was amplified by PCR used the primers Mus_Npm_Fwd aataatggatccatggaagactcgatggatatgg & Mus_Npm_Rev aataatgaattcttaaagagatttcctccactgcc and cloned into the pBABE Puro vector between the BamHI and EcorI restriction sites. Primers used for mutagenesis of Ser48 to Ala MusNpm_S48A_sense cagttgtcattaagaacggtcgcgttaggagcaggggcaaaagat & MusNpm_S48A_antisense atcttttgcccctgctcctaacgcgaccgttcttaatgacaactg. Primers used for the mutagenesis of Ser48 to Glu MusNpm_S48E_sense ccagttgtcattaagaacggtcgagttaggagcaggggcaaaagatg and MusNpm_S48E_antisense catcttttgcccctgctcctaactcgaccgttcttaatgacaactgg. Site directed mutagenesis was achieved using the Quikchange II kit (Agilent) according to the manufacturer's instructions. Human NPMc was cloned between XHOI and HIND III sites of pBabe puro vector. Primers used for NPMc include NPMc_pbabe_FWD aataatggatccATGGAAGATTCGATGGACATGG & NPMc_pbabe_Rev aataatgaattcctattttcttaaagagacttcctccactgccagacagagatcttgaatagcctcttg. Primers used for RFP and ZSGreen NPM mutant variants include RFP-NPMwt - NPM_ red_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPM_ red_REV

aataataagcttTTAAAGAGACTTCCTCCACTGCC; RFP-S48A -NPM- NPM_ red_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPM_ red_REV aataataagcttTTAAAGAGACTTCCTCCACTGCC; RFP-S48E-NPM NPM_ red_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPM_ red_REV aataataagcttTTAAAGAGACTTCCTCCACTGCC; ZSG-NPMcWT NPMcyto_ green_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPMcyto_ green_REV aataataagcttctattttcTTAAAGAGACTTCCTCC; ZSG-S48A-NPMc NPMcyto_ green_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPMcyto_ green_REV aataataagcttctattttcTTAAAGAGACTTCCTCC; ZSG-S48E-NPMc NPMcyto_ green_FWD aataatctcgagctATGGAAGATTCGATGGACATGG & NPMcyto_ green_REV aataataagcttctattttcTTAAAGAGACTTCCTCC

2.6 – Cell Irradiation

Cells were irradiated at the indicated doses while in mid log growth phase using a Mark 1 cesium irradiator (J.L. Shepherd, San Fernando, CA) at a dose rate of 1.7 Gy/min. Non-irradiated control plates were placed on a cold metal plate identical to those found in the irradiator as a control for heat shock effects on AKT phosphorylation.

2.7 – 2D Clonogenic assay

In all clonogenic survival experiments, (200-400) cells were plated from single cell suspensions and allowed to adhere to culture dishes prior to irradiation and / or inhibitor exposure. Inhibitor treatment was initiated 1 hour prior

to irradiation and maintained for 24 hours. After the treatment interval, the medium was replaced with drug-free medium. Control cultures also underwent medium replacement at the same time to control for this manipulation. Cells were irradiated with a Mark 1 cesium irradiator (J.L. Shepherd) at a dose rate of 1.7 Gy/min. Colonies were stained with crystal violet solution and counted 10 to 15 days after irradiation. The surviving fraction was derived using the formula:

(# Colonies / # of cells plated) $_{\text{irradiated}}$ ***/*** (# Colonies / # of cells plated) $_{\text{unirradiated}}$.

Each point on the survival curve represents the mean surviving fraction from at least three dishes. Clonogenic survival curves are representative of independent replicate experiments.

2.8 – Cell lysis

Whole cell lysates were prepared by lysing cells with 1% NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 % (v/v) NP-40, 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate and 'Complete' proteinase inhibitor cocktail EDTA free (1 tablet/10 ml lysis buffer (Roche)). Lysates were rotated end over end for 30 min at 4ºC and centrifuged (20,817 x g, 10 min) before the addition of NuPage sample buffer or SDS-PAGE sample buffer (1x concentration, 62.5 mM Tris-HCl pH 6.8, 25 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol blue).

For immunoprecipitation, lysates were pre-cleared (4 ºC, 1 Hr) with protein-G coupled to magnetic beads (Millipore), prior to incubation with antibody conjugated to protein-G magnetic beads. Lysates and antibody coupled beads were rotated

end over end at 4°C for at least 3 Hrs. Immunoprecipitates were washed (4 x 1ml) with lysis buffer minus the protease and phosphatase inhibitors. Immunoprecipitated proteins were boiled in SDS-PAGE sample buffer for western blot analysis.

Nuclear lysates were prepared as described previously (Schreiber et al., 1989) with additional modifications. Briefly, 3-5 x 10^6 cells were trypsinised and harvested by centrifugation (500 x g, 5 min) , washed twice in TBS and re-suspended in 1-2 ml ice cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) by gently pipetting in a 1 ml tip. The cells were left on ice for 15 min to swell, after which 75 µl of 10 % NP-40 was added. The tube was vigorously vortexed for 10 sec and centrifuged at 500 x g for 2 min. The supernatant, which constitutes the cytoplasmic fraction, was removed. The nuclear pellet was re-suspended in 150 µl ice-cold lysis buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 0.5 mM EDTA, 0.5% (v/v) NP-40, 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate and 'Complete' proteinase inhibitor cocktail EDTA free (1 tablet/10 ml lysis buffer) and the sample sonicated. The nuclear extract was centrifuged (20,817 x g, 15 min, 4^0C) and the supernatant containing the nuclear extract was used as an in-put for immunoprecipitation or added to SDS-PAGE sample buffer as described above.

2.9 – Protein concentration determination

Protein concentrations of cell lysates were measured by Bradford assay using Coomassie Blue (Sigma- Coomassie protein assay reagent) according to established protocols. In short, protein standards were prepared using BSA (0,

250, 500, 1000, 1500 and 2000 µg/mL BSA). 5µL of the each standard and 5µL of a 1:50 dilution cell lysate was mixed with 250 µL of the assay reagent in a 96 well plate and the absorbance read on a plate reader (PolarSTAR Omega). The absorbance was read at 595nm (analysed on MARS Data Analysis Software-BMG Labtech) and a graph of absorbance plotted for the protein and standards. To determine the protein concentration of a sample from it absorbance, the standard curve was used to find the concentration of standard that would have the same absorbance as the sample. Protein concentrations were also measured using a Nanodrop 1000 Spectrophotometer. The values obtained were used to normalise the protein concentrations prior to loading on gels for Western blot analysis.

2.10 – Sodium Dodecyl Sulphate –Poly-Acrylamide Gel production

Poly acrylamide gels were cast using the Bio-Rad mini-PROTEAN tetra electrophoresis system (Bio-Rad Hercules, CA). 1.5 mm glass spacer plates were aligned with glass cover plates and clamped to provide a water tight seal. Separating/resolving gel solution was then prepared as detailed in table 2.11.1.

Table 2.10.1. Reagent volumes for poly acrylamide gels.

Reagent	**12% Resolving gel**	**10% Resolving gel**	**4 % stacking Gel**
d H_2O	3.3 mL	4 mL	3.6 mL
30 % Acrylamide : Bis-Acrylamide 37.5:1	4 mL	3.3mL	650 µL
1.5 M Tris pH 8.8	2.5 mL	2.5 mL	-
1.0 M Tris pH 6.8	-	-	600 µL
10 % (w/v) SDS	100 µL	100 µL	50 µL
TEMED	10 µL	10 µL	5.0 µL
10 % (w/v) Ammonium Persulphate (APS)	100 µL	100 µL	50µL

A 50mL stock solution of the base components (all components excluding TEMED and 10 % APS) of the gels in table 2.11.1 was stored at +4 °C. When required, a suitable volume of the stock solution was decanted and the catalyst and cross linking reagents TEMED and 10 % APS added. Resolving gel was poured between the glass plates to a point approximately 6 mm below the edge of the cover glass and was then overlaid with 1.5 mL, 20 % ethanol to ensure a level interface between stacking and resolving gels, and allowed to solidify (approximately 5 minutes). The ethanol was then poured off and the gel surface washed briefly with dH_2O. 4% stacking gel was then be made up as detailed above (table 2.10.1) and poured on top of the resolving gel. A well former is then inserted into the acrylamide solution and allowed to solidify (approximately 5

minutes). Gels were then used in downstream applications such as SDS-PAGE and western blotting detailed below.

2.11 – Sodium Dodecyl Sulphate –Poly-Acrylamide Gel Electrophoresis

Cells were washed twice with ice cold PBS and lysed by scrapping in ice cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 % (v/v) NP-40, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na_3V0_4, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate and 'Complete' proteinase inhibitor cocktail EDTA Free (1 tablet / 10ml lysis buffer). Lysates were rotated end over end (4ºC, 30 min), centrifuged (21000 x g. 15 min), diluted in the appropriate volume of sample buffer (Laemli buffer- 60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 minutes. The samples were then loaded onto the SDS-PAGE gels and run at a constant voltage of 120 V at room temperature.

2.12 – Semi-Native Poly-Acrylamide Gel Electrophoresis (Semi-Native-PAGE)

Cells were washed twice with ice cold PBS and lysed by scrapping in ice cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 % (v/v) NP-40, 1 mM EGTA, 50 mM NaF, 1 mM Na_3V0_4, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate and 'Complete' proteinase inhibitor cocktail EDTA Free (1 tablet / 10ml lysis buffer). Lysates were rotated end over end (4ºC, 30 min), centrifuged

(21000 x g. 15 min), diluted in the appropriate volume of NuPage sample buffer (samples were not boiled) and immediately loaded onto 10 % Bis-Tris NuPAGE gels or Native NuPAGE gels. Gels were run at a constant voltage (100 V) at 4ºC before transfer to Nitrocellulose membrane (transfer buffer maintained at 4^0C).

The isoelectric point is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI (isoelectric point) and negatively charged at pH values above their pI. Standard Native-PAGE requires most proteins to have an acidic or slightly basic pI (~3–8) in order to be able to migrate from the positive to the negative pole. NPM has a pI previously identified as 5.1 (Yun et al., 2003) and was run on standard Native gel systems used in the experiments.

2.13 – Western blotting

Immunoprecipitates and cell lysates were resolved on 4-12% Bis-Tris Nu-PAGE gradient gels (Invitrogen) or on SDS-PAGE gels and transferred onto PVDF (polyvinylidene fluoride) (Millipore) or Nitro-cellulose (Millipore) membranes. Membranes were blocked with either 5 % non-fat milk in PBS 0.1% Tween 20, 5% (w/v) Bovine Serum Albumin or ready to use blocking buffer for the Licor system. The primary antibody was incubated overnight at 4^0C. The membranes were washed four times with PBS 0.1% Tween 20 before incubation with HRP-conjugated or Fluorescent (Licor) secondary antibody for 2 hours. Following this, the membranes were further washed at least six times before the application of standard enhanced chemi-luminescence substrate (Pierce) or high sensitivity substrate (Millipore) and exposed to X-ray film (Kodak) or developed in the Licor

system. The membranes developed with the Licor system were maintained in the dark at all times from the time they were incubated with the secondary antibody.

Membranes were stripped using 2% w/v SDS, 62.5 mM Tris pH 6.8 stripping buffer along with 2-Mercaptoethanol (0.7% of the final solution). Following incubation for 30min at 50^0C, the membranes were washed three times in PBS 0.1% Tween 20 and blocked with the specific blocking buffer before proceeding as above.

2.14 – Immuno-fluorescent staining of tissue sections and cells

Frozen tissue sections: The slides were fixed in acetone or 4% Paraformaldehyde for 10 minutes at room temperature. They were then washed in PBS and non-specific binding blocked with 3% Normal Bovine Serum (NBS) + 0.1% Triton X100 for 20 minutes. Primary antibody at a concentration of 1:100 was added to the sections on the slides and incubated overnight at 4^0C. The slides were washed with PBS. Secondary fluorescent labelled antibody at a concentration of 1/500 was subsequently added to the slides and incubated for 1 hour. Finally the slides were washed and viewed under the fluorescence microscope.

In vitro- adherent cells: Cells were grown on coverslips, fixed in 0.2% Paraformaldehyde and the above steps repeated.

In vitro- suspension cells: Suspension cells were harvested, washed in TBS and cells re-suspended in Cytospin buffer (10mM Tris pH 7.5, 10mM NaCl, 5mM $MgCl_2$). Glass slides were mounted on Cytofunnels (Thermo Scientific) and 2 x 10^5 cells per condition added to each well of the funnel. The Cytospin funnels

were then spun on the Thermo Scientific Cytospin 4 centrifuge at 4000 rpm for 4 minutes, the slides retrieved and air-dried before fixing and processing as mentioned above.

2.15 – Immunoprecipitation and co-Immunoprecipitation

Co-immunoprecipitation from whole cell lysates: Cells were harvested and washed with PBS. They were lysed in 0.5% or 1% (v/v) NP40 lysis buffer (20 mM Hepes pH 7.5, 0.5% NP40, 150 mM NaCl, 0.5 M EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 0.5 mM Na_3VO_4 and 1 x EDTA free protease inhibitors (Roche)). Lysates were cleared by centrifugation at 21000g for 15 mins. Lysates were pre-cleared (4 ºC, 1 Hr) with protein-G coupled to magnetic beads (Millipore), prior to incubation with antibody conjugated to protein-G magnetic beads. Lysates and antibody coupled beads were rotated end over end at 4°C for at least 3 Hrs. Immunoprecipitates were washed (4 x 1ml) with lysis buffer (20mM Hepes, 1% NP40, 150mM NaCl, 0.5M EDTA) minus the protease and phosphatase inhibitors. Immunoprecipitated proteins were boiled in SDS-PAGE sample buffer for western blot analysis. IP incubations lasted for 3 hours at 4^0C, rotating at 40 rpm.

Co-immunoprecipitation from nuclear extracts: Nuclear lysates were prepared as described previously (Schreiber et al., 1989) with additional modifications. Briefly, 3-5 x 10^6 cells were trypsinised and harvested by centrifuging at 500g for 5min. The pellet was re-suspended in TBS (Tris Buffered Saline) and centrifuged at 500g for 5 minutes. The cells were again pelleted by centrifugation and the pellet re-suspended in 1-2 ml cold buffer A (10mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1mM DTT; 0.5mM PMSF) by gently pipetting in a 1ml tip. The cells were left on ice for 15 minutes to swell, after which 25ul of 10% Nonident

NP-40 (Fluka) was added. The tube was then vigorously vortexed for 10 seconds and centrifuged at 500g for 2 min. The supernatant, which constitutes the cytoplasmic fraction, was removed. The nuclear pellet was re-suspended in 150µl ice-cold lysis buffer (150 mM NaCl; 20mM Hepes pH 7.5; 0.5mM EDTA; 0.5% NP40; protease and phosphatase inhibitors) and the sample sonicated. The nuclear extract was centrifuged at 21000g for 15min at 4^0C and the supernatant was used to perform the IP as described above.

2.16 - Quantitative Real Time Polymerase Chain Reaction (qRT PCR)

PSN1 and T24 cell monolayers were treated with MK-2206 or DMSO control before harvesting. Samples were prepared for quantitative RT-PCR using Power SYBR® Green Cells-to-CT™ Kit (Life Technologies), according to the manufactures protocol. The Real-Time PCR Cycling Conditions were as follows: Holding Stage, 95^0C for 10min (x1), Cycling Stage: Step 1- 95^0C for 15 sec and Step 2- 60^0C for 1 min (x 50), Melt Curve Stage (continuous): Step 1- 95^0C for 15 sec, Step 2- 60^0C for 1 min, Step 3- 95^0C for 30 sec and Step 4- 60^0C for 15sec. 18S was used as an internal control to normalize all data. The following primers were used: p53 FW: ACGCTTCCCTGGATTGGCAGC R: GAGGGGGCTCGACGCTAGGA, p14ARF FW: CTACTGAGGAGCCAGCGTCTA R: CTGCCCATCATCATGACCT and 18S FW: AGTCCCTGCCCTTTGTACACA R: GATCCGAGGGCCTCACTAAAC. The experiments were carried out in triplicate for each data point.

2.17 - Polymerase Chain Reaction (PCR)

PCR was used to amplify the coding sequence of p14ARF in the PSN1 cells. Primers were designed using PRIMER-BLAST design software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and PCR reactions set up in 200µl PCR tube using Qiagen HotStarTaq polymerase and buffers.

Table 2.17.1 – Optimised PCR reaction for amplification of p14ARF

10x PCR buffer	5 µl
dNTPs (10 mM)	1.5 µl
MgSO4 (50 mM)	0.5 µl (optimised)
Forward primer (5 µM)	0.75 µl
Reverse primer (5 µM)	0.75 µl
Template (200 ng/µl)	1 µl
10x enhancer	5 µl
dH_2O	35 µl
Platinum *pfx* polymerase	1 µl

Table 2.17.2. Thermal cycles for PCR amplification

Segment	**Cycles**	**Temperature**	**Time**
1	1	95 °C	900 seconds
2	35	94 °C	60 seconds
		56 °C	60 seconds
		72 °C	60 seconds
3	1	72°C	600 seconds

Table 2.17.3 – Primer sequences for amplification

Primer	Sequence
1-p14ARF_F	GCACTTGCCCTTCCAGGTAT
1-p14ARF_R	CAACATGTCTGGGCCTCTGT
2-p14ARF_F	ACGTGGCTTTAAGGTCTGGG
2-p14ARF_R	CCAGTTCCAAGCTGGAGAGG

2.18- IHC staining and analysis of tumour xenografts

Immuno- Peroxidase staining on frozen tissue, Formalin Fixed Paraffin Embedded tissue and Tissue Micro Arrays:

Standard immunohistochemistry protocols were followed for all IHC studies. All formalin fixed paraffin embedded tissues were de-paraffinised in Xylene followed by gradient ethanol washes. Antigen retrieval was done by boiling in 10mM citrate buffer (pH 6.8). Endogenous peroxidise was blocked with 3% Hydrogen Peroxide (H2O2) prior to all immuno-peroxidase staining protocols. Non-specific binding of secondary antibody was blocked using 3% Normal Serum of the animal of origin of the corresponding secondary antibody. The tissue sections were then incubated with the required primary antibody overnight at 4^0C. They were then washed with PBS thrice and incubated with the biotinylated secondary antibody for 30 minutes, followed by washing and incubation with Avidin Biotin Complex (ABC). The final

staining of the Peroxidase conjugated antibodies and the Avidin Biotin Complex (ABC) were done by using the chromogen 3,3'- Diaminobenzidine (DAB).

2.19 – Xcelligence cell proliferation assay

KPC mice derived cells were plated at a density of 1.5 x 10^4 cells/ml in an E-Plate 16 (ACEA Biosciences,Roche) according to manufacturer's instructions. The growth characteristics were measured using an xCELLigence RTCA DP (ACEA Biosciences,Roche) analyser which recorded the growth in terms of cell index, which is a dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status.

Cell Index $_i$= $(R_{tn}-R_{t0})/F_i$ where i= 1,2,3 F_1=15Ω, F_2=12Ω, F_3=10Ω and n=0,1,2,…N(time points).

2.20 – Resazurin proliferation assay

5 x 10^3 cells per well was plated in 24 well plates in multiples of 6 wells per condition. 24 hours later, media was changed and 1ml of fresh media containing Resazurin at 10µg/ml was added per well and left to incubate for 2 hours. The remaining steps were done away from direct light. 2 hours after incubation, 200µl of the media from each well was withdrawn and added to a 96 well plate. Plates are covered in foil to avoid exposure to direct light and were read on a plate reader with the parameters: Excitation wavelenght-544, Emission wave-length-590-10 and Gain-1000.

2.21 – 3D clonogenic assay

3D colony assay of the KPC mouse derived cells was adapted from a previously described protocol for 3D culture of mouse pancreatic cells (Enge et al., 2009). Cells were re-suspended at a density of 2.5×10^3 cells/0.5 mL in methylcellulose-based colony culture medium. In short, 1 mL of the culture mixture contained DMEM, 1% (wt/vol) methylcellulose (Sigma), 5% (vol/vol) Matrigel (BD Bioscience), 50% (vol/vol) conditioned media from KPC mouse cells in culture, 5% (vol/vol) FCS, 10 mmol/L nicotinamide (Sigma), 10 ng/mL human recombinant activin-βB (R & D Systems), 0.1 nmol/L exendin-4 (Sigma), and 1 ng/mL vascular endothelial growth factor–A. The cells were treated with MK2206 (1μM, 24hrs) prior to irradiation with a Mark 1 cesium irradiator (J.L. Shepherd) at a dose rate of 1.7 Gy/min for a total of 6 Gy. Colonies were counted after 15 days using a Nikon Eclipse TE2000-E microscope.

2.22 – In vivo PSN-1 xenografts

All animal procedures were performed in accordance with current UK legislation under an approved project license. Female athymic nude mice (BALB/c nude) (Harlan) were divided into groups receiving injections subcutaneously into the flank with 1×10^6 PSN-1 HRE luc human PDC cells with 4×10^6 LTC-14 (stellate cells). Animals were assigned randomly into different groups, to receive carrier, 60 mg/kg, and 120 mg/kg of MK-2206 s/c on three alternate days and 320 mg/kg of MK-2206 s/c once, in the first experiment. Animals were assigned randomly into different groups in the second experiment to receive either carrier or 60 mg/kg of MK-2206 s/c on two alternate days followed by a single dose of 6 Gy

radiation under anaesthesia on day 4. Tumour growth was measured regularly by callipers. Tumour hypoxia was measured on Day 1 and Day 4 under anaesthesia in the IVIS imaging system.

2.23- Extraction of plasma derived circulating cell free tumour DNA (cfDNA)

DNA extraction from plasma was performed using a Qiagen Circulating Nucleic Acid extraction kit, according to manufacturer's protocol. Extracted DNA was quantitated using PicoGreen assay. Standard DNA stock (100ug/ml) is prepared by diluting to appropriate concentration. Reagents are added in serial dilution in following volumes to produce the standard curve,

Row	DNA Concen. (ng/ul)	Volume DNA standard (ul)	Volume 1xTE buffer (ul)
A	0	0	100
B	0.0025	1.25	98.75
C	0.005	2.5	97.5
D	0.01	5	95
E	0.025	12.25	87.75
F	0.05	25	75
G	0.1	50	50
H	0.2	100	0

2ul of each DNA sample in duplicate is added to 98ul 1xTE buffer to make up 100ul of 1:50 dilution. The samples are loaded in adjacent columns of a 96 well plate and a 1:200 concentration PicoGreen (Invitrogen) solution added to each well before being read on an Optima Plate reader (Excitation 485nm and Emission 538nm).

2.24- Digital PCR for Mutation analysis of ctDNA

Digital Polymerase Chain Reaction (DPCR) is a refinement of conventional Polymerase Chain reaction, where a single sample is partitioned into multiple reactions which help very sensitive analysis of samples that have rare alleles and very low copies of the gene. The Quantstudio 3D is a Thermo Scientific/Life Technologies manufactured DPCR system that uses sealed chips to partition each sample into 20,000 individual reactions in individual microscopic wells. Taqman assays are used to probe the samples which are then read on the Quantstudio system and quantified into absolute gene copy numbers. This technique can be used to multiplex up to two separately tagged (FAM and VIC) probes and is sensitive for identification of specific gene mutations using mutation specific probes.

cfDNA was extracted from 1ml of plasma as described in the previous section and the reaction set up as follows according to manufacturer's directions:

Material	Volume (µL)	Stock	Final
Digital PCR Master Mix 2X	17.4	2X	1X
Taqman Assay(20X)	1.7	20X	1X
Diluted DNA	3.5	10ng/µL	1ng/µL
Water	12.2	-	-

Table 2.24.1: Optimised DPCR reaction for identification of HBB, KRAS-G12V mutants and KRAS-G12D mutants (for a total of 34.8µL for 2 chips/sample)

Stage	**Temperature**	**Time**	**Cycles**
Hot Start	96°C	10 min	1
Anneal/Extend	56°C	2 min	39 cycles
Denature	98°C	30 sec	
Final extension	60°C	2 min	1
Hold	10°C	Infinity	1

Table 2.24.2: Thermal cycles for DPCR amplification of rare mutant alleles

2.25- Fluorescence Activated Cell Sorting (FACS) - cell cycle analysis

2 x 10^5 cells were plated into 60mm tissue culture dishes and incubated overnight to allow cells to reach mid log phase growth prior to inhibitor treatment and irradiation. Cells were treated with inhibitors prior to irradiation with x-rays using an RS-225 x-ray cabinet (Gulmay Medical Ltd, Surrey U.K.) at a dose rate of 2.77 Gy

/ min. Cells were trypsinised at the specified time points post irradiation using 0.05 % Trypsin / EDTA (GIBCO-BRL). The resultant cell suspensions were centrifuged at 1100 rpm for 5 minutes at 4°C, washed once with PBS and slowly re-suspended in ice cold 70 % ethanol, to avoid cell clumping. Cell suspensions were subsequently centrifuged at 2000 rpm for 10 minutes and washed once with PBS prior to re-suspension in 200 µg/mL RNase A diluted in PBS containing 50 µg/ml Propidium Iodide. Cells were incubated for 30 minutes at room temperature. Samples were protected from light prior to analysis, which was done within 2 hours of staining. Cytometry and analysis was accomplished using a CyAn FACS with Modfit LT analysis software.

2.26 - Statistical analysis

A two-tailed Student's *t* test was used as indicated in figure legends to indicate the probability of variation and p values of < 0.05(*), <0.01(**) and <0.001 (***) were considered significant. Measurements of $p14^{ARF}$ nucleolar intensity were taken from normally distributed populations. However, owing to the variability of the sample sizes between groups the t-test used accounts for the unequal variance. Calculations were performed using the following unpaired t-test equation.

$$t = \frac{\overline{X}_1 - \overline{X}_2}{s_{\overline{X}_1 - \overline{X}_2}}$$

Where the denominator allows for the unequal variance to be calculated as follows;

$$s_{\overline{X}_1 - \overline{X}_2} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}.$$

$\bar{x}_1$ = Mean of group1; $\bar{x}_2$ = Mean of group 2

n_1 = population size of group 1; n_2 = population size of group 2

The value for variance (s^2) can be calculated as below, and is equal to the standard deviation squared;

$$s^2 = \frac{1}{n}\sum_{i=1}^{N}(x_i - \overline{x})^2.$$

Values obtained for t were then referred to a t-table to obtain the p-values quoted using infinity as the value for degrees of freedom.

Error bars were plotted as ± SEM. SEM was calculated using the following formula;

$$SEM = \frac{SD}{\sqrt{n}}$$

Where *n* = sample size (number of cells) and SD = standard deviation derived using the following formula;

$$SD = \sqrt{\frac{1}{n}\sum_{i=1}^{N}(x_i - \overline{x})^2}.$$

Mantel-Cox log rank was employed to determine the significance of variation in survival of mice harboring xenograft tumours (time to sacrifice).

To study clinical data, the same was downloaded from cBioPortal for Cancer Genomics and analysed with SPSS 21.0 software. The Shapiro-Wilk test was used to assess distribution of datasets, whereby the null hypothesis of normal distribution was rejected for all datasets tested ($p<0.05$). Correlation analysis was carried out using the non-parametric Spearman-Rho test.

2.27- Ethics

All animal experiments were conducted in accordance with current UK legislation under an approved project license.

All anonymised healthy volunteer samples were obtained with informed consent, following Institutional guidelines and approved by the UK Health Department Ethics Research Service. ARC 2 trial material was retrospectively stored anonymised samples and covered under the trial ethics guidelines.

Chapter 3

AKT mediated regulation of Nucleophosmin and ARF

Chapter 3 – AKT mediated regulation of Nucleophosmin and ARF

3.1 – Introduction

Studies in our lab as well as work from previous authors (Prevo et al., 2008) had shown that tumour cells that were carrying p53mut could be sensitised to radiation following inhibition of the PI3K/AKT pathway. In order to identify the link between the two pathways, we initially looked at AKT interacting proteins and identified NPM by mass spectrometry of AKT immunoprecipitates. This was also further verified by western blots, where NPM was found to interact with active AKT. While the interaction between AKT and NPM has been previously documented (Lee et al., 2008), the precise role for this interaction has not been elucidated. Furthermore, NPM is a key regulator of p14ARF activity and could explain the relationship between AKT and mutant p53.

Previous work done in our lab had shown that NPM could be detected in immuno-precipitates of a pan-AKT substrate antibody from T24 cell lysates (fig. 9.1.A). Further in-silico analysis identified the RXXS as a non-consensus AKT substrate motif at Serine 48 (S48) of the N-terminal of NPM. The RXXS motif had been previously reported to be a bona-fide AKT substrate recognition motif (Maddika et al., 2008) and we further confirmed the phosphorylation at Ser48 by kinase assays. To further study the importance of the AKT phosphorylation site on NPM, an antibody was raised against a synthetic peptide spanning residues 45-56 of NPM (RTVSLGAGAKDE) incorporating phospho-Serine at position 48 (pS48-NPM), which could detect the levels of phospho-Ser48-NPM (fig. 9.1.B).

Following on from the initial data that we had and that was available in the public domain, this study aimed to identify the mechanisms that were underlying the

PI3K/AKT-NPM-mutp53 interaction and to utilize this information as a possible therapeutic target (Hamilton et al., 2014).

3.2 – Validation of the anti-Phospho-S48 NPM antibody

In order to study the AKT phosphorylation site on NPM, an anti pS48-NPM antibody was raised against a synthetic peptide spanning residues 45-56 of NPM (RTVSLGAGAKDE) incorporating phospho Serine (underlined- fig. 3.2.A) at position 48. Peptide synthesis and immunizations were carried out by Eurogentec. The anti pS48-NPM antibody from terminal bleeds of immunised rabbits, was affinity purified against the phospho-peptide. In order to confirm the recognition of the AKT phosphorylation site on NPM, lysates from T24 cells treated with PI-103 and HT1080 isogenic cell lines (SG1- $NRAS^{wt}$;$NRAS^{Q61K+ve}$ and SG2- $NRAS^{wt}$; loss of $NRAS^{Q61K+ve}$) were probed with the antibody against pS48-NPM and the western blot analysis showed a decrease in the levels of pS48-NPM that reflected the decrease in NPM-AKT interaction seen in the AKT immuno-precipitates (fig.3.2.B). In order to confirm that the antibody was recognizing the valid phospho-protein and not a decrease in the total NPM levels, we treated T24 cell lysates with the Mn^{2+}-dependent protein phosphatase 'λ-phosphatase' which has activity towards phosphorylated serine, threonine and tyrosine residues and saw that the antibody specifically recognized the phospho-protein and not the total protein (fig. 3.2.C). In order to further validate the antibody, we stained breast and squamous cell cancer tissue microarray (TMA) sections which had been blocked with the phospho-peptide corresponding to the recognition site of the anti-body. As

expected, the unblocked sections showed a clear staining for pS48-NPM, whereas the peptide-blocked sections did not show any staining (fig.3.2.D).

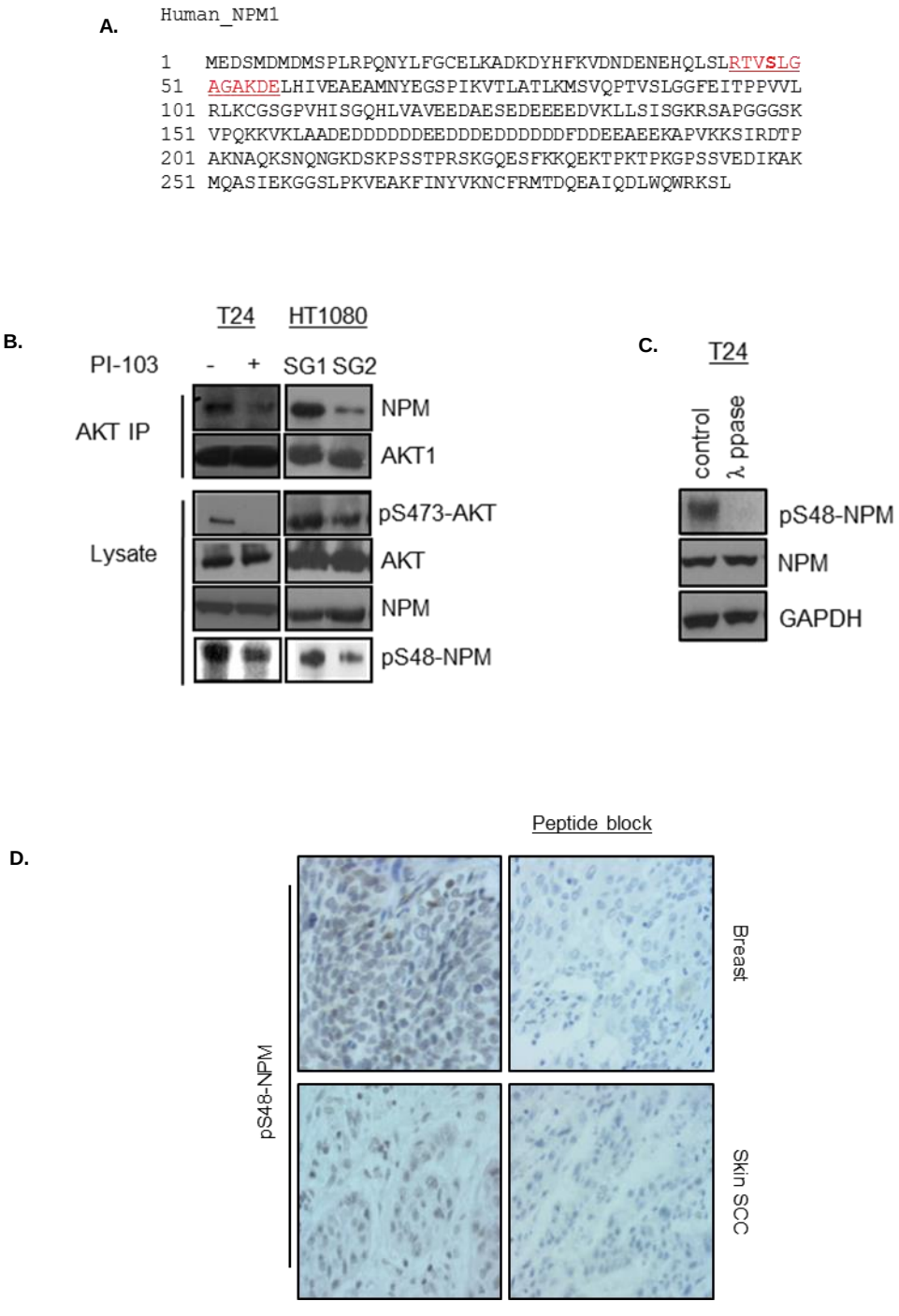


Fig. 3.2: Validation of the anti-phospho-S48 NPM antibody- A) panel showing the peptide coverage of the human NPM including S48 (highlighted in red) B) T24 cells treated with PI-103 (0.4 μM) or (Right) the isogenic cell lines HT1080-SG1 ($NRAS^{wt}$;$NRAS^{Q61K+ve}$) and SG2 ($NRAS^{wt}$; loss of $NRAS^{Q61K+ve}$) (Plattner et al., 1996). Immunoprecipitates and lysates were blotted with the indicated antibodies

(Figure courtesy of Dr. Garth Hamilton). C) Detection of pS48-NPM by an antibody raised against a synthetic peptide spanning residues 45-56 of NPM (RTVSLGAGAKDE) and incorporating phospho Serine at position 48. The pS48-NPM signal was lost on treatment of the lysate with **λ** Phosphatase, without affecting the levels of total NPM D) TMA's (US Biomaxx) were pre-incubated in the presence or absence of the immunogenic peptide used to raise the phospho-Ser48 NPM antibody before staining with anti-phospho-Ser48 NPM. Representative western blot images from experimental replicates of n=3

3.3 – AKT phosphorylates NPM at S48 and regulates its tertiary structure

The above mentioned data had confirmed the AKT mediated phosphorylation of NPM at S48. In order to understand the impact of this phosphorylation on NPM, we analysed the crystal structure of the protein. We found that the S48 lies within the conserved AKDE loop of NPM which significantly contributes to the interactions at the NPM pentamer : pentamer interface (Lee et al., 2007). The phosphorylation of S48 was incompatible with incorporation into the pentameric ring due to steric clashes at the monomer-monomer interface (fig. 3.3.A and fig. 3.3.B). Furthermore, recent mathematical modeling predicted that S48 should be important for controlling the NPM monomer: oligomer equilibrium (Mitrea and Kriwacki, 2012). To address whether the phosphorylation at S48 would regulate NPM oligomerisation, *Npm*$^{-/-}$; *p53*$^{-/-}$ MEF were reconstituted with NPM-WT, a non-phosphorylatable NPM-S48A or a phosphomimetic derivative NPM-S48E and lysates from the reconstituted MEFs were resolved under native conditions. In agreement with previous studies (Enomoto et al., 2006), NPM was detected as both a monomer and an oligomer (fig. 3.3.C and fig. 3.3.D). The non-phosphorylatable NPM-S48A on the other hand appeared more oligomeric, while the phosphomimetic mutant, NPM-S48E, although less stable, was exclusively

monomeric, as has previously been reported for NPM mutants that cannot oligomerize (Enomoto et al., 2006). Interestingly, we also observed that under mild denaturing conditions NPM-WT monomers could be seen, which became more evident under increasing denaturing conditions while the NPM-S48A oligomers were more resistant to denaturing conditions (fig. 3.3.E) and maintained most of their oligomeric fraction.

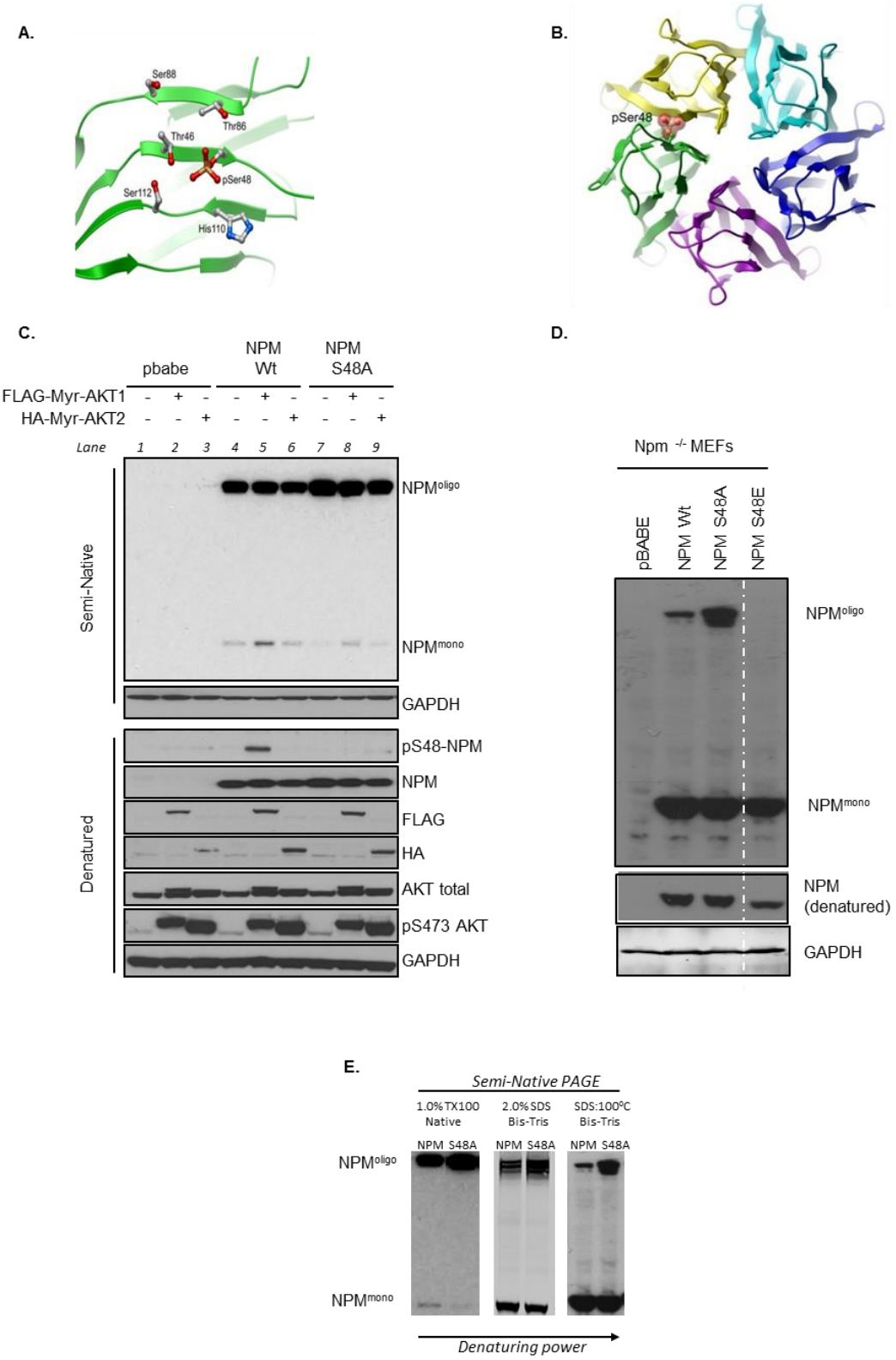


Fig: 3.3: AKT regulates NPM tertiary structure. A) Model of the NPM monomer surface indicating the position of S48 B) Diagram of the NPM pentameric ring (top view) showing a space filling model of phosphorylation at S48, highlighting a steric clash with the neighbouring subunit C) *Npm-/-, p53-/-* double null MEFs infected with pBabe retrovirus expressing FLAG-tagged-myristoylated (myr)-AKT1 or HA-tagged-myr-AKT2 in combination with NPM-WT or NPM-S48A as indicated. NPM

oligomerisation status was determined by semi native gel electrophoresis and denatured lysates, which were probed with the indicated antibodies. D) *Npm*$^{-/-}$*; p53*$^{-/-}$ MEFs infected with retrovirus expressing control pBABE vector, pBABE-NPM, pBABE NPM-S48A or pBABE NPM-S48E. Lysates were separated under semi native gel electrophoresis or denaturing conditions (Bottom) and probed with the indicated antibodies E) Lysates from cells as in (C) were processed under different progressively stringent denaturing conditions and separated by gel electrophoresis and probed for Nucleophosmin. Representative western blot images from experimental replicates of n=5

We next wanted to determine if AKT was regulating the oligomerisation of NPM. The reconstituted MEFs described above were co-infected with constitutively active Myristoylated-AKT1 (Myr-AKT1) and Myristoylated-AKT2 (Myr-AKT2) (fig. 3.3.C). Myr-AKT has a 14 amino acid Myristoylation group attached to it, that targets the AKT to the cell membrane leading to constitutive phosphorylation and activation (Kohn et al., 1996). In the presence of Myr-AKT1 there was an increase in the monomeric fraction of NPM corresponding to the increase in the S48 phosphorylation (lane 5, fig.3.3.C) which is not seen in the presence of the non-phosphorylatable NPM variant. We thus confirmed that AKT mediated phosphorylation of S48 played a role in the regulation of the NPM tertiary structure.

3.4 - AKT phosphorylation regulates NPM tertiary structure in tumour cells

Having confirmed that AKT mediated phosphorylation regulates NPM tertiary structure in the genetically modified MEFs, we next wanted to see if the same was the case in cancer cells. We used T24 bladder cancer cells carrying a p53 mutation (Y126*) as also an activating mutation in the HRAS gene (G12V)

which results in activation of the PI3K/AKT pathway (http://www.lgcstandards-atcc.org/~/media/0A1DDC31011D45F2BCF969CE5FE50B66.ashx). The cells were transfected with siRNA against AKT1 and the lysates probed by western blot. As seen in the MEFs, T24 cells show monomeric and oligomeric fractions of nucleophosmin. Knock-down of AKT1 was associated with a significant increase in the oligomeric fraction of NPM and a corresponding decrease in its monomeric fraction. This change corresponded to the decrease in the pS48-NPM levels, confirming that AKT played a role in the regulation of NPM oligomerisation in cancer cells in a S48 dependent manner.

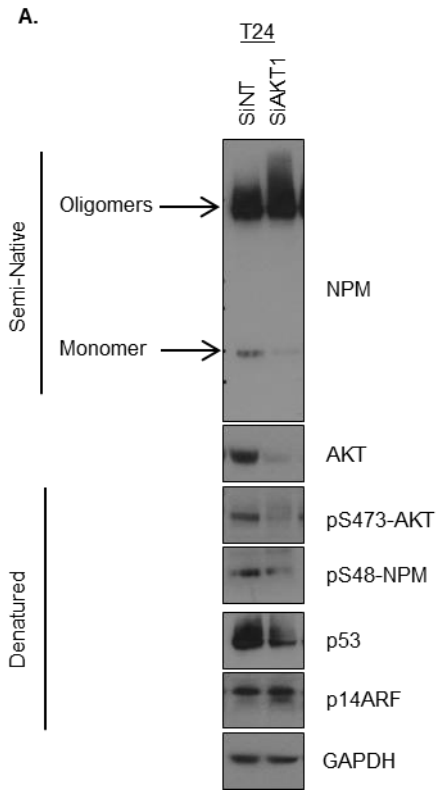


Fig. 3.4.A: AKT phosphorylation regulates NPM tertiary structure in tumour cells A) T24 cells were transfected with siRNA against AKT1 and lysates separated under semi native gel electrophoresis or denaturing conditions and probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

3.5 – MK2206, an AKT inhibitor, can modulate NPM tertiary structure

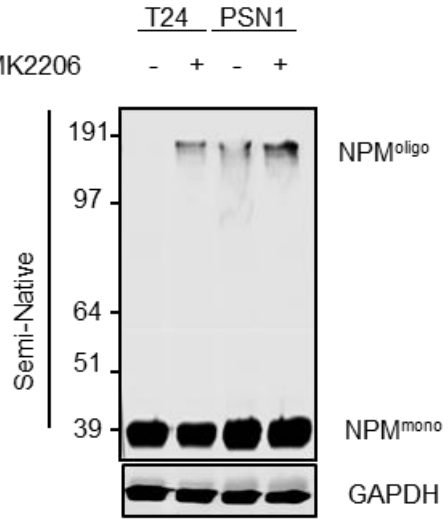


Fig. 3.5.A: MK2206 modulates NPM tertiary structure A) T24 and PSN1 cells treated with MK-2206 (5 μM, 24 hrs) and semi-native PAGE of whole cell lysates probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

MK2206 is a PH domain specific AKT inhibitor that is undergoing clinical trials. We decided to use MK2206 due to its high specificity to AKT without affecting other members of the PI3K/AKT pathway including mTOR. We treated T24 and PSN1 ($KRAS^{V12}$ mutant and point mutation at 132 of p53) cancer cell lines with MK2206. Treatment of the cells with the drug resulted in a characteristic increase in the oligomeric fraction of Nucleophosmin which recapitulated the effects seen previously with the MEFs as well as the siRNA mediated knock-down of AKT (fig.3.4), indicating that AKT specific inhibitors are effective in our system. Interestingly the decrease in the monomeric fraction of the NPM appears mild compared to the significant increase in the oligomeric fraction possibly reflecting the dynamic nature of the NPM turnover in cells.

3.6 – RAS functions through AKT to regulate NPM tertiary structure

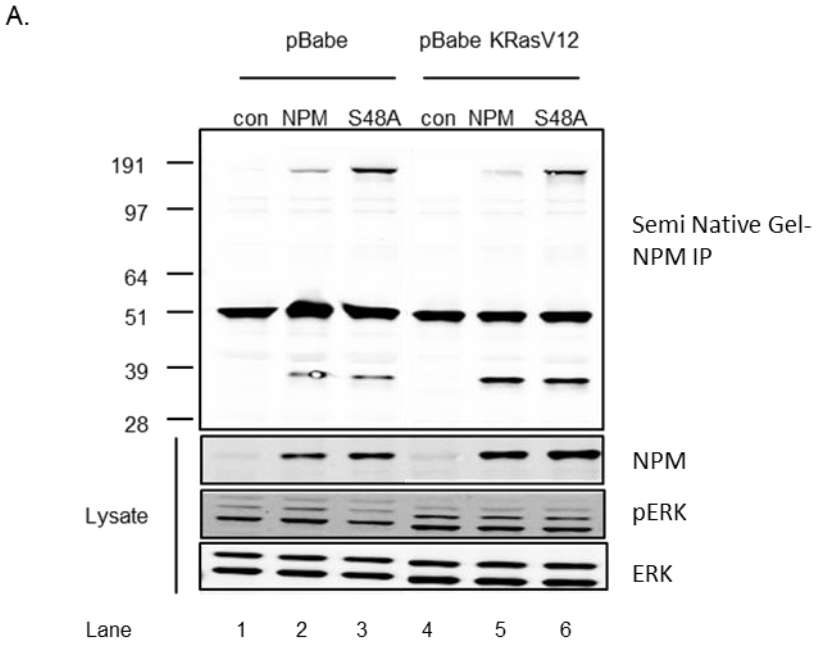


Fig. 3.6.A: Ras regulates NPM tertiary structure: A) *Npm*$^{-/-}$; *p53*$^{-/-}$ MEF co-infected with KRAS^{V12} and NPM-WT or NPM-S48A as indicated. NPM oligomerisation status was determined by semi native gel electrophoresis and denatured lysates, which were probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

The PI3K/AKT pathway is a well-studied effector of the RAS pathway and activating mutations of RAS can increase signalling via the PI3K/AKT pathway. This is important in the context of cancer cells due to the frequency of these mutations. We were thus interested in identifying if RAS mutations would have an effect on NPM oligomerisation. In order to assess this, *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs reconstituted with NPM-WT and NPM-S48A were co-infected with the constitutively active KRAS^{V12} mutant variant. As seen previously with the constitutively active AKT expression, we could observe an increase in the monomeric fraction of the NPM (Lane 5, Fig. 3.6) even with minimal increase in RAS activity based on the increased ERK phosphorylation. The above data

indicated that upstream activating mutations like RAS mutations which activates AKT, can regulate the oligomerisation of NPM.

3.7 - Phosphorylation of S48-NPM regulates NPM and ARF localisation in MEFs

Previous reports have shown that NPM isolated from the nucleolus is predominantly oligomeric in nature (Chan and Chan, 1995). Since phosphorylation of S48-NPM influences NPM oligomerisation we next wished to address if S48 was regulating NPM localization as well. The S48 lies in a characterised NES which is required for the sub-cellular localisation of NPM (Bolli et al., 2009) and we hypothesised that the phosphorylation may play a role in nucleolar localisation. In order to address this, we used the *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs reconstituted with NPM-WT and NPM-S48A and co-infected with myr-AKT1 and stained for NPM and pS48-NPM.

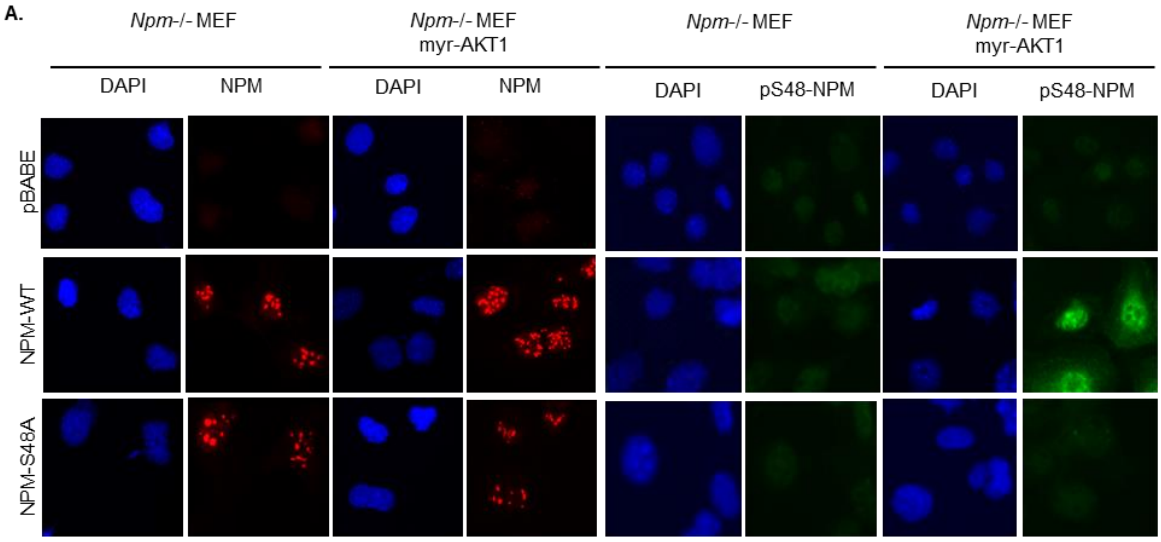


Fig. 3.7.A: *Npm*$^{-/-}$; *p53*$^{-/-}$ MEF reconstituted with NPM-WT & NPM-S48A and co-infected with myr-AKT1. Cells stained for NPM and pS48-NPM. Representative Immunofluorescence images from experimental replicates of n=3

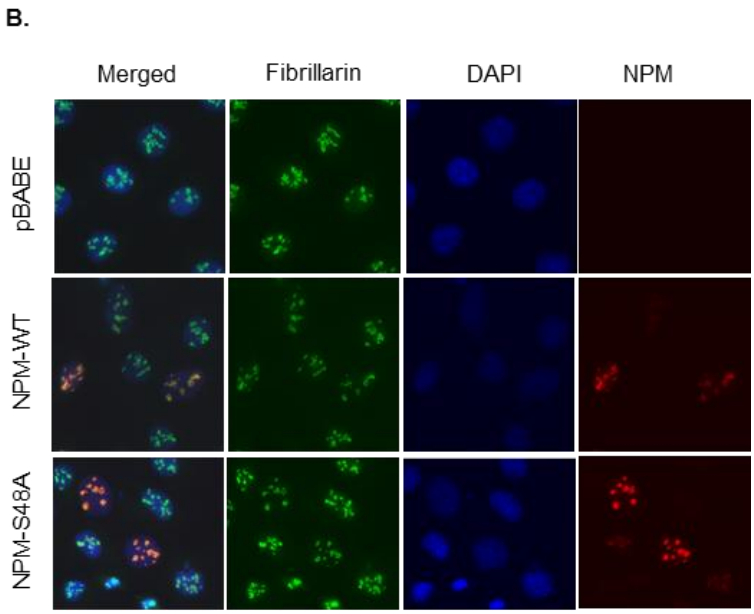


Fig. 3.7.B: *Npm*$^{-/-}$; *p53*$^{-/-}$ MEF reconstituted with NPM-WT and NPM-S48A. Cells stained for NPM and the nucleolar marker Fibrillarin. DNA stained with DAPI. Representative Immunofluorescence images from experimental replicates of n=3

The NPM-WT and the NPM-S48A expressing MEFs show localisation of NPM to specific nuclear foci in the absence of constitutively active AKT1 (fig. 3.7.A) which was confirmed to be nucleolar by Fibrillarin staining (fig. 3.7.B). On the other hand, the presence of myr-AKT1 in the NPM-WT MEFs resulted in diffuse NPM staining and highly fragmented NPM foci which were characteristically different from the NPM-S48A MEFs that predominantly maintain their NPM foci. NPM has been known to form stable oligomers and localise with the fibrillar proteins of the nucleolus, but additional pools of NPM have also been known to cycle through the various cellular compartments (Grisendi et al., 2006). Furthermore, in contrast to the total NPM staining, the pS48-NPM is not localised to the nucleoli and is instead distributed throughout the nucleus and cytoplasm in the NPM-WT MEFs, in agreement with previous reports that non-oligomeric NPM is deficient in nucleolar targeting (Bolli et al., 2009). This would suggest that S48-NPM and

importantly the AKT mediated phosphorylation at S48-NPM, does play a role in regulating the oligomerisation and localisation of NPM in cells.

The regulation of S48-NPM phosphorylation and subsequently the NPM localisation raises the question of whether this mechanism may also govern NPM interacting proteins. As mentioned previously, NPM plays an important role in sequestering ARF into the nucleolus and thereby its activity in the cell especially in the regulation of p53 stability (Enomoto et al., 2006; Gjerset, 2006; Gjerset and Bandyopadhyay, 2006; Itahana et al., 2003; Korgaonkar et al., 2005; Kuo et al., 2004). Previous reports have also shown that restricting NPM oligomerisation by site directed mutagenesis can restrict ARF transport to the nucleolus (Enomoto et al., 2006; Itahana et al., 2003). This raised the interesting prospect that since phosphorylation of S48-NPM can regulate NPM oligomerisation, it could in turn affect ARF localisation in the cells. To test this, we used the *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs described above and infected them with the NPM-WT and NPM-S48A variants. ARF could be seen to bind to both the NPM variants, but the cells carrying NPM-S48A have elevated ARF levels, which suggested its increased stabilization (fig. 3.7.D). This also suggested that S48 does not directly regulate the association of ARF to NPM, but rather regulates its stability through regulation of NPM oligomerisation. Localization of ARF to the nucleolus has been reported to stabilize it and protect it from ubiquitin ligase mediated degradation (Chen et al., 2010a), further suggesting that it is the localization with NPM at the nucleolus that promotes the ARF stability. Further examination of the cells on fluorescence microscopy showed very low and diffuse staining for ARF in the *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs, but reconstitution with NPM-WT showed characteristic ARF co-localization with the NPM as well as foci formation (fig. 3.7.C). Expression of the NPM-S48A

variant showed similar foci formation, but interestingly, these cells also showed increased immunofluorescence intensity (fig. 3.7.C and fig. 3.7.E). Taken together with the protein levels described above, this suggested that the increased ARF stabilisation was due to its increased localisation with NPM at the nucleolus, which prevented its ubiquitination and turn over. To test the effect that phosphorylation of S48-NPM would have on ARF localisation, we further expressed the phospho-mimetic S48E variant in the MEFs. The NPM-S48E were less stable than the other variants due to their inability to form oligomers and this also makes comparison of ARF protein levels in the lysates difficult. In spite of this, Immunofluorescence images showed diffuse staining of ARF in the NPM-S48E cells (fig. 3.7.C), and its inability to form nucleolar foci. Furthermore, expression of myr-AKT1 in the different variants of these cells resulted in increased monomeric NPM in the NPM-WT variants and quite interestingly, these cells were also exquisitely sensitive to AKT1 as we saw a subsequent diffuse staining of ARF in the nucleus (fig.3.7.C). On the other hand, ARF remained nucleolar and stable in the non-phosphorylatable NPM-S48A variants.

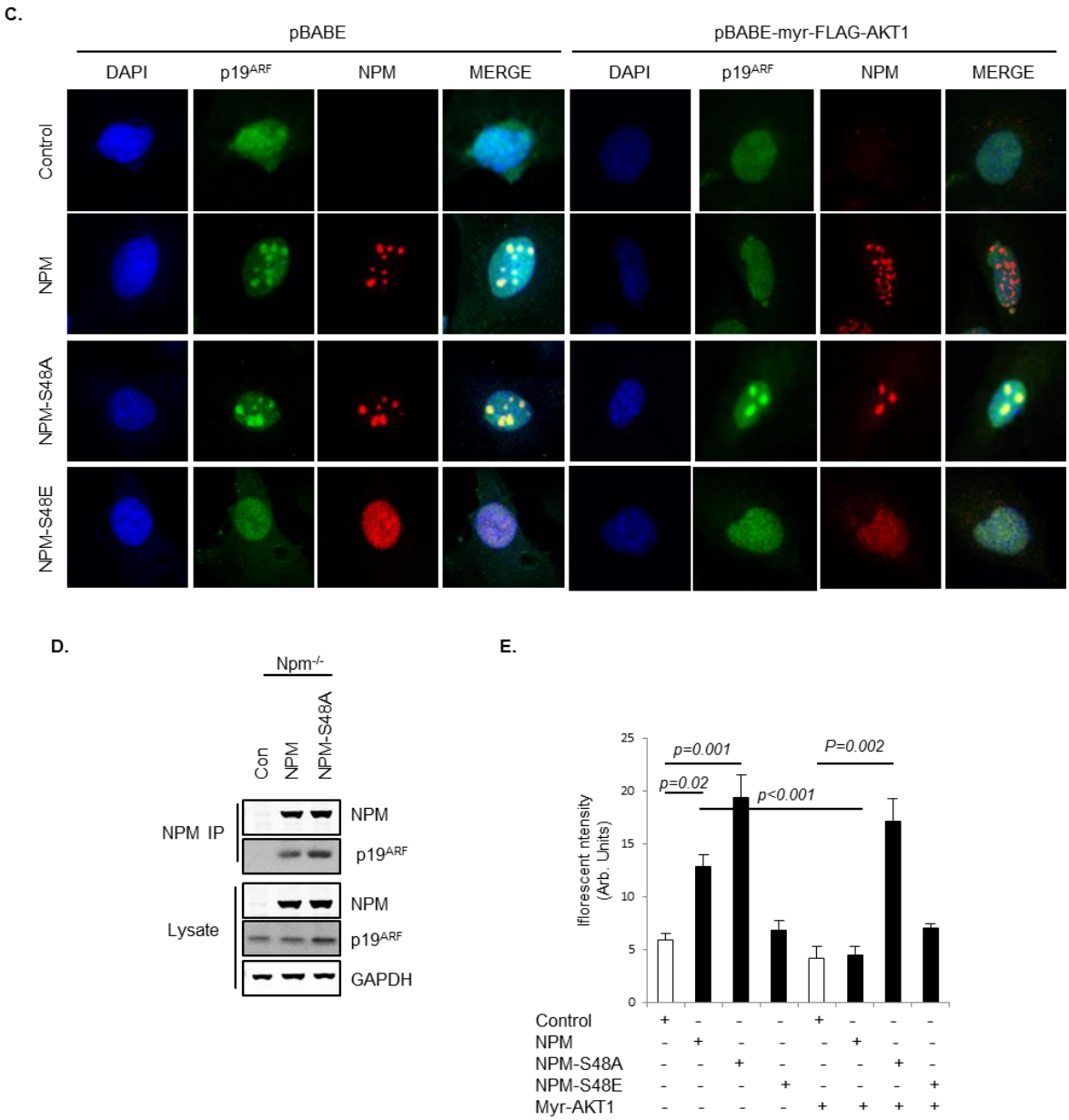


Fig. 3.7 C,D,E: C) *Npm-/-, p53-/-* MEFs were infected with pBABE retrovirus expressing FLAG-tagged-myr-AKT1 in combination with NPM-WT, NPM-S48A or S48E as indicated. Cells were fixed and stained with DAPI, anti-NPM and anti p19ARF D) NPM immunoprecipitates and whole cell lysates from *Npm-/-;p53-/-* MEFs expressing human NPM or NPM-S48A were probed with the indicated antibodies E) Graph shows quantification of p19ARF staining intensity in Immunofluorescence images using ImageJ. Representative Immunofluorescence and western blot images from experimental replicates of n=3

RAS as previously described, activates AKT and regulates the NPM oligomerisation. In order to validate the above mentioned NPM-ARF regulation as

an oncogenically driven event, we co-expressed K-Ras^{V12} in the *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs reconstituted with the NPM-WT, NPM-S48A and NPM-S48E variants (fig.3.7.F). Immunofluorescence analysis showed a similar S48 dependent ARF localisation as described with the myr-AKT1 cells.

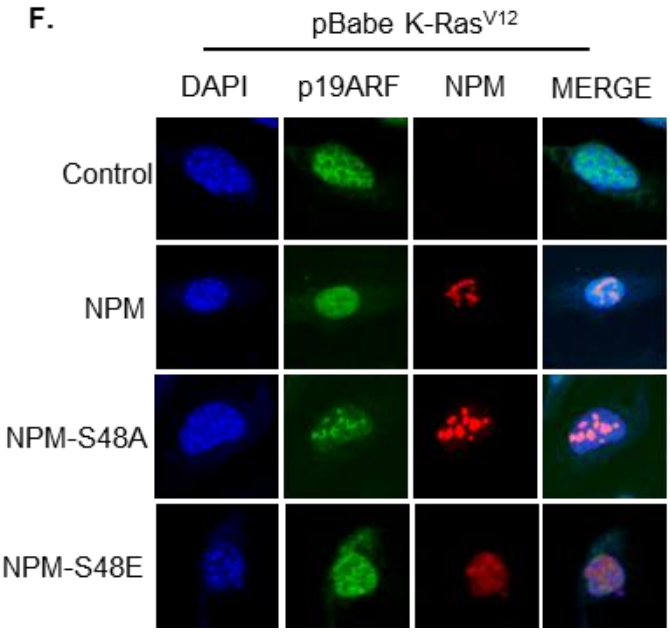


Fig.3.7.F: *Npm-/-, p53-/-* MEFs were infected with pBABE retrovirus expressing control empty vector, NPM-WT, NPM-S48A and S48E as indicated along with K-Ras^{V12} mutant variant. The cells were fixed and stained for p19^{ARF} (green) and NPM (red). Representative Immunofluorescence images from experimental replicates of n=3

Taken together, the above results suggested that AKT mediated phosphorylation of S48 regulated NPM and ARF localisation. The data also suggested that a stable pool of NPM persists in the oligomeric form , which could possibly be due to the inability of AKT to access and phosphorylate the S48 buried in the oligomer, while the monomeric cycling pool and newly synthesised NPM can be phosphorylated and prevented from forming oligomers. This would in turn play a role in the regulation of NPM-ARF interaction and disruption of nucleolar localisation.

3.8 - Phospho S48-NPM subcellular localisation in tumour cells

To investigate the AKT mediated phosphorylation of S48-NPM and the resulting regulation of NPM localisation in tumour cells, we examined the T24 cells described previously. These cells have activated AKT due to the activating HRAS mutations and this result in high levels of pS48-NPM which is seen both in the cytoplasm and the nucleus. The monomeric pS48-NPM does not localise to the nucleolus (phase contrast image in fig. 9.2 and nucleolin co-staining in fig. 3.8) and is instead seen staining both the nucleus and the cytoplasm as seen in the MEFs expressing the NPM-S48E.

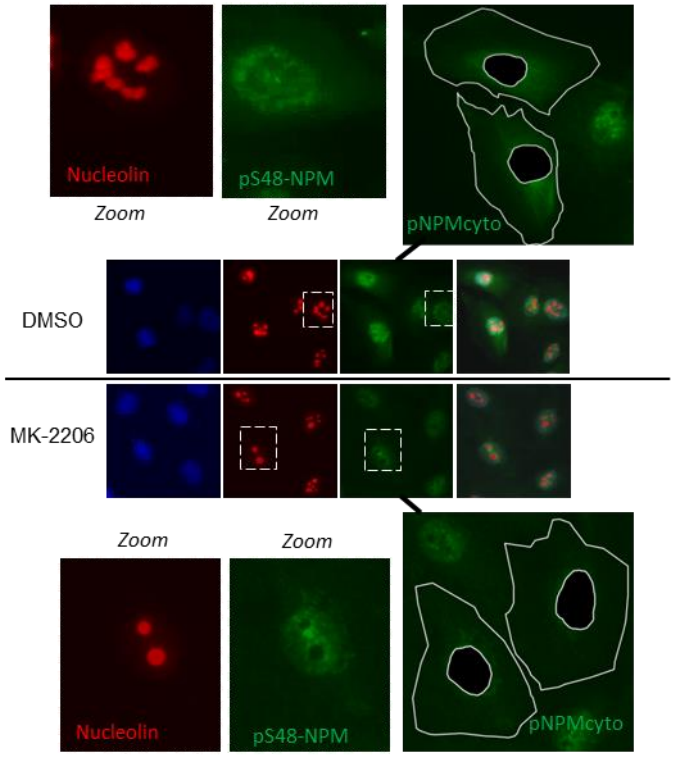


Fig.3.8: pS48-NPM localisation in T24 cells: A) T24 cells were treated with MK2206 (5μM, 24 hrs) or DMSO as indicated and stained for Nucleolin and pS48-NPM. Panels show enlarged images of Nucleolin and pS48-NPM staining in cells as well as cytoplasmic pS48-NPM staining in cells where the nuclei in the images have been electronically blacked out to highlight the cytoplasmic staining. Representative Immunofluorescence images from experimental replicates of n=3

Inhibiting AKT in these cells (using either PI-103 or MK2206) resulted in an overall decrease in the pS48-NPM staining, especially the cytoplasmic fraction of the monomeric NPM (fig. 9.2 and fig. 3.8). This confirmed in tumour cells, the previous description of monomeric NPM localisation that was seen in the MEFs.

3.9 – AKT inhibition regulates NPM-ARF localisation in tumour cells

We next wanted to investigate if AKT regulates NPM mediated ARF localisation in tumour cells in the manner that was characteristically seen in the reconstituted *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs in previous sections. Due to the activating HRAS mutations and AKT activation, T24 cells have phosphorylated S48-NPM (fig. 3.4.A), and inhibition of AKT results in an increase in the oligomeric fraction and a concomitant decrease in the monomeric fraction of NPM (fig. 3.4.A, fig. 3.5.A and fig. 3.9.A). Interestingly, cell lysates separated on semi-native gels showed an ARF separation pattern that was similar to that of NPM suggesting a co-migration. Immunofluorescence imaging for p14ARF in these cells also showed a diffuse nuclear staining that was similar to the *Npm*$^{-/-}$; *p53*$^{-/-}$ MEFs or those reconstituted with the NPM-S48E. Exogenous expression of NPM-S48A in these cells resulted in the formation of ARF nucleolar foci, which was not seen on expression of NPM-WT due to the endogenous AKT activity (fig.3.9.B). On the other hand, inhibition of AKT with PI-103 or MK2206 resulted in re-localization of ARF to the nucleoli (fig.3.9.C), confirming the role that AKT plays in the regulation of ARF localization.

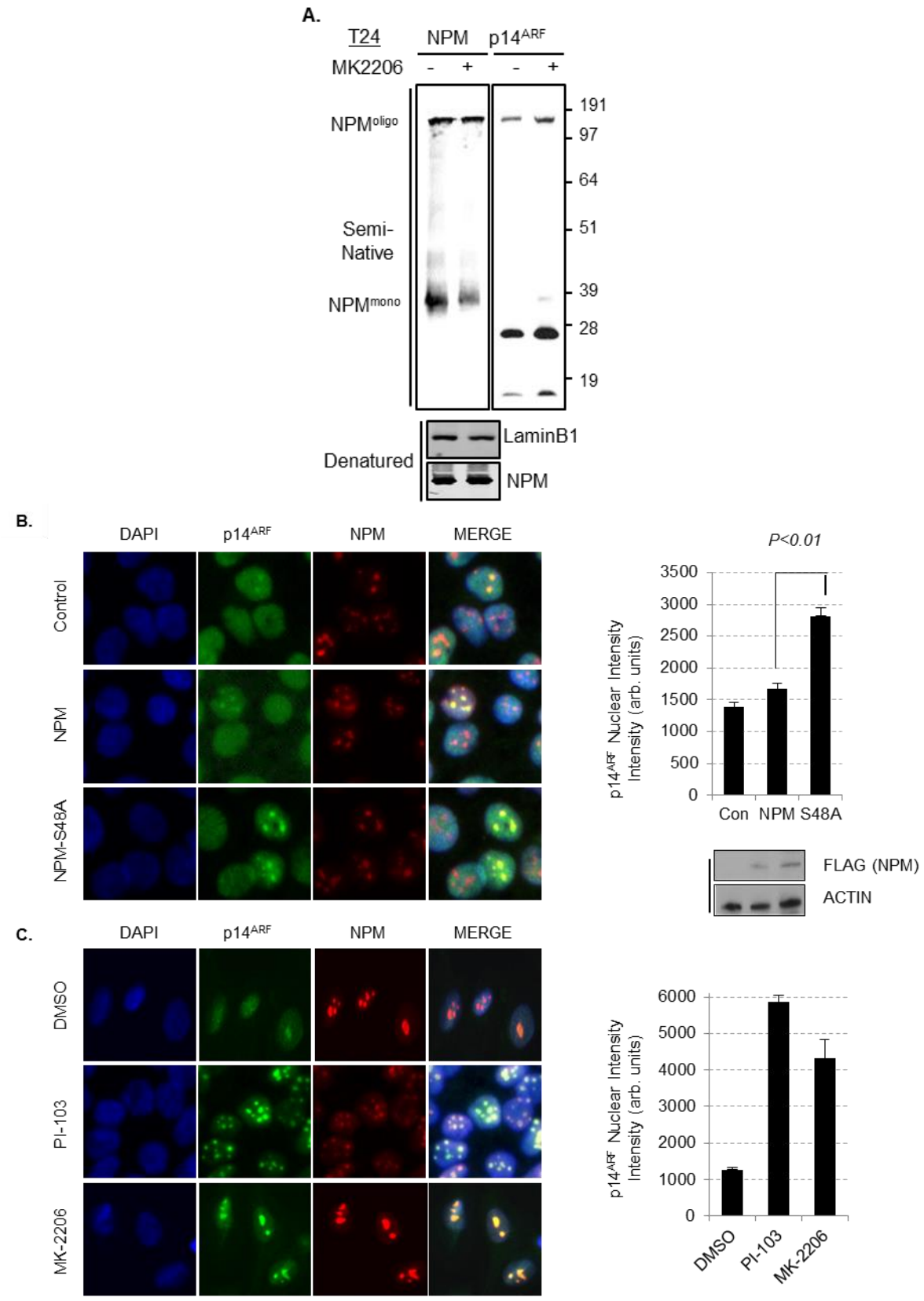


Fig. 3.9: AKT inhibition regulates NPM-ARF localisation in T24 cells. A) Nucleoplasmic fractions from T24 cells that were treated with MK-2206 (5 μM, 24 hrs) were subjected to semi-native gel electrophoresis and probed by western blot

with the indicated antibodies B) T24 cells were transfected with empty vector (control), FLAG-NPM or FLAG NPM-S48A or (C) treated with DMSO, PI-103 (0.4 μM), or MK-2206 (5 μM) for 24 hrs. Cells were stained for NPM (red) and p14ARF (green). Each graph represents the quantification of p14ARF staining intensity in the nucleolus and was performed by In Cell Analyser 1000 automated epifluorescence microscope. Data are represented as mean ± SEM. Representative western blot and Immunofluorescence images from experimental replicates of n=3

The re-localization of ARF to the nucleoli is also seen following the inhibition of AKT in other cells where AKT is active such as the non-small cell lung cancer cell-line H1299 that carry RAS mutations (fig. 3.9.D).

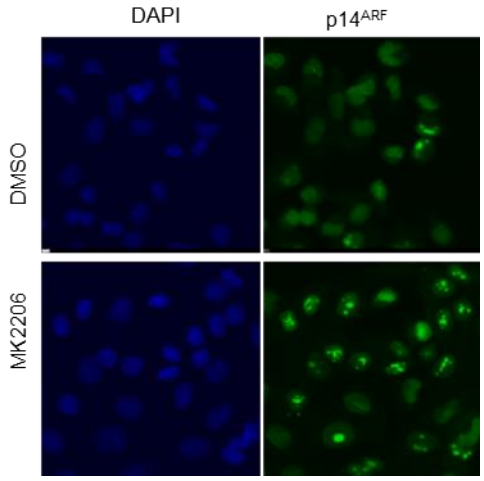


Fig. 3.8.D: H1299 cells treated with MK2206 (5μM, 24 hrs) or DMSO and stained for $p14^{ARF}$ (green). Representative Immunofluorescence images from experimental replicates of n=3

3.10 – AKT inhibition and NPM-ARF nucleolar localisation

The NPM mediated localisation of ARF to the nucleolus has been reported to protect and stabilise ARF levels in the cell (Chen et al., 2010a; Colombo et al., 2005a; Colombo et al., 2006; Kuo et al., 2004) by preventing ubiquitination and degradation. Since ARF was localised to the nucleolus upon AKT inhibition, we

next addressed if the ubiquitination of ARF was also affected during the same process. Inhibiting AKT using siRNA or by inhibitors resulted in the stabilisation of ARF levels in the cells (fig. 3.4.A and fig.3.9.A). Furthermore as seen in fig.3.9.A, sequestration of ARF to the nucleolus correlated with increased co-migration of ARF and oligomeric NPM as well as decreased ARF ubiquitination (fig.3.10.A) and a concomitant increase in the total ARF protein level in cell lysates. This increase was not transcription dependent (fig.3.10.B) thus confirming the post-translational stabilisation of the ARF protein by nucleolar sequestration.

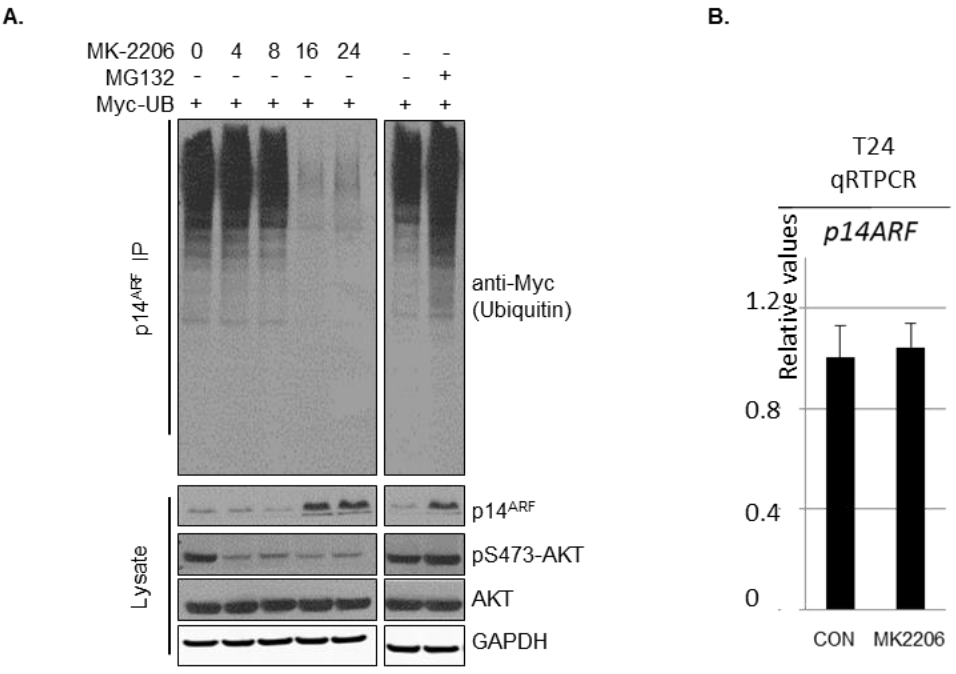


Fig. 3.10: AKT mediated NPM-ARF localisation to the nucleolus protects ARF from degradation A) Ubiquitination assay of p14ARF in H1299 cells transfected with Myc-tagged ubiquitin treated with DMSO, MG-132 (10 μM, 16 hrs) or MK-2206 (5 μM) for the times indicated (figure courtesy of Dr. Garth Hamilton) B) Relative levels of p14ARF mRNA in T24 cells in the presence of MK-2206 (5 μM, 24hrs) determined by qRTPCR. Representative western blot images from experimental replicates of n=3

3.11 – MDM2-ARF interaction is constitutive in cells with active AKT

The data presented suggests that AKT mediated phosphorylation and a subsequent decrease in the oligomeric fraction of NPM, also promotes nucleoplasmic localisation of ARF. The most important function of ARF as described in previous chapters is its role as an inhibitor of the E3 ubiquitin ligase MDM2, which in turn results in the stabilisation of the p53 protein (Kamijo et al., 1997; Sherr, 2006). NPM mediated sequestration of ARF to the nucleolus has been reported to increase MDM2 activity in the nucleus (Korgaonkar et al., 2005) and likewise, re-localisation of ARF to the nucleoplasm has been shown to increase ARF-MDM2 interaction (Lee et al., 2005).

In agreement with previous reports (Llanos et al., 2001), we saw that MDM2 was predominantly seen in the nucleoplasm in T24 cells (fig. 3.11.A and B) and even though ARF was translocated to the nucleolus along with NPM on AKT inhibition, MDM2 levels remained relatively stable in the nucleoplasm and there was no visible staining of MDM2 at the nucleolus. Previous studies have also shown that MDM2 is phosphorylated at S166 and S186 by AKT, which results in its trafficking to the nucleus (Mayo and Donner, 2001; Ogawara et al., 2002). Upon expression of the constitutively active MDM2-S166D:S186D (MDM2-DD) in T24 cells, we do indeed see nuclear localisation of MDM2, but this did not affect ARF localisation to the nucleolus after AKT inhibition (fig. 3.11.C). Interestingly, MCF7 cells that lack ARF when treated with AKT inhibitors show a decrease in MDM2 activity characterised by an increase in the levels of its substrate protein p53 levels. But when ARF is expressed exogenously, there is an increase in p53 stability, which is lost following treatment with the AKT inhibitor (fig.3.11.D). Taking all the above into account, it would suggest that NPM constitutively associates with ARF in the

nucleus and shuttles between the nucleolus and nucleoplasm depending on its oligomeric state, while at the same time the MDM2 activity which may be promoted by AKT, is dependent on the ARF status in the nucleus.

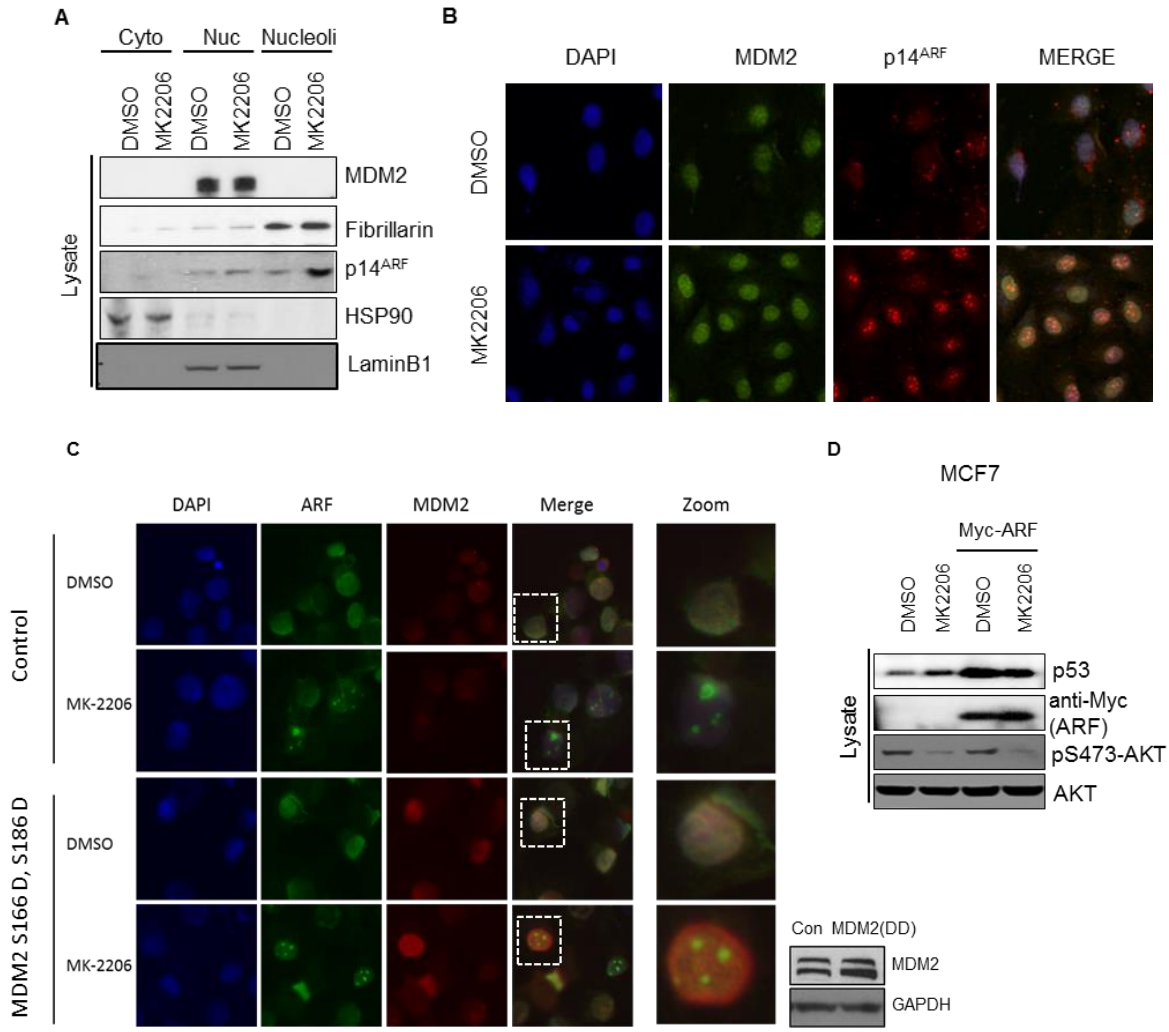


Fig. 3.11.A-D AKT inhibition and MDM2/ARF localisation A) western blots of cytoplasmic, nuclear and nucleolar fractions (obtained by ultracentrifugation of sonicated nucleoplasmic lysates) from T24 cells. Subcellular fractions were analysed by western blot and probed with the indicated antibodies B) T24 cells treated with DMSO or MK-2206 (5 μM, 24 hrs) and the localization of p14ARF (red) and MDM2 (green) determined by immunofluorescence. Nuclei were stained with DAPI C) T24 cells were transfected with pCDNA3 empty vector or double phopsho-mimetic MDM2 S166D,S186D and treated with DMSO or MK2206 (5μM). The cells were trypsinised and spun onto glass slides using a Thermo Scientific Cytospin 4 (1200RPM for 4 minutes), fixed and stained for $p14^{ARF}$ (green) and MDM2 D) MCF7 cells were transfected with Myc tagged ARF, treated

with MK2206 (5μM,24 hrs) or DMSO and probed by western blot as indicated. Representative western blot and Immunofluorescence images from experimental replicates of n=3

In order to further investigate whether and how the NPM/ARF interaction described above regulated MDM2, we performed large-scale purification of ARF from HeLa nuclear lysates in collaboration with Dr. Grigory Dianov's lab and identified a high molecular weight complex that elutes after ion exchange and size exclusion separation, containing both NPM and MDM2 in a highly purified protein fraction (fig. 9.3). This indicated a strong molecular interaction between the constituents of the complex. But from the data shown above where AKT does not influence nucleolar translocation of MDM2, this also suggested that MDM2 cannot associate with the NPM/ARF oligomers and be trafficked to the nucleolus. Thus AKT inhibition cannot per se influence the ability of NPM protein to bind to MDM2 or ARF, but instead regulate the oligomerisation of NPM which can accommodate ARF and traffic it to the nucleolus, but not so in the case of MDM2.

In order to examine if NPM-ARF-MDM2 tripartite complex can exist in the non-nucleolar nucleoplasm and to study if the AKT mediated phosphorylation had any direct effect on the interaction, we immuno-precipitated FLAG tagged NPM from *Npm-/-, p53-/-* MEFs that had been reconstituted with NPM-WT, NPM-S48A and NPM-S48E. The complexes were eluted with FLAG peptide and the NPM-MDM2 complexes further isolated by immuno-precipitation of MDM2 from the FLAG elute. Interestingly ARF was present in the NPM-MDM2 complexes (fig.3.11.E), although to different levels due to the inherent differential stability of NPM, suggesting that the AKT phosphorylation site itself does not regulate the interaction.

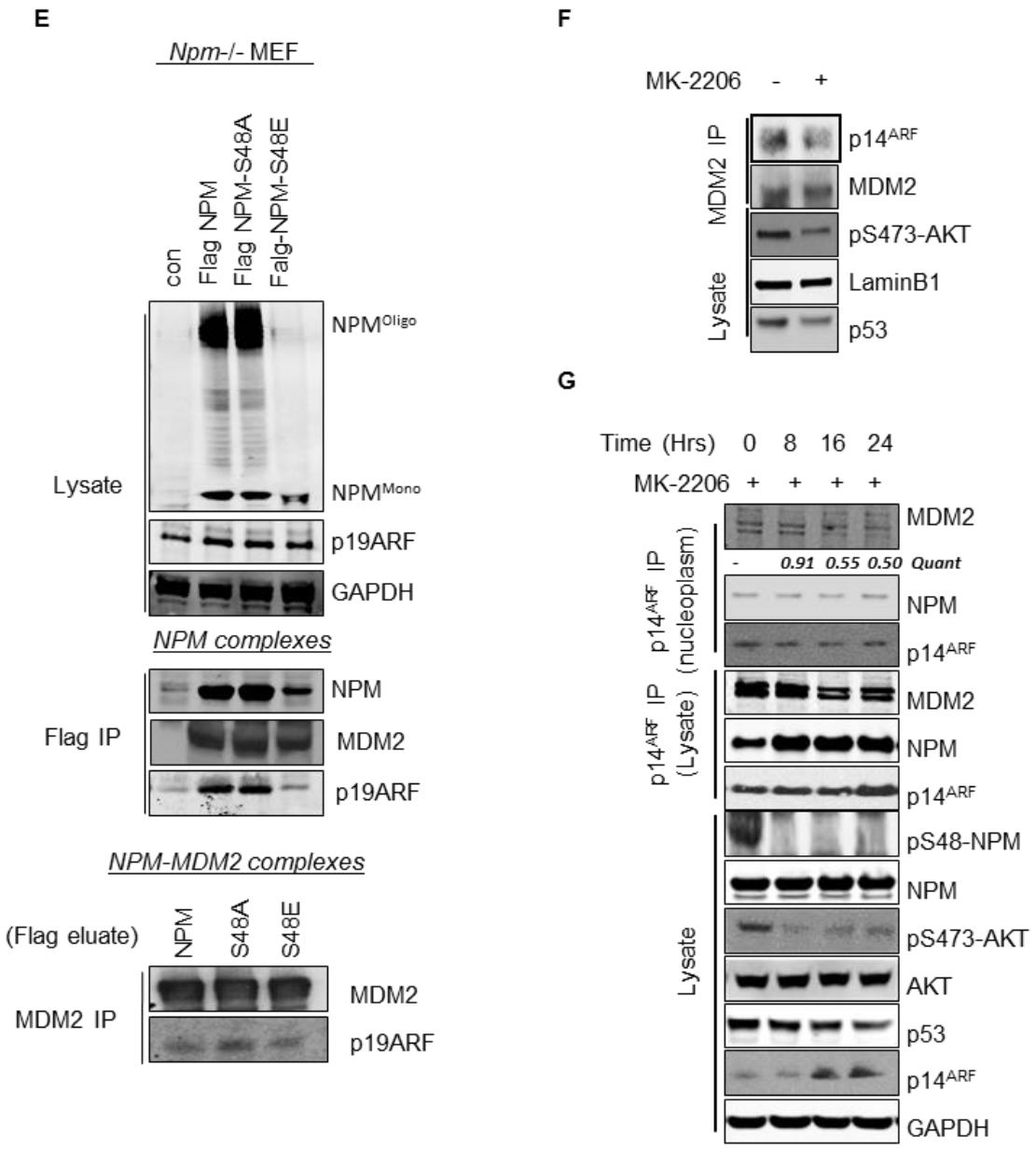


Fig. 3.11.E-G: E) *Npm-/-, p53-/-*double null MEF were infected with pBABE retrovirus empty vector and pBABE expressing FLAG-tagged-NPM-WT, NPM-S48A or S48E as indicated. Immunopurification of Flag-NPM was done by pulling down with a Flag specific antibody (middle panel) followed by elution of complexes by using the Flag peptide and subsequent immunopurification of endogenous MDM2 (lower panel). F) Nuclear immunoprecipitates of MDM2 from T24 cells treated with MK-2206 (5 μM, 24 hrs). Immunoprecipitates and lysates were blotted with the indicated antibodies. G) T24 cells were treated with MK-2206 (5 μM) as indicated. p14ARF was immunoprecipitated from whole cell lysates and nuclear extracts and the association with NPM and MDM2 determined by western blot. Immunoprecipitates and lysates were blotted with the indicated antibodies. Representative western blot images from experimental replicates of n=3

We next wanted to see if the AKT mediated regulation of the NPM oligomerisation and ARF localisation to the nucleolus had an effect on ARF association with MDM2. Lysates from T24 cells were immuno-precipitated for endogenous MDM2 and were seen to associate with ARF (fig. 3.11.F). Upon treatment with MK2206 the composition of the complexes shifted with exclusion of MDM2 from the complex and a concomitant increase in the NPM-ARF interaction (fig.3.11.F and G), which corresponded to the increase in the accumulation of ARF at the nucleoli (fig. 3.9.C).

3.12 – Chapter 3 discussion

The constitutive AKT activation characteristic of many tumours as a result of activating pathway mutations or inactivation of the regulatory upstream components, as well as p53 mutations have been described to co-occur in many *in vivo* models. The exact mechanism behind this event has not been described previously nor the clinical significance or the therapeutic implications of this interaction dealt with by previous authors. In this study, we aimed to answer this very interesting question which could explain the discrepancies in many previous reports.

p53 protein levels are characteristically regulated by the E3 ubiquitin ligase MDM2 and MDM2 itself is regulated by the tumour suppressor p14ARF. Our initial studies looking at AKT interacting proteins identified NPM as one of the candidate proteins. NPM is known to act as a chaperone that regulates p14ARF and other important proteins. Furthermore, previous authors had shown that siRNA or

aptamer mediated inhibition of NPM (Jian et al., 2009; Qi et al., 2008) resulted in delocalisation of p14ARF from the nucleolus and a subsequent stabilisation of p53 protein levels. This made NPM an interesting candidate to continue investigating further. The initial work done in our lab showed that AKT interacts with NPM and also phosphorylates the S48 moiety of NPM. In silico analysis revealed that phosphorylation of S48 by AKT would result in an inability of the NPM protein monomers to form stable oligomers due to steric hindrance. Furthermore, since this site was known to be located in a Nuclear Export Sequence region of NPM, we hypothesised that phosphorylation would have an effect on the nuclear localisation of NPM.

The data presented in this chapter builds on our initial work which identified the AKT mediated phosphorylation site on NPM. The antibody raised against the S48 phosphorylation site was first validated and was found to be highly specific to the phosphorylation site in both cancer and non-cancer cell lines (fig.3.2). We then proceeded to demonstrate using different NPM genetic variants that AKT mediated phosphorylation of S48-NPM resulted in impaired oligomerisation of NPM (fig.3.3) and that constitutive AKT activity (which is commonly seen in many cancers) resulted in a perturbation of the oligomeric fraction of the wild-type NPM. The oligomeric form of NPM has been described to be localised to the nucleolus (Enomoto et al., 2006) and in agreement with those reports, we saw that the oligomeric fractions of both wild-type and the non-phosphorylatable NPM do localise to the nucleolus. This was also demonstrated in cancer cells treated with the AKT inhibitor MK2206, where inhibition of AKT and subsequent inhibition of the phosphorylation of S48-NPM resulted in increased NPM oligomerisation and localisation to the nucleolus. Interestingly, we also saw that ARF localisation and

stability correlated with the NPM localisation and NPM sensitivity to AKT activity. The principle functional role attributed to ARF has been on regulation of the MDM2 (Kamijo et al., 1997; Sherr, 2006). We found that phosphorylation of S48-NPM which leads to the restriction of NPM and ARF to the nucleoplasm resulted in increased ARF-MDM2 interaction. Interestingly we also saw that NPM, ARF and MDM2 can exist as a tripartite complex in the non-nucleolar nucleoplasmic compartment (fig.3.11), but that the S48-NPM phosphorylation itself does not influence this interaction. The inhibition of AKT activity was associated with localisation of ARF to the nucleolus, which was observed to lead to a decrease in the interaction between ARF and MDM2 in the nucleoplasm due to the decreased pool of active ARF in the nucleoplasm. The decreased ARF-MDM2 interaction and the subsequent increase in the MDM2 activity in the nucleoplasm was assessed in further experiments and is described in the proceeding chapter.

Thus we proved that AKT mediated phosphorylation of S48-NPM plays an important role in the regulation of NPM oligomerisation, which in turn is important for regulating ARF localisation. ARF localisation to the nucleolus following AKT inhibition decreases the ARF-MDM2 interaction in the nucleoplasm and consequently increases the MDM2 activity. This in turn would be expected to increase the activity of MDM2 and play a role in the regulation of the stability of its substrates especially p53.

Chapter 4

AKT regulates mutant p53 via Nucleophosmin

Chapter 4 – AKT regulates mutant p53 via Nucleophosmin

4.1 – Introduction

We demonstrated in chapter 3 that AKT mediated phosphorylation of S48 of NPM regulated its oligomerisation and consequent localisation in the cell. Inhibition of AKT using siRNA as well as pharmacological agents promoted oligomerisation of NPM and its translocation to the nucleolus which in turn regulated the tumour suppressor ARF. We also showed that the localisation of ARF to the nucleolus and the subsequent decrease in the nucleoplasmic pool resulted in a decrease in the ARF- MDM2 interaction. We next wanted to investigate how this regulation would influence MDM2 activity on its substrates, especially p53. Since mutant p53 retains many of the regulatory components possessed by its wild type variant (Suh et al., 2011), we were interested to know whether AKT mediated regulation of NPM and MDM2 would also affect p53mut protein levels. Should inhibition of AKT activity regulate MDM2 activity and decrease p53mut levels, we were keen to determine whether this could be utilised to mitigate p53mut ‘gain of function’ and thereby improve therapeutic responses.

4.2 – Inhibition of AKT increases MDM2 activity

We saw in section 3.10 that inhibition of AKT resulted in a decrease in the ARF-MDM2 interaction in the nucleus, due to the increased translocation of ARF to the nucleolus. The decrease in the ARF-MDM2 interaction was associated with an increase in the MDM2 activity. H1299 cells transfected with wild type p53 and HA-tagged ubiquitin showed low basal MDM2 activity (fig. 4.2.A & B). Inhibition of AKT using MK2206 resulted in restriction of ARF to the nucleolus and an increase in MDM2 activity characterised by increased auto-ubiquitination of MDM2 as well as MDM2 mediated p53 ubiquitination (determined by sensitivity to the MDM2 inhibitor Nutlin 3a) (fig. 4.2.B).

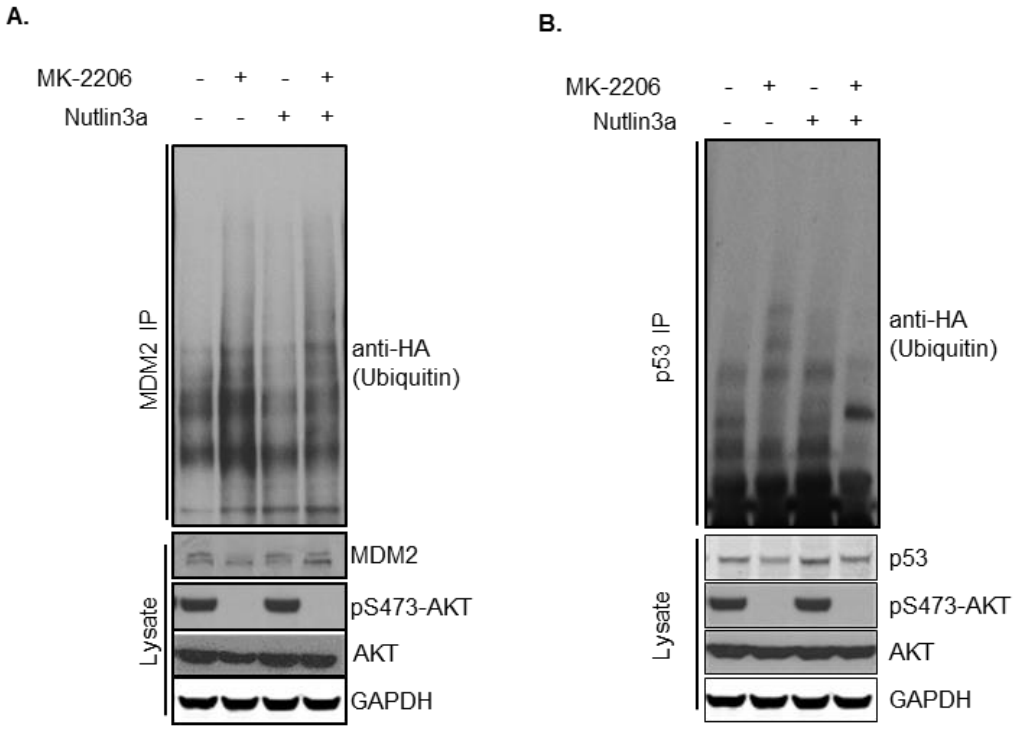


Fig. 4.2 AKT inhibition increases MDM2 activity in H1299 cells A) MDM2 and (B) p53 ubiquinitation assay in H1299 cells transfected with wild type p53, HA-tagged ubiquitin and treated for 16 hrs with DMSO, MK-2206 (5 μM) or Nutlin3A (5 μM) as indicated. Immunoprecipitates and whole cell lysates were probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

The above data confirmed our hypothesis that AKT inhibition was indeed increasing the MDM2 activity and promoting MDM2 mediated ubiquitination of its substrates.

4.3 – Inhibition of AKT decreases the p53mut stability

Since the inhibition of AKT promotes ARF localisation to the nucleolus and consequently an increase in MDM2 activity, we next wanted to see how this would affect p53mut stability. T24 cells (expressing an in-frame deletion of Tyr126 of p53) has high endogenous p53 expression, but inhibiting the AKT activity using siRNA or an AKT inhibitor showed a decrease in the p53 levels (fig. 3.4.A and 3.10.F). MDM2 has been shown to degrade p53mut *in vivo* (Terzian et al., 2008) and so we hypothesised that the AKT mediated localisation of ARF and the subsequent regulation of MDM2 would regulate the levels of p53mut. An antibody that specifically recognizes p53mut in the native state (clone Ab240) showed on immunofluorescence microscopy, a considerable decrease in the p53mut staining in T24 and PSN1 cells treated with MK2206 (fig. 4.3.A).

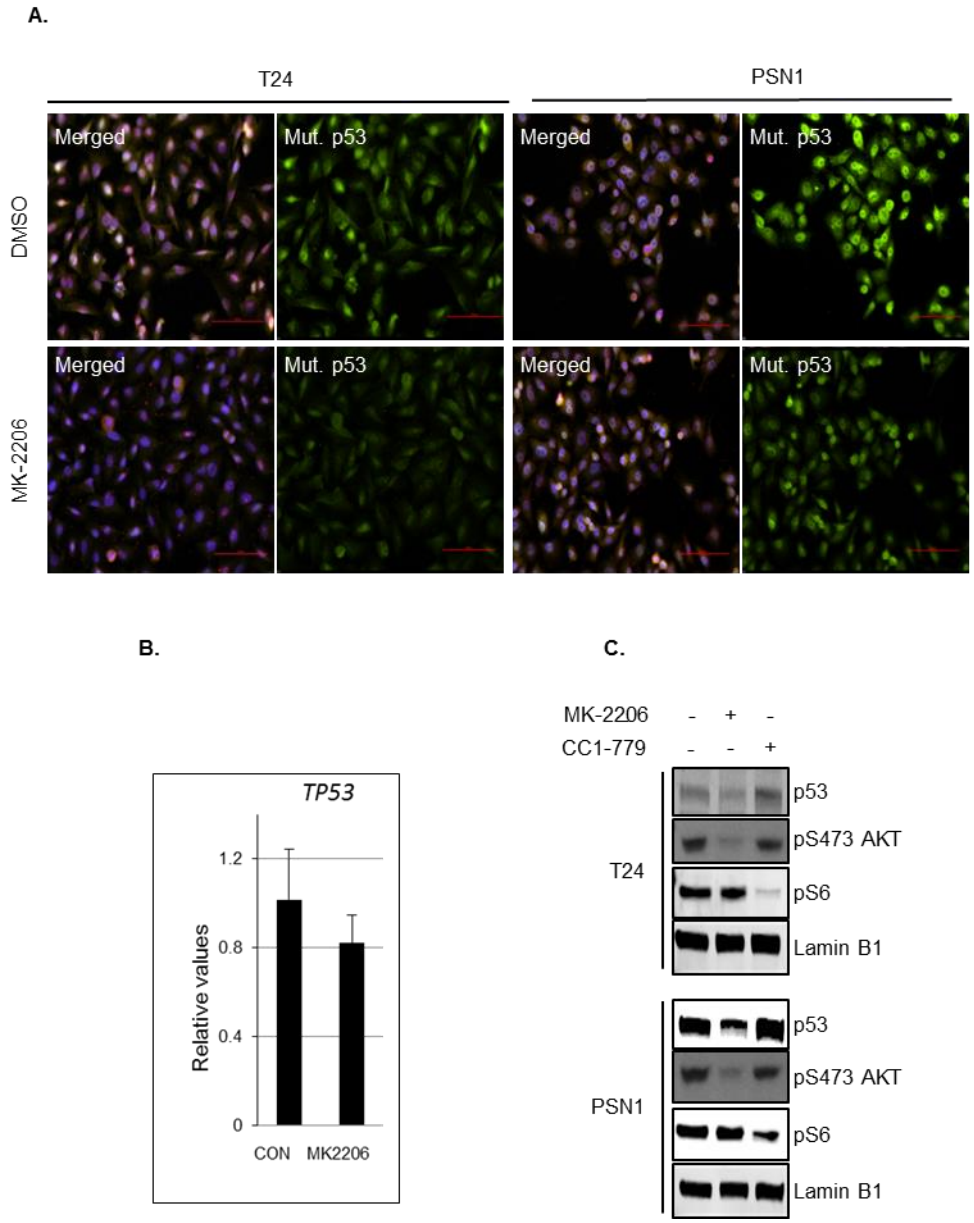


Fig. 4.3.A-C AKT inhibition decreases p53mut stability A) T24 cells and PSN1 cells were treated with MK-2206 (5 µM) or DMSO for 24 hrs. Cells were fixed and stained with DAPI and anti-mutant p53 (Ab240 clone). B) Relative levels of p53 mRNA in T24 cells in the presence of MK-2206 (5 µM, 24hrs) determined by qRTPCR. C) T24 cells and PSN1 cells were treated with DMSO control, MK-2206 (5 µM) or CCI-779 (1 µM) for 16 Hrs. Nuclear extracts were prepared from the cells and probed with the indicated antibodies. Representative Immunofluorescence and western blot images from experimental replicates of n=3

The decrease in p53mut in response to AKT inhibition was not a transcriptional change, as seen from the p53 transcript levels (fig. 4.3.B), but rather a post-translational change due to increased ubiquitination and degradation. The effects were also not due to downstream translational signals as treatment with the mTOR inhibitor did not show an alteration in the p53mut stability (fig. 4.3.C). Other p53mut variants like the R175H and R248W 'hotspot' mutants expressed in H1299 cells also showed increased ubiquitination following AKT inhibition (fig.9.4), further validating our finding that AKT inhibition and increased MDM2 activity decrease p53mut stability.

4.4 – Inhibition of AKT decreases p53mut stability in a NPM-ARF dependent manner

In order to confirm that the above mentioned decrease in p53mut stability was a direct effect of AKT inhibition, we examined T24 cells pre-treated with or without MK2206 for p53mut stability following the addition of the protein translation inhibitor cyclohexamide. In the cells treated with MK2206, the half-life of p53mut was reduced when compared to the control cells (fig.4.4.A), indicating that the turnover was accelerated following AKT inhibition. Similar results were also obtained on S^{35} Met/Cys pulse chase analysis (fig.9.5)

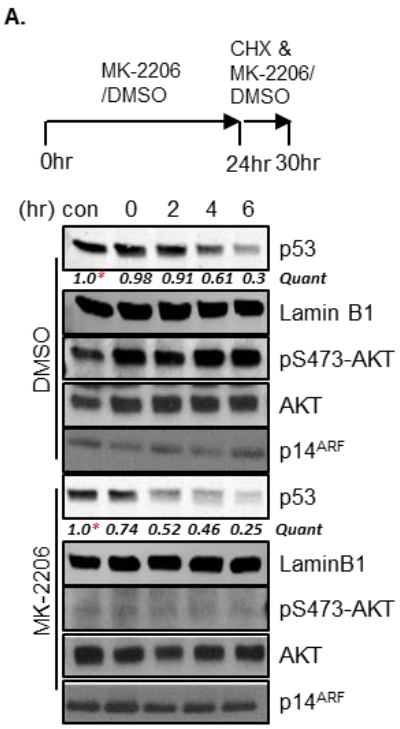


Fig. 4.4.A A) T24 cells were pre-treated with MK-2206 (5 μM) or DMSO for 24 hrs before the addition of fresh media containing cyclohexamide (100 μM) (CHX) in combination with MK-2206 (5 μM) or DMSO for the times indicated. Nuclear extracts were prepared from treated cells and blotted with the indicated antibodies. Representative western blot images from experimental replicates of n=3

Since our previous findings suggested that oligomerisation of NPM regulated ARF localisation, MDM2 activation and decrease in p53mut stability, we reasoned that direct perturbation of the NPM oligomerisation would also affect p53mut stability. Following treatment of T24 cells with an NPM oligomerisation inhibitor NSC348884, which inhibits formation of NPM oligomers, we saw that the effects on p53mut levels following siRNA as well as MK2206 mediated AKT inhibition, were abrogated (fig. 4.4.B and C). Furthermore, the stability of p53mut was reduced by siARF and promoted by Nutlin 3a independent of NSC348884 (fig. 4.4.B and C), while converesely, *Npm*$^{-/-}$*, p53*$^{-/-}$ MEFs transfected with p53^{R248H} and NPM-WT showed increased p53mut stability in the presence of myr-AKT1

(fig. 4.4.D). The S48A variants on the other hand showed increased basal $p53^{R248H}$ levels which was not affected by myr-AKT1.

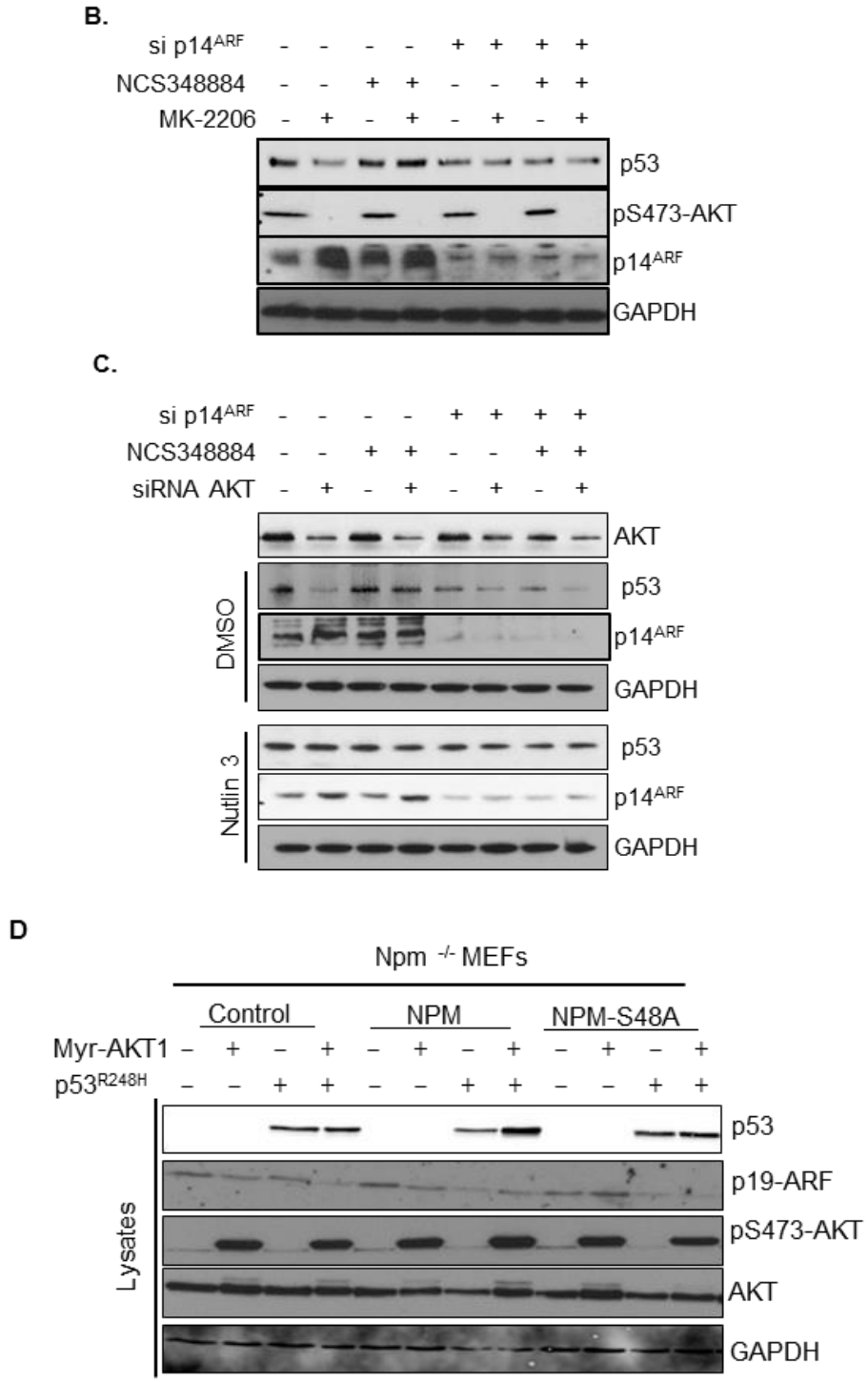
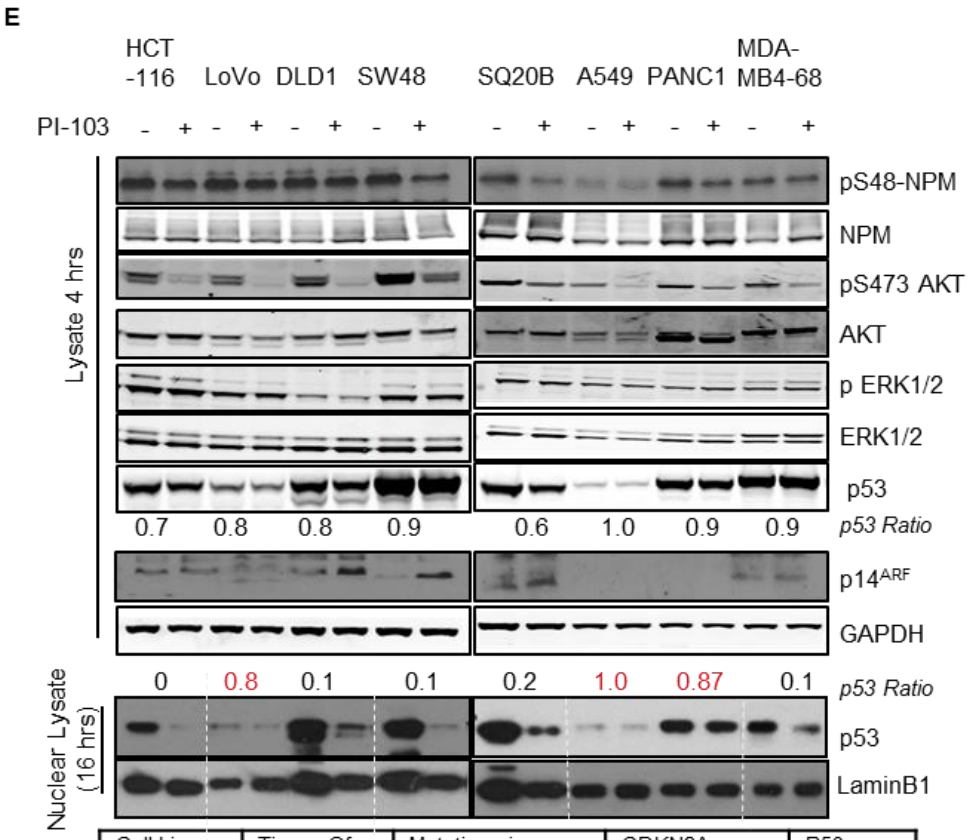


Fig. 4.4.B-D: B) T24 cells were transfected with non-targeting control or p14ARF siRNA and treated with DMSO, MK-2206 (5 μM) or the NPM oligomerisation inhibitor NSC348884 (4 μM) (Qi et al., 2008) as indicated C) T24 cells were transfected with non-targeting control, AKT1, or p14ARF siRNA. Cells were

treated with NCS348884 (4 μM), Nutlin3a (5 μM) or DMSO as indicated. Whole cell lysates were probed with the indicated antibodies. D) *Npm*$^{-/-}$, *p53*$^{-/-}$ MEFs were infected with pBABE retrovirus expressing control empty vector, NPM-WT and NPM-S48A along with Myr-AKT1 and p53^{R248H} and probed by western blot as indicated. Representative western blot images from experimental replicates of n=3

The above mentioned findings suggest that disruption of NPM quaternary structure is sufficient to stabilize p53mut and functions via ARF and MDM2. Finally, to assess if AKT mediated regulation of ARF localisation was a general mechanism in cells to regulate p53 stability, we examined a number of cell lines treated with PI-103. Short exposure to the drug (4 hours) resulted in a decrease in the pS48-NPM levels which was also accompanied by a decrease in the p53 levels in the SQ20B cells. Longer exposure to PI-103 (16 hours) resulted in a decrease in stability of p53 in all the cells except the CDKN2A null cells A549 and PANC1, as well as the CDKN2A methylated LoVo cells (fig. 4.4.E). Taking all the above data into consideration, we were able to confirm our hypothesis of AKT regulating NPM oligomerisation and subsequently the ARF and MDM2 mediated stabilisation of p53mut in the cancer cells.



Cell Line	Tissue Of Origin	Mutations in EGFR/RAS/ PI3K/AKT	CDKN2A ARF Status	P53 Status
T24	Bladder	HRAS G12V		Δ Y126
PSN1	Pancreatic	KRAS G12		K162N
HCT-116	Colon	KRAS G12D PIK3CA H1047R		WT
LOVO	Colon	KRAS G13D	Methylated	WT
DLD1	Colon	KRAS G13D		S241F
SW48	Colon	EGFR G719S		R248W
SQ20B	Head and neck	Hyperactive EGFR		insertion Y126
A549	Lung	KRAS G12S	Deleted	WT
PANC1	Pancreas	KRAS G12D	Deleted	R273H
MDA-MB-468	Breast	PTEN		R280K
SKMEL28	Skin	EGFR P753S		L145R

Fig. 4.4.E: Cell lines were treated with PI-103 (0.4µM) for 4 hours (upper panel) or 16 hours (lower panel). Whole cell (upper) and nuclear lysates were western blotted with the indicated antibodies. Levels of p53 were normalized relative to the GAPDH or Lamin B1 loading control and expressed as a ratio of the p53 levels present in DMSO treated cells. All mutational data were obtained from the COSMIC database http://www.sanger.ac.uk/genteics/CGP/cosmic and ICR (Institute for Cancer Research) database https://cansar.icr.ac.uk/cansar/cell-lines.

4.5 – AKT inhibition sensitises p53 mutant tumour cells to radiation

The data presented until now would suggest that inhibiting AKT promotes increased turnover of p53mut in cancer cells. Inhibiting p53mut function has been an area of interest for many years, as p53 mutations are associated with gain of function phenotypes that accelerate tumour development and resistance to treatment (Freed-Pastor and Prives, 2012). Since mutations in p53 have been associated with resistance to DNA damage inducing treatments including ionizing radiation (IR) (Lee and Bernstein, 1993) we reasoned that inhibition of AKT and the subsequent decrease in p53mut should revert this resistance to IR.

Treatment of T24 and SQ20B cells with PI-103 decreased the pS48-NPM and p53 levels with a concomitant increase in the sensitivity of the cells to IR (fig. 4.5.A and B). Similarly, inhibition of AKT with MK2206 in T24 and PSN1 cells increased the sensitivity of the cells to IR reflecting lower p53mut levels (fig. 4.5.C). Direct ablation of p53mut using siRNA targeting p53 in T24 cells also showed a similar increase in sensitivity to IR with decreased clonogenic survival (fig. 4.5.D). Taking all the data together, it suggested that the AKT and NPM mediated regulation of ARF localisation regulated p53mut levels and sensitivity to IR. Interestingly, siRNA mediated silencing of p14ARF in T24 cells increased their sensitivity to IR, further confirming that p14ARF behaved as an oncogene and promoted resistance in p53mut cells (fig. 4.5.E).

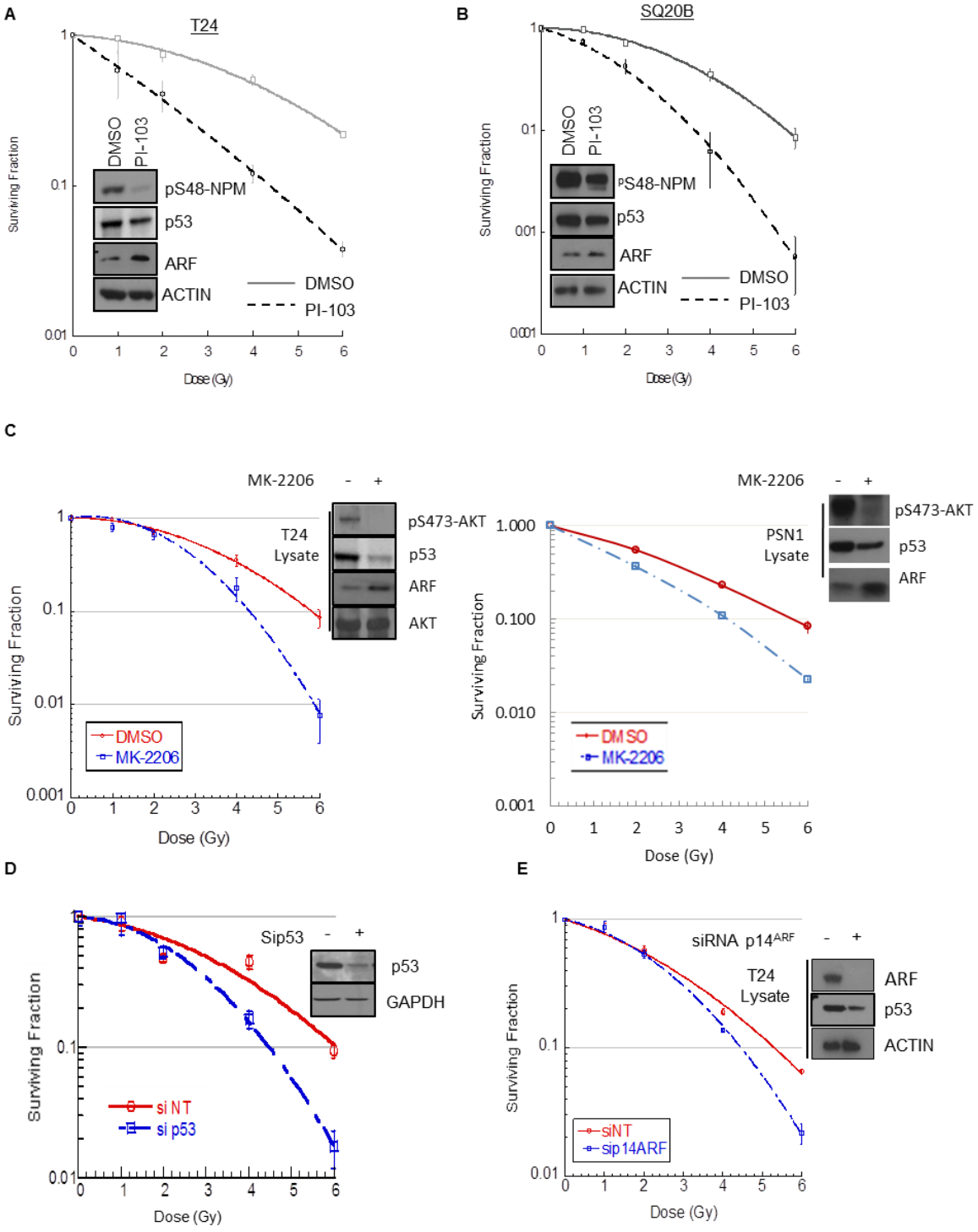


Fig. 4.5 AKT inhibition sensitises p53mut tumours to IR: A and B) Clonogenic survival assay and western blots of whole cell lysates of (A)T24 cells and (B) SQ20B cells in the presence of PI-103 (0.4 μM) or DMSO control and irradiated at the indicated doses C) Clonogenic survival of T24 and PSN1 cells following treatment with ionizing radiation at the indicated doses. Cells were pre-treated with MK-2206 (5 μM) or DMSO before irradiation. Whole cell lysates were blotted with

the indicated antibodies D) T24 cells were transfected with siRNA against p53 or a non-targeting control and irradiated at the indicated doses E) As in (D) except cells were transfected with non-targeting (NT) control or p14ARF siRNA before irradiation. Whole cell lysates were blotted with the indicated antibodies.

Nucleoplasmic ARF has been reported to be targeted for ubiquitination by the E3-ligase ULF (Chen et al., 2010a) and we reasoned that manipulation of ULF levels would regulate ARF levels and alter the sensitivity to IR. Indeed, siRNA mediated silencing of ULF increased ARF levels and also resulted in a decrease in sensitivity characterised by increased clonogenic potential (fig. 4.5.F).

F.

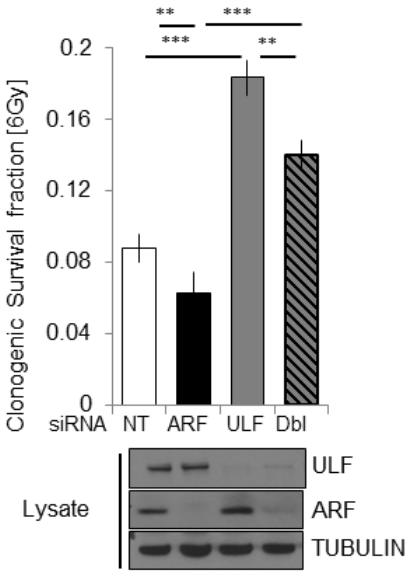


Fig. 4.5.F) T24 cells were transfected with siRNA against $p14^{ARF}$ or ULF individually or in combination as indicated. Cells were irradiated (6 Gy) and surviving fraction determined (bars, ** > 0.01; *** > 0.001, student *t*-test). Experimental replicates of n=3

In order to confirm that the phosphorylation of S48-NPM was responsible for mediating resistance to IR in p53mut cells, we expressed siRNA resistant NPM-WT and NPM-S48A in T24 cells after first ablating the endogenous NPM in them.

In agreement with the data presented, the S48A variants showed increased sensitivity to IR (decreased clonogenic survival), which corresponded to the increased ARF foci formation at the nucleolus (fig.9.6). This further suggested that AKT mediated phosphorylation of S48-NPM promotes ARF mediated increase in p53mut stability which in turn results in decreased sensitivity to IR.

4.6 – AKT inhibition relieves p53mut mediated repression of p73

The p53mut gain of function has been described in previous chapters and an important gain of function phenotype associated with p53mut is the repression of other tumour suppressor transcription factors, especially, the p53 family member p73 (Brosh and Rotter, 2009; Muller and Vousden, 2013). Since p73 shares many transcriptional targets with p53, repression of p73 by the p53mut protein results in dominant-negative suppression of the classical p53 targets (Irwin et al., 2003). Therefore we reasoned that the p53mut mediated repression of p73 maybe the reason for decreased sensitivity to IR and that the reduction of p53mut stability following inhibition of AKT maybe removing this repression of p73 and sensitising the cells. Interestingly, treatment of T24 and DLD1 cells with the inhibitors PI-103 or MK2206 resulted in a decrease in the p53 levels and a concomitant increase in the p53/p73 target genes BAX and p21 (fig.4.6.A,B and C). We confirmed that the induction of p21 was p73 transcription dependent by silencing p73 in T24 cells, which resulted in a characteristic decrease in the p21 expression (fig.4.6.B and C).

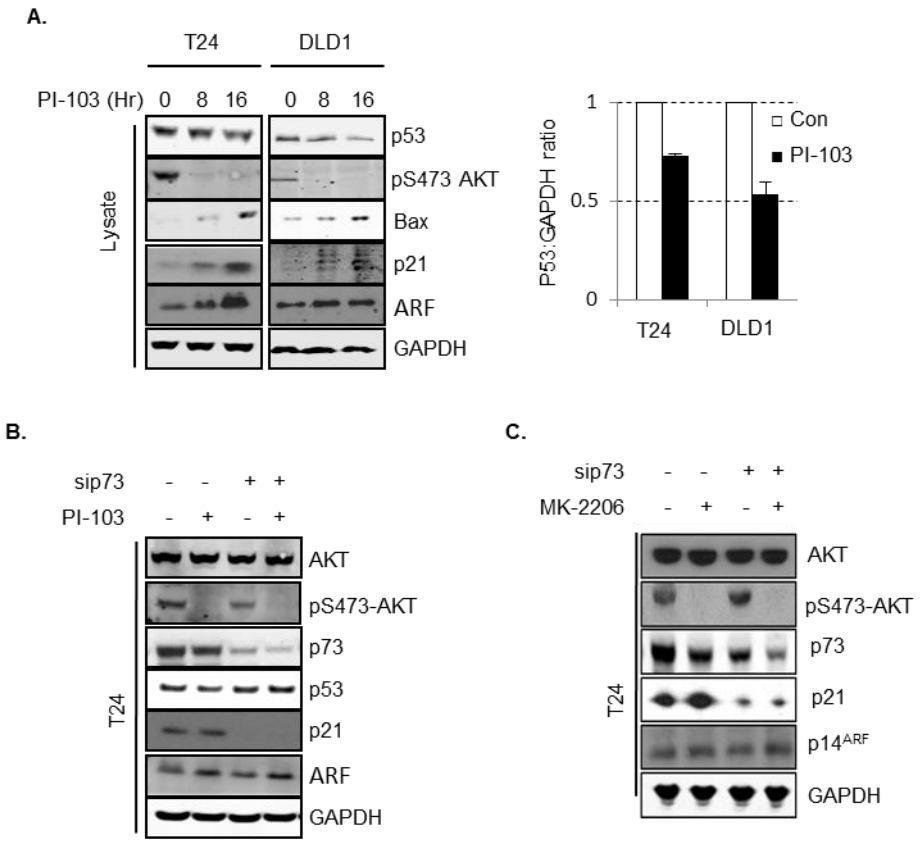


Fig.4.6.A-C: AKT inhibition relieves p53mut mediated repression of p73 A) T24 cells (p53Mut) and DLD1 cells (p53Mut) were treated with PI-103 (0.4 μM) for the indicated times. Bars indicate relative level of p53 at 8 hours. Whole cell lysates were blotted with the indicated antibodies B) T24 cells transfected with NT or p73 siRNA against p73 were treated with PI-103 (0.4 μM) for 16 hrs. Whole cell lysates were blotted with the indicated antibodies C) T24 cells transfected with siRNA against p73 or a non-targeting control and were treated with MK-2206 (0.5 μM) for 16 hrs. Whole cell lysates were probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

p73 activates p21 mediated senescence has been described earlier, and in line with the existing information, the induction of p21 in the T24 and DLD1 cells was accompanied by a G1/S cell cycle arrest (fig.4.6.D) and an induction of senescence as seen by an increase in the senescence marker β-galactosidase (fig.4.6.E). Furthermore, the induction of senescence was significantly increased following IR in the cells that had been pre-treated with MK2206 suggesting in

combination with the data presented above, that the AKT inhibition decreases the p53mut levels and subsequently the repression of p73, thereby promoting the p73 mediated increase in senescence.

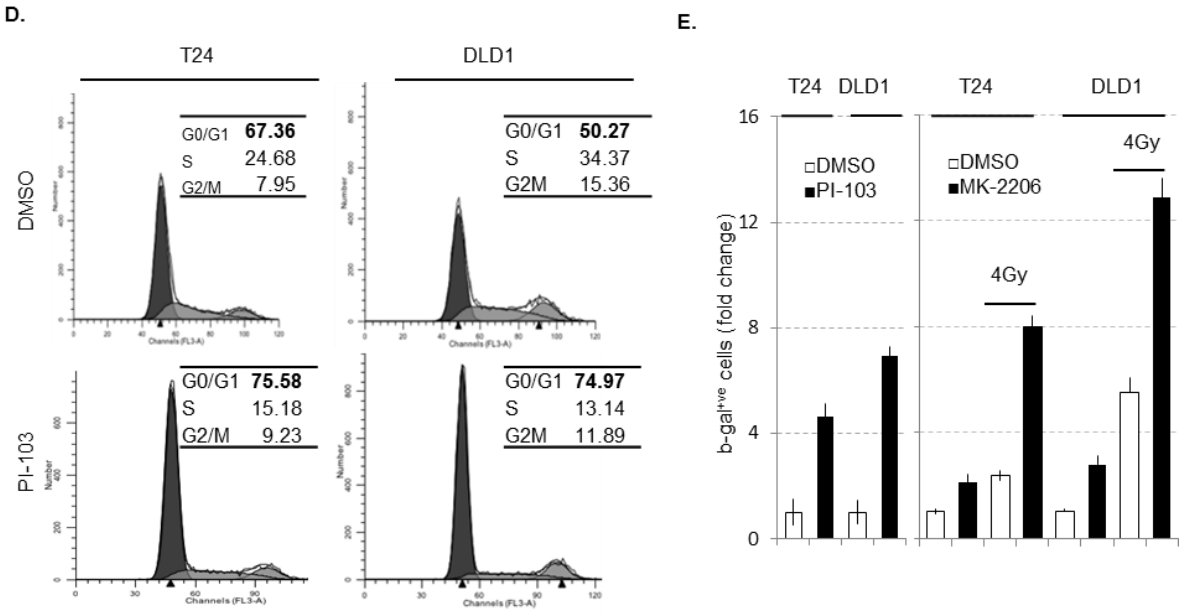


Fig.4.6.D and E: D) T24 and DLD1 cells were treated with PI-103 (0.4 μM) or DMSO and stained with propidium iodide prior to FACS analysis of cell cycle profiles. E) T24 and DLD1 cells were treated with PI-103 (0.4 μM) as above (left) or MK-2206 (5 μM) for 24 hrs and then exposed to single dose of ionizing radiation (IR) (4 Gy). Cells were maintained in culture media in the absence of drug for 5 days and senescent cells determined by staining for β-galactosidase activity. Bars indicate fold increase over background.

4.7 – AKT mediates regulation of p53mut in KPC mouse pancreatic cancer derived cells

Pancreatic cancers are invariably associated with activating KRAS mutations and are also quite frequently associated with concomitant p53 mutations, with many *in vivo* models having been developed to investigate this. In order to confirm all the data presented in the preceding sections, we took advantage of pancreatic tumour cell-lines derived from the $KRas^{G12}$ Pdx1-cre,

$p53^{R172H}$ (KPC) mouse models of pancreatic ductal adenocarcinoma. Pancreatic cancer tumour cells were derived by the collaborating lab of Dr. Owen Sansom at the Beatson Institute, Glasgow, from KPC ($KRas^{G12D}$: $p53^{R172H}$), *Trp53*flox ($KRas^{G12D}$: $p53^{fl}$), and ARF-/- ($KRas^{G12D}$: $p53^{R172H}$:$ARF^{-/-}$) mice which have been described previously (Hingorani et al., 2003; Jackson et al., 2001; Jonkers et al., 2001; Kamijo et al., 1997; Olive et al., 2004). The cells showed similar growth and survival characteristics (fig.4.7.A), but not surprisingly, only the ARF positive cells showed stabilization of p53mut (fig.4.7.B). Interestingly, we also confirmed that the p53mut was degraded by the endogenous MDM2, since treatment of the cells with Nutlin 3a stabilized the p53mut protein irrespective of the ARF status of the cells.

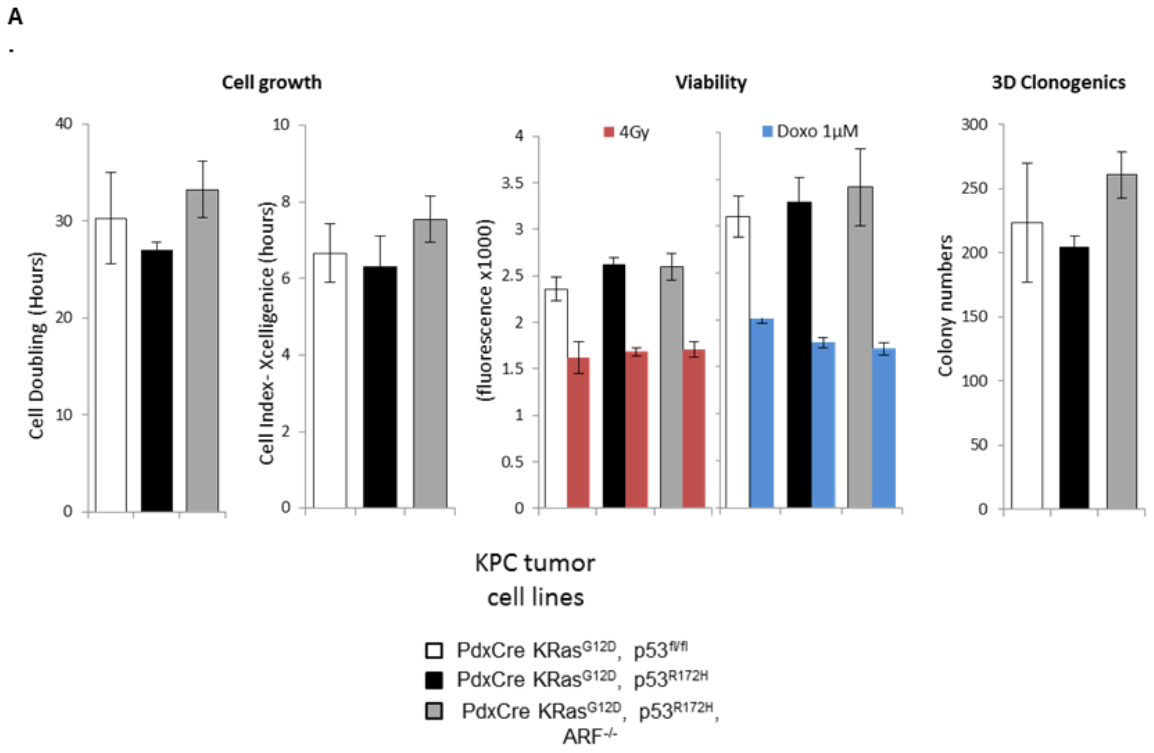


Fig. 4.7.A: KPC mouse derived pancreatic cancer cell lines- growth characteristics

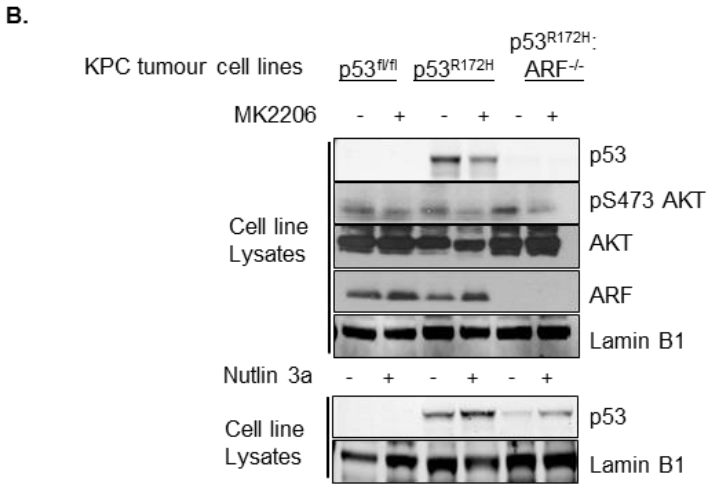


Fig. 4.7.B: p53mut stability is influenced by ARF and MDM2: KPC mice derived KRAS^{G12D} p53 Floxed (p53Fl/Fl), KRAS^{G12D} p53^{R172H} ARF+/+ and KRAS^{G12D} p53^{R172H} ARF-/- pancreatic tumour cells were treated with MK2206 (1μM), Nutlin3A (5μM) or DMSO as indicated. Whole cell lysates were probed with the indicated antibodies. Representative western blot images from experimental replicates of n=3

4.8 – Colony formation of KPC mouse pancreatic cancer cells is sensitive to AKT inhibitors

The ARF and MDM2 mediated decrease in p53mut following AKT inhibition and the consequent increase in sensitivity seen in tumour cells were further tested in the KPC mouse derived cells.

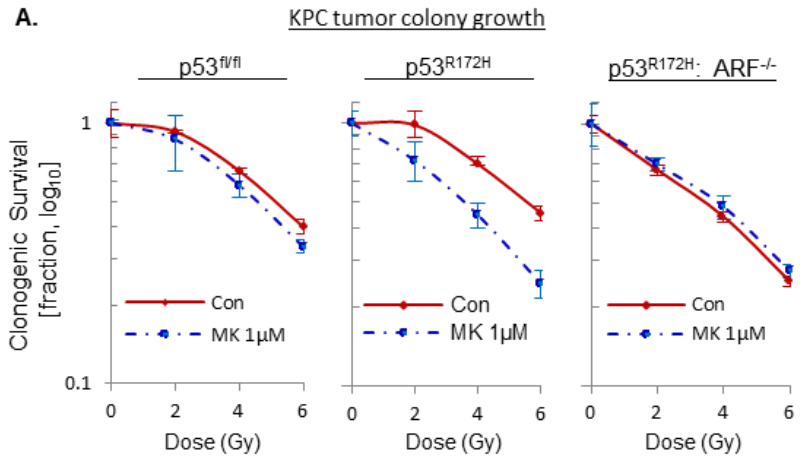


Fig.4.8.A: AKT inhibitors sensitise only KPC mouse cells positive for ARF to IR: Clonogenic survival of KPC mice derived KRAS^{G12D} p53 Floxed (p53Fl/Fl),

$KRAS^{G12D}$ $p53^{R172H}$ ARF+/+ and $KRAS^{G12D}$ $p53^{R172H}$ ARF-/- pancreatic tumour cells following treatment with radiation at the indicated doses. Cells were pre-treated with MK2206 (1µM) or DMSO before irradiation

Clonogenic assays on the KPC derived pancreatic tumour cells showed increased sensitivity to IR following AKT inhibition, only in the case of the p53mut positive cells that also possessed functional ARF. The ARF null cells showed no increase in sensitivity nor did the p53 floxed cells, indicating that the effects of AKTi were dependent on p53mut and ARF. This was strong evidence to support our mechanism where tumour cells that possess a mutant p53 gene suppress responses to IR only in an ARF and AKT dependent manner.

4.9 – AKT inhibitors restricts KPC pancreatic cancer 3D spheroids

The growth characteristics and sensitisation of the $p53^{R172H}$ (ARF^{+ve}) cells to IR following AKT inhibition was not just a feature restricted to 2D colony forming potential. The same increase in sensitivity was also characteristically seen in 3D spheroid growth with the p53mut and ARF positive cells alone showing increased sensitivity to IR (fig.4.9.A) following AKT inhibition. An interesting feature noted was that the $p53^{R172H}$ (ARF^{+ve}) colonies showed typical morphological patterns characteristic of invasiveness suggesting the more invasive nature of the cells that possess high levels of p53mut(fig.4.9.A).

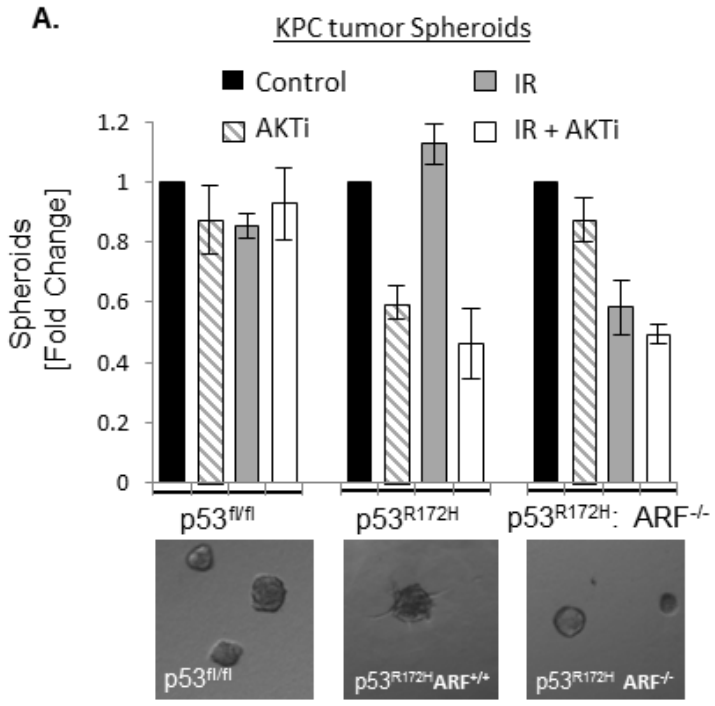


Fig.4.9.A: 3D growth of KPC mouse cells: Bars showing 3D clonogenic survival of KPC mice derived KRAS^{G12D} p53Fl/Fl, KRAS^{G12D} p53^{R172H} ARF+/+ and KRAS^{G12D} p53^{R172H} ARF-/- pancreatic tumour cells following treatment with 6Gy radiation. Cells were pre-treated with MK2206 (1µM) before radiation. Lower panel shows representative images of 3D colonies with the p53^{R172H} (ARF^{+ve}) colonies showing the 'spiky' invasive morphology. Experimental replicates of n=3

4.10 – Chapter 4 discussion

The regulation of NPM oligomerisation, the subsequent localisation of ARF and the modulation of its activity on MDM2 were explored in the previous chapter. In this chapter, we looked at the downstream effects of this regulation, with a focus on the MDM2 substrate p53. We first examined the increased MDM2 activity following AKT inhibition by studying its effect on the MDM2 substrates. A clear increase in the MDM2 activity following AKT inhibition was seen; with both wild type p53 as well as MDM2 itself showing increased ubiquitination (fig. 4.2). Interestingly, we also found that p53mut protein including different "hot-spot"

mutant variants, showed a similar increase in ubiquitination and a consequent decrease in stability, confirming previous *in vivo* reports where MDM2 was shown to regulate p53mut (Terzian et al., 2008). This was also quite clearly an NPM-ARF dependant post-translational effect and discounted the possibility of translation or transcription mediated changes (fig. 4.3). The results seen with HA- Ubiquitin (figs. 4.2 and 9.4) can be improved using His tagged Ubiquitin as they are stable under denaturing conditions and can potentially avoid the drawbacks associated with epitope tags like HA (Haemagglutinin) which can cause denaturation of the anti-tag antibodies (under denaturing conditions) and result in refolding of proteins and potentially allowing non-covalent interactions to occur. Furthermore, the NPM-ARF dependent effects observed were strengthened by the finding that inhibition of ARF and NPM reverses the effects seen on p53mut stability. Examination of a panel of cell lines with different p53 and ARF status showed that the cells having functional ARF alone exhibited a decrease in p53 stability irrespective of the mutation status (fig.4.4).

Since p53mut stability was clearly affected by AKT inhibition, the next logical step was to study the effect that the decrease in p53mut stability was having on the tumour cells. p53 mutant cells have been reported to be resistant to treatment with IR (Lee and Bernstein, 1993) due to its dominant negative effects (Freed-Pastor and Prives, 2012) and a reduction in p53mut stability has been hypothesised to increase the sensitivity of p53mut tumours to treatment. Interestingly, we saw an increase in therapeutic sensitivity (fig. 4.5) in p53mut cells treated with PI3K/AKT inhibitors. We were able to demonstrate that this was an NPM-ARF and p53mut dependent effect, as inhibiting p53mut or ARF and modulating the NPM in cells (fig. 4.5) increased their sensitivity to IR. It has been previously demonstrated that

p53mut can bind to and repress other transcription factors including other members of the p53 family of tumour suppressive transcription factors (Muller and Vousden, 2013) which include p73 and p63 and that a decrease in the p53mut levels re-established the sensitivity to treatment. The decrease in p53mut stability that we see following AKT inhibition in ARF competent cells relieved the repression primarily on p73, leading to a p73 mediated activation of the senescence genes and a consequent initiation of senescence as well as G1/S cell cycle arrest. Since our initial findings were reported on cancer cells which could be argued to have other stochastic genomic alterations that could be responsible for the effects we see, we decided to look at pancreatic cancer cells derived from the KPC mouse which were identical except for their p53mut and/or ARF status. Interestingly, but as expected, AKT inhibition in the p53mut cells sensitised them to IR only in the presence of a functional ARF, clearly validating our model at least in the *in vitro* setting. We thus confirm our hypothesis that AKT inhibition results in a NPM-ARF mediated increase in MDM2 activity which subsequently leads to a decrease in p53mut and a relief in the repression of p73. Consequently, tumour cells show increased sensitivity to concomitant treatment with radiation, highlighting the exciting prospect of using combination therapy with AKT inhibitors and DNA damaging agents to sensitise p53mut tumours in the *in vivo* setting.

Chapter 5

AKT mediated regulation of p53mut sustains p53mut tumours *in vivo*

Chapter 5 – AKT mediated regulation of p53mut sustains p53mut tumours *in vivo*

5.1 – PSNI xenografts in nude mice

The data presented in previous chapters indicated that AKT regulates NPM oligomerisation and localisation. It also suggested that AKT inhibition resulted in NPM and ARF localisation to the nucleolus, which in turn promoted increased MDM2 activity. This would result in increased ubiquitination and turnover of MDM2 substrates, primarily p53. We demonstrated that this same mechanism functions in p53mut cells, where AKT inhibition resulted in a decrease in p53mut levels and a relief in the repression of p73, which in turn sensitised tumour cells to DNA damaging therapies like IR. To verify our observations and to determine if AKT inhibition could sensitise tumours to IR, we decided to study a pancreatic tumour model in the *in vivo* system. We used a PSN1 pancreatic adenocarcinoma mouse xenograft tumour model which included stromal support to appropriately replicate the human disease (Mantoni et al., 2011). The tumours once established were treated sub-cutaneously with MK2206 at doses of 60mg/kg, 120mg/kg and 320mg/kg and alternate day scheduling as described previously (Cheng et al., 2012; Cherrin et al., 2010; Gorlick et al., 2012; Hirai et al., 2010), harvested and subject to further analysis. We were keen to identify the lowest dose that could produce adequate AKT inhibition since we wanted to use the drug as a sensitising agent rather than as monotherapy for further *in vivo* studies in combination with other agents. The data obtained confirmed our initial hypothesis and led us to analyse various clinical samples and clinical data-sets to confirm the significance of our findings.

5.2 – MK2206 regulates NPM oligomerisation and ARF localisation in PSN1 xenografts

The inhibition of AKT and a subsequent decrease in the stability of p53mut in PSN1 cells have been described in previous sections. In order to confirm our findings in the *in vivo* setting, we established PSN1 xenografts on the flanks of athymic nude mice, which included stromal support with stellate cells to replicate the human disease. The tumours were treated with DMSO or MK2206 at the doses and schedules as given below, once they reached the randomisation volume of 80-100mm^3.

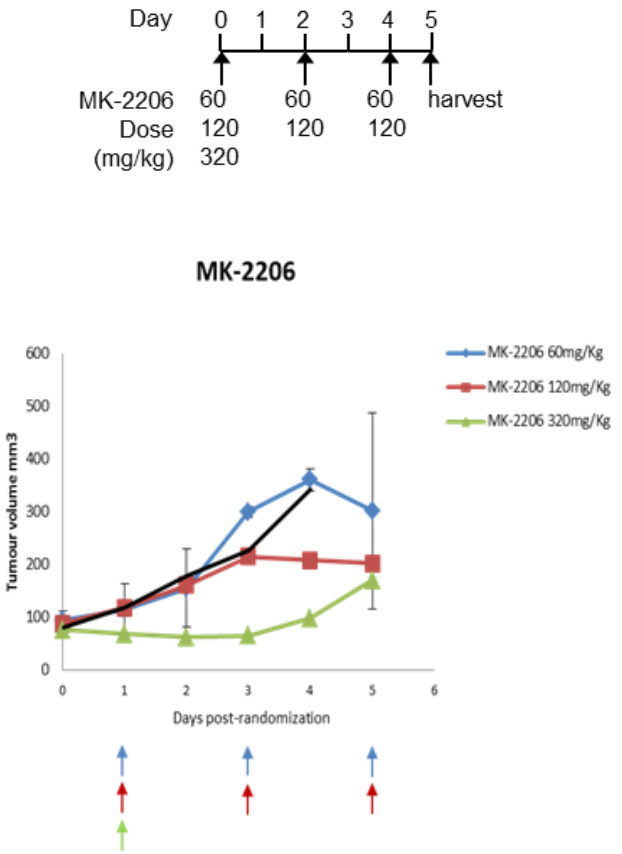


Fig.5.2.A: Panel above showing dosage and frequency of treatment with MK2206 (60 mg/kg-320 mg/kg) as indicated or β-cyclo-dextrin (1.5 mg/ml) carrier on PSN1 xenografts (PSN1 cells co-injected with LTC-14 stellate cells) established in the flank of athymic nude mice. Lower below showing tumour volumes measured from the day of randomisation till the day of harvest.

The animals were sacrificed and tumours harvested the day after the last treatment with MK2206. Tumour lysates from both the 60mg/kg and the 120mg/kg treatment arms showed a decrease in the pS473-AKT and pS48-NPM, which also correlated with the enhanced oligomerisation of NPM and a significant increase in p21 (fig.5.2.B). Not surprisingly, the larger single dose of 320mg/kg did not show detectable AKT inhibition as the tumours were harvested on day 5 after the single dose of the drug, by which time the drug would have been metabolised and the AKT inhibition lost. Interestingly, although not part of the original study aims, a follow-up of the tumour sizes on the 5 days of the experiments showed no significant differences between the control, 60mg/kg and the 120mg/kg arms (fig.5.2.A), while the higher single dose of 320mg/kg alone showed a growth delay.

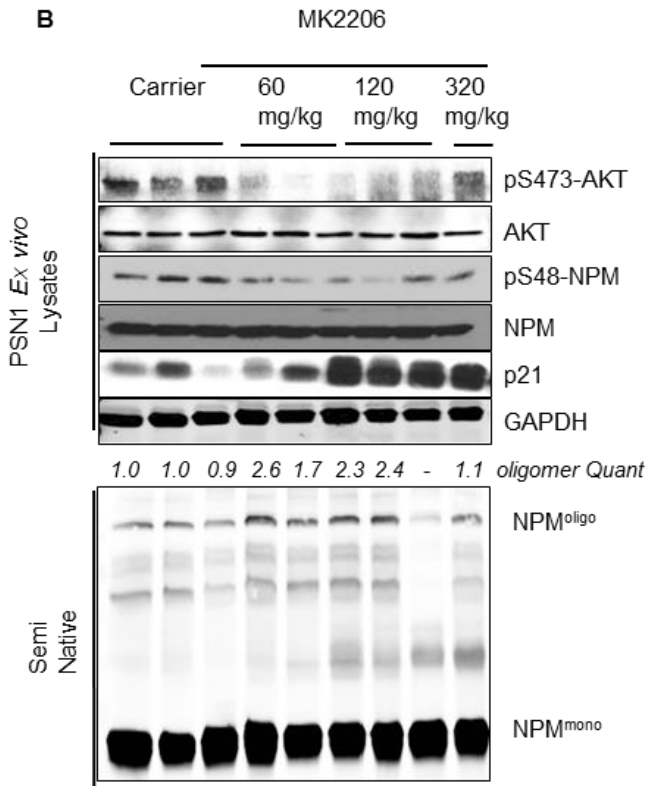


Fig.5.2.B: PSN1 Xenograft tumours treated with MK2206 (60 mg/kg, 120mg/kg and 320 mg/kg) or β-cyclo-dextrin (1.5 mg/ml) carrier were lysed and lysates probed by western blot with the indicated antibodies

Along with the changes in the NPM oligomerisation, treatment of the tumours with MK2206 was associated with the re- localisation of ARF to the nucleolus (fig.5.2.C), recapitulating our *in vitro* findings.

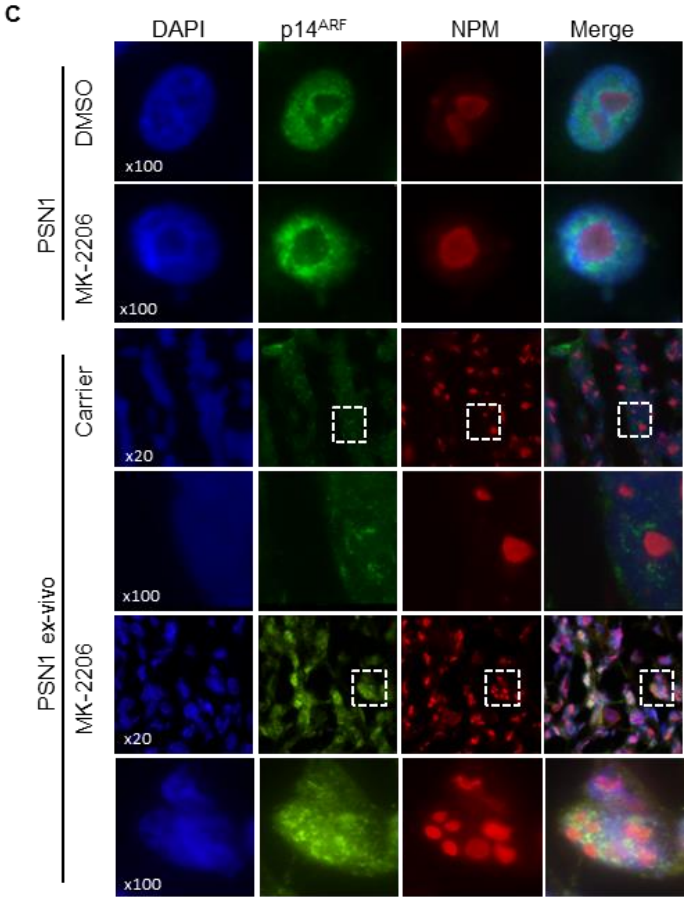


Fig.5.2.C: Sections of PSN1 xenografts (lower panel) and *in-vitro* PSN1 cells (upper panel) fixed and stained with anti-NPM (red) and anti-p14ARF (green).

5.3 – AKT inhibition regulates mutant p53 levels in PSN1 xenografts

We had shown in our *in vitro* studies that the changes in the NPM oligomerisation and the increased re-localization of ARF to the nucleolus following treatment with MK2206 was associated with an increase in the MDM2 activity and a decrease in the stability of its substrate p53. We saw a similar effect in the PSN1

xenografts, where the treatment of the tumours with MK2206 resulted in a decrease in the p53mut stability. Tumour sections stained with the p53 specific DO1 antibody or the p53mut specific Ab240 antibody showed a decrease in protein levels characterised by a decrease in staining on immunofluorescence. Immuno-Histo Chemistry (IHC) also revealed a decrease in the p53 levels in the tumour sections of the MK2206 treated animals that corresponded to the decrease in the pS473-AKT and the pS48-NPM levels.

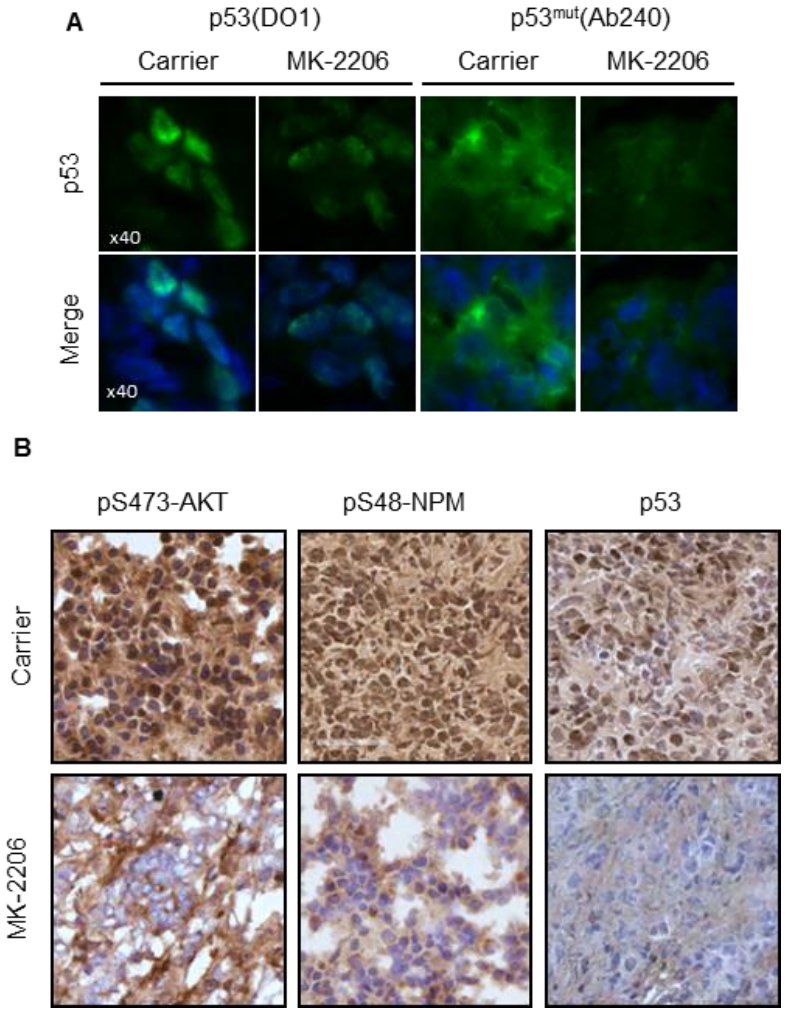


Fig. 5.3.A and B: AKT inhibition regulates mutant p53 levels in PSN1 xenografts A) PSN1 xenografts treated with MK2206 or carrier were stained with DAPI, anti-p53 (DO1) or p53mut (Ab240) (B) PSN1 xenografts treated with MK-2206 (60 mg/kg) or carrier were stained by immunohistochemical methods with anti-pS473-ATK, anti-pS48-NPM or p53

5.4 – MK2206 sensitises PSN1 xenograft tumours to radiation and increases survival in mice

The AKT inhibition and subsequent decrease in p53mut that reinstates the tumour suppression and DNA damage responses and also helps sensitise tumour cells *in vitro* to IR, was described above. To test the synergistic effects seen *in vitro*, we selected the minimal dose of MK2206 that we tested *in vivo* and which showed reduction in AKT activity (60mg/kg). We then proceeded to set up another PSN1 xenograft study where we treated the tumours with two alternate day doses of 60mg/kg MK2206 and a single dose of IR (6 Gray, XRT) according to the schedules shown in fig.5.4.A.

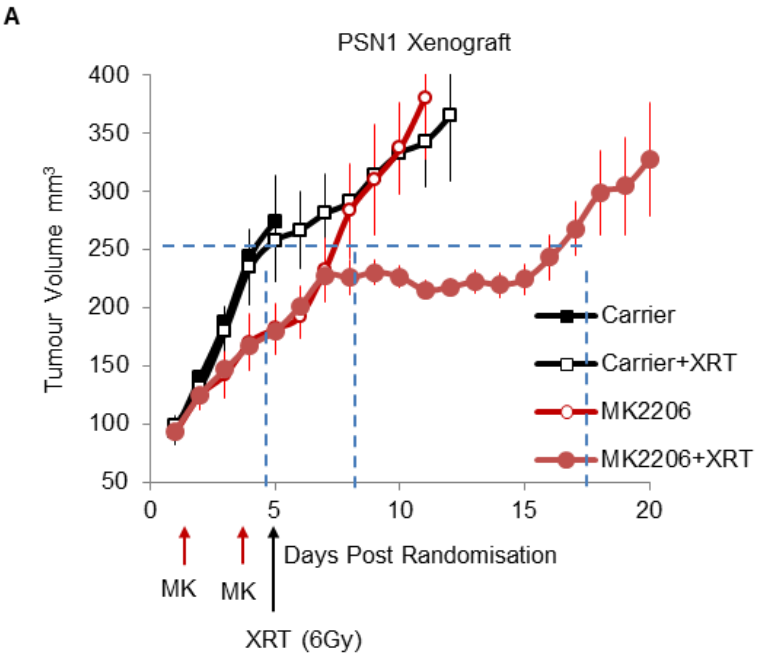


Fig.5.4.A: PSN1 xenografts established in the flank of athymic nude mice were injected subcutaneously with two alternate day doses of MK2206 (60 mg/kg) or carrier. Mice were subsequently treated with a single dose of IR (6 Gy) and tumour volumes measured regularly with callipers. Dash lines indicate tumour growth differential at 250 mm^3.

MK2206 induced tumour growth delay of 3.3 days compared to the control animals (fig.5.4.A), although overall survival (measured by the surrogate of 4 times the tumour volume from time of randomisation and initiation of treatment), was not affected. In contrast, the treatment arm with the combination of MK2206 and XRT resulted in an additional growth delay of 9.2 days and 12.5 days compared to the XRT and MK2206 mono-therapy arms (fig.5.4.A). This also resulted in a significant increase in survival of the treated animals (fig. 5.4.B and fig. 9.7).

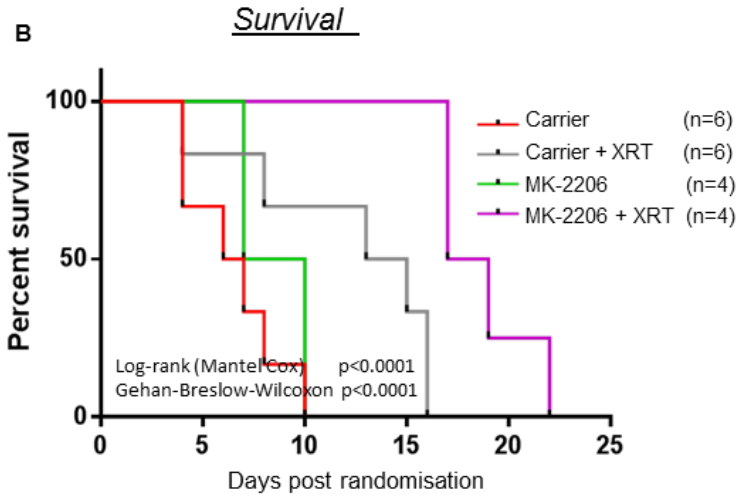
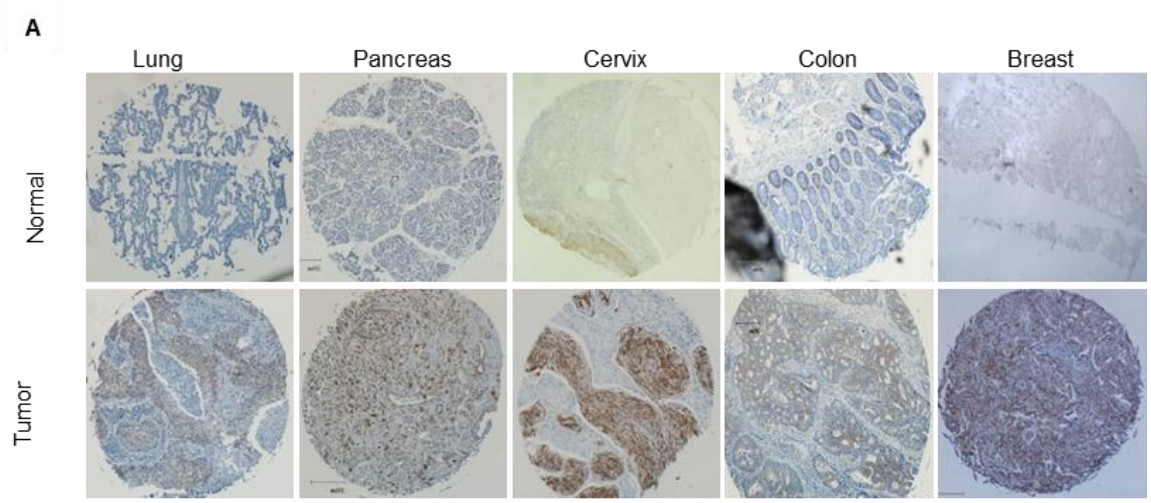


Fig.5.4.B: Survival data (4X initial tumour volume) of the mice with PSN1 xenografts treated with two alternative day doses of MK2206 (60 mg/kg) or β-cyclo-dextrin (1.5 mg/ml) carrier with or without a single dose of IR (6 Gy). Mice were sacrificed when any single dimension reached 12.5 mm.

5.5 – pS48-NPM is expressed in human tumours

The *in vitro* and *in vivo* data presented established our hypothesis that phosphorylation of S48-NPM by AKT promoted its localisation to the nucleoplasm, which in turn resulted in the nucleoplasmic translocation of ARF. The subsequent inhibition of MDM2 activity and stabilisation of p53mut consequently resulted in treatment resistance. To address if this was a common phenomenon seen in

cancers, we next decided to look at the S48-NPM expression in human tissue samples, both from normal tissue and tumour tissue. We addressed the pS48-NPM levels by IHC on commercially available tissue micro-array sections of a number of tumours that are frequently associated with mutational events that result in the activation of the PI3K/AKT pathway. The tissue sections from lung, pancreas, colon and breast stained positive for pS48-NPM in more than 50% of cases and were scored as low, moderate and high (fig.5.5.A).



Tissue	Lung	Pancreas	Cervix	Colon	Breast
Total (n)	43	40	200	69	144
pS48-NPM^{+}	21	20	130	44	117
% positive	48.8	50	64	63.8	80.7
Low	16	10	70	33	40
Moderate	5	6	31	7	51
High	0	4	29	4	26

Fig.5.5.A: pS48-NPM expression in human tumours: Tissue micro array sections (US Biomax) of both normal and tumour derived tissue from lung, pancreas, cervix, colon and breast were stained for pS48-NPM. All images are 5 x magnifications and scale bars represent 200 µm. Total numbers of samples analysed by automated Aperio scanning and those with low, moderate or high degrees of staining are shown in table in the lower panel.

The tissue sections on the TMA's were not separately verified by a histo-pathologist and results seen may be confounded by the presence of aberrant tissue samples that do not correlate with the supposed tissue of interest.The cervical cancer sections were included as additional positive controls since Human Papillomavirus (HPV) infected tissue is known to over-express NPM and also displays increased AKT activity (McCloskey et al., 2010). Fig.5.5.B shows pancreatic cancer TMAs where the pS48-NPM antibody specifically identifies pS48-NPM without cross-reacting with total NPM. This data alongwith the cell-line data presented in previous chapters suggested that phosphorylation of S48-NPM and the regulation of NPM quaternary structure was perturbed in a number of solid tumours.

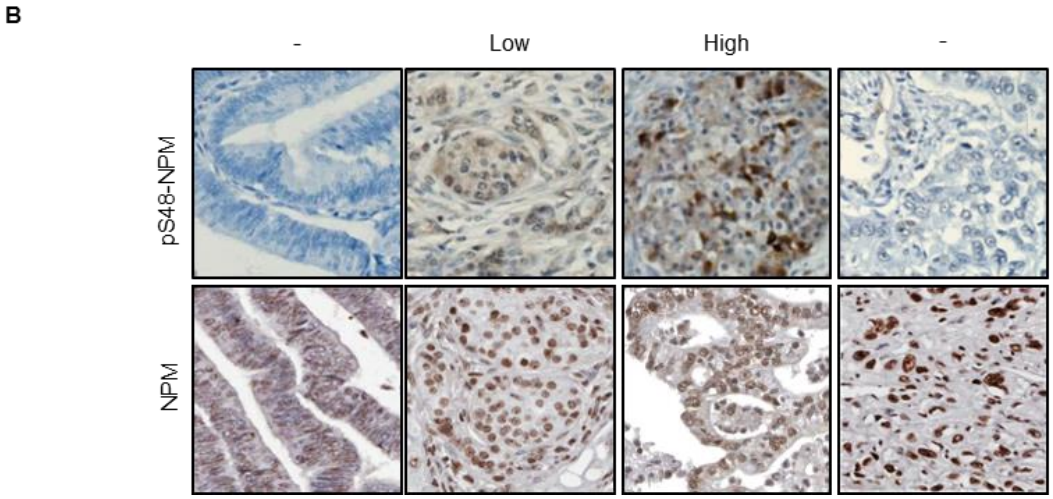
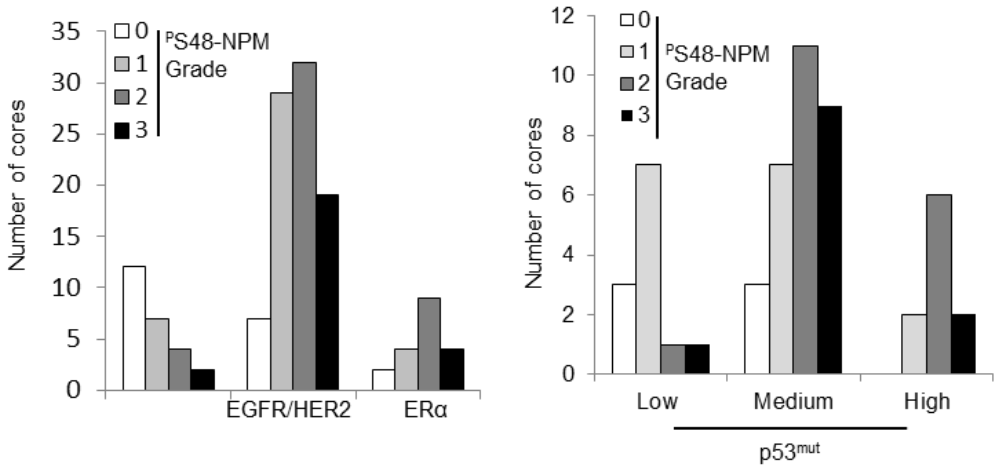


Fig.5.5.B: TMA from pancreatic tumors were stained for pS48 NPM (top) or total NPM (bottom).

5.6 – pS48-NPM and p53 expression correlates with upstream mutations that activate PI3K/AKT in breast cancers

The therapeutic resistance and the associated prognostic significance of p53mut stability has been demonstrated in breast cancers (Walerych et al., 2012). Likewise, the EGFR/Her2 and Estrogen Receptor α (ERα) positive breast cancers have been shown to have elevated AKT activity (She et al., 2008b). Taking these into consideration, we aimed to identify if there was a correlation between pS48-NPM and p53 staining, EGFR/HER2 status and tumour stage in the breast cancer cohort.

A



B

TNM / EGFR/HER2+	Tis	T2/T3	T4
p53 -	5.1%	79.5%	12.8%
p53+	3.8%	73.0%	23.2%

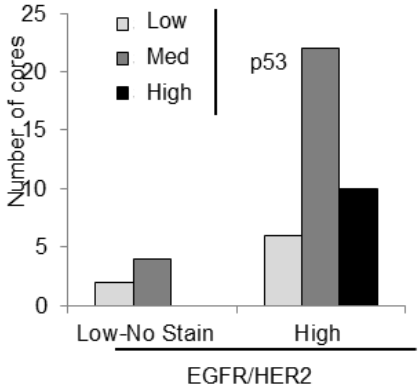


Fig.5.6.A and B: A) (Left) Data from the breast tumour microarray demonstrating the degree of pS48-NPM staining in those cores annotated as EGFR/HER2 positive (low and high) or estrogen receptor (ER) +ve. (Right) Phospho-S48-NPM staining in cores scored low, medium or high for p53. B) Data from the breast tumour microarray, demonstrating the correlation of EGFR/HER2 positivity and low, medium or high p53 staining.

The highest levels of pS48-NPM staining correlated with EGFR/HER2 positivity as well as increased p53 staining (indicative of p53mut) (fig.5.6.A). Furthermore, the increased p53 staining (p53mut) was also associated with advanced stage disease (fig.5.6.B). Previous studies have shown that not all EGFR/HER2 or ERα

positive breast cancers have p53 mutations. However co-occurrence of both are associated with advanced stage disease (Coates et al., 2012). Interestingly, our data suggested that aberrant AKT activity stabilised p53mut activity, which was in contrast with previous reports that indicated AKT activity reduced p53 levels by nuclear translocation of MDM2.

		AKT activity			
		pS473	pT308	pS473	pT308
p53 vs AKT activity	Split *CDKN2A* (mRNA)	(<50)		(>50)	
	p53 (protein) correlation	-0.281	-0.284	-0.092	-0.016
	sig. (2 tailed)	0.014	0.013	ns	ns
	N	76	76	326	326
	Split *CDKN2A* (mRNA)	(<100)		(>100)	
	p53 (protein) correlation	-0.276	-0.203	-0.014	0.22
	sig. (2 tailed)	0.0002	0.006	ns	ns
	N	180	180	222	222
	Split *TP53* (mutation)	(no Mut)		(Mut)	
	p53 (protein) correlation	-0.236	-0.132	-0.07	-0.069
	sig. (2 tailed)	4.4×10^{-5}	0.023	ns	ns
	N	295	295	107	107

		pS473-AKT	pT308-AKT	p53 (protein)
AKT activity vs AKT substrate	pRAS40 (phospho) correlation	0.491	0.474	0.275
	sig. (2 tailed)	1×10^{-13}	1×10^{-13}	1.99×10^{-8}
	N	402	402	402

		p53 Status					
		all p53		non-Mut		Mutant	
AKT activity vs p53	Split *CDKN2A* (mRNA)	(<50)	(>50)	(<50)	(>50)	(<50)	(>50)
	pRAS40 (protein) correlation	0.017	0.327	0.079	0.240	-0.248	0.314
	sig. (2 tailed)	ns	1.5×10^{-9}	ns	0.0002	ns	0.002
	N	76	326	62	233	14	93
	Split *CDKN2A* (mRNA)	(<100)	(>100)	(<100)	(>100)	(<100)	(>100)
	pRAS40 (protein) correlation	0.143	0.37	0.134	0.296	0.001	0.314
	sig. (2 tailed)	ns	1×10^{-8}	ns	0.0003	ns	0.005
	N	180	222	152	143	28	79

Table.5.6: AKT activity positively correlates with p53 protein levels in ARF (CDKN2A) expressing breast cancers (TCGA). (Table courtesy of Dr. Anna Grawenda).

To confirm if the discrepancy between our findings and previous reports was due to the presence of ARF, we examined AKT activity and p53 levels in a large cohort of invasive breast cancer in The Cancer Genome Atlas (TCGA) database where the ARF (CDKN2A) expression was known. The correlation of p53 protein levels

with the activity of AKT was studied in 402 breast cancer patients from the TCGA database for whom complete information on protein and gene expression levels together with *TP53* mutational status was available. The data was downloaded from cBioPortal for Cancer Genomics (Cerami et al., 2012; Gao et al., 2013) and analysed with SPSS 21.0 software (analysis done by Dr. Anna Grawenda). The Shapiro-Wilk test was used to assess distribution of datasets, whereby the null hypothesis of normal distribution was rejected for all datasets tested ($p<0.05$). Correlation analysis was carried out using the non-parametric Spearman-Rho test.

The p53 protein expression was found to negatively correlate with increased AKT activity where the CDKN2A activity was below the background levels. The negative correlation was lost with increased CDKN2A expression or when mutations were present (Table 5.6.A). To test the relationship with AKT activity, we looked at the phosphorylation of the AKT substrate PRAS40 which has been used previously in the clinic to monitor AKT activity (Yap et al., 2011). We found that the AKT activity correlated with the PRAS40 phosphorylation and also positively correlated with the p53 levels. This positive correlation was seen in the case of both p53 and p53mut, but interestingly was restricted to patients where the CDKN2A expression was detectable above the background levels. Furthermore, the correlation was lost in the CDKN2A absent cases. This data was significant as it confirmed in patients the dual role of AKT to act as both a positive and negative regulator of p53 depending on the expression of ARF (the presence of ARF promoting stabilisation and the absence promoting increased MDM2 mediated p53 degradation in the nucleus).

5.7 – AKT activity correlates with pS48-NPM and p53 expression in a CDKN2A dependent manner in pancreatic cancers

The pancreatic tumour sections in the TMA discussed above indicated 50% pS48-NPM positivity even though most pancreatic cancers are known to harbour KRAS mutations involving the G12, G13 and Q61 residues of KRAS which would be expected to increase AKT activity uniformly. Not surprisingly, since these mutations result in a range of amino acid substitutions, it can result in differential downstream pathway activation (Pylayeva-Gupta et al., 2011) and pancreatic tumours harbouring the KRAS mutations have indeed been associated with variable AKT activity (Kennedy et al., 2011). In order to further address the relationship between AKT activity (pS473-AKT), pS48-NPM levels and p53 levels in pancreatic cancers, we obtained fresh Pancreatic TMAs and stained them for the three markers. 50% of the tumour sections were found to be on the lowest quartile of pS48-NPM staining and displayed low AKT activity, possibly due to variable AKT activation by the different RAS mutations. Significantly the pS48-NPM levels and pS473-AKT were seen to correlate, with the highest correlation seen in the nucleus (fig. 5.7).

We have seen in previous chapters that the RXXS motif corresponding to the S48 moiety of NPM lies in a characterised Nuclear Export Sequence. Interestingly, we found that when we compared the cytoplasmic pS48-NPM to elevated p53 (suggestive of p53mut), 81 of the 122 tumours were positive for p53 and significantly correlated with the pS48-NPM cytoplasmic levels (fig.5.7- panel on the right).

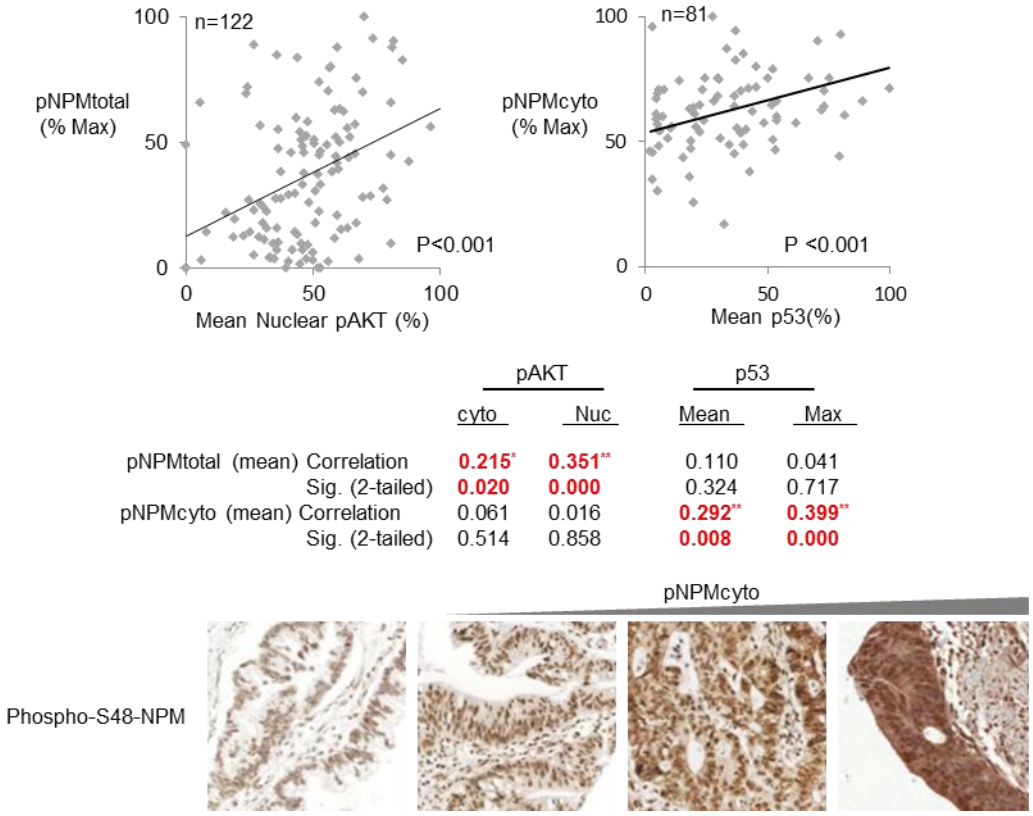


Fig. 5.7: Correlation between pS48-NPM and AKT/p53 expression in pancreatic cancers A) Scatter plots demonstrating bivariate correlation of automated total phospho-S48-NPM and nuclear phospho-S473-AKT staining in human pancreatic ductal adenocarcinomas (n=122, left), or cytoplasmic phospho-S48-NPM in samples with p53 positivity (n=81, right). Below, representative images for comparison of variation in phosphoS48-NPMcyto scoring of pancreatic ductal adenocarcinoma tumour micro array sections.

The INK4A/ARF locus has been known to be inactivated by methylation or deletion in pancreatic cancers (Bardeesy et al., 2006).While deletion of the INK4A locus can result in loss of both splice variants, quite often, the functional loss of p14ARF may be independent of p16 or vice-versa especially in the presence of inactivating methylation events (Esteller et al., 2000; Freedberg et al., 2008). Taking this into account, we obtained mRNA from a subset of the pancreatic tumours for which tissue samples were available. We found that 26 out of the 40 tissue samples available were positive for ARF mRNA transcript levels and in

these ARF positive tumours, there was a significant correlation between pS48-NPM and p53 staining levels (fig.9.8), suggesting that ARF was essential for this observation.

5.8 – Chapter 5 discussion

The regulation of NPM oligomerisation by AKT and the subsequent role that this regulation plays in maintaining the stability of p53mut has been dealt with in previous chapters. We showed that inhibiting the AKT activity in p53mut cells using different modalities resulted in an NPM-ARF-MDM2 mediated decrease in p53mut stability. The decrease in p53mut in the cells released the dominant negative effects of the mutant protein, manifested as a decrease in the repression of p73 and a consequent increase in the sensitivity to DNA damaging treatments including IR. Having confirmed our mechanism using various modalities, we next decided to study the effect of AKT inhibition on xenograft tumours in mice and the utility of combining AKT inhibitors with IR for treatment. We further analysed patient derived tumour samples for identifying a correlation between AKT activity, NPM expression and p53 expression in different solid tumour types.

We used the PSN1 pancreatic adenocarcinoma cells along with stromal support (stellate cells) to set up a mouse xenograft tumour model to appropriately replicate the human disease (Mantoni et al., 2011). Different doses of the AKT inhibitor were used as has been previously described and the tumours harvested and analysed for the protein expression by various methods. In a manner recapitulating the *in vitro* findings that were described previously, we found that AKT inhibition resulted in a decrease in the pS48-NPM levels, an increase in the

oligomerisation of NPM and a consequent increase in the localisation of ARF to the nucleolus. Quite interestingly, we saw that this was associated with a decrease in the levels of p53mut protein and a subsequent increase in the p21 expression levels, further confirming our *in vitro* data. Since monotherapy using AKT inhibitors have been reported to have minimal anti-tumour effects especially in the context of activating mutations in the mitogen signalling pathways (Hirai et al., 2010; Ihle et al., 2009), we decided to combine treatment with MK2206 and IR in the same tumour model and to study their effect on tumour growth. As with previous reports, AKT inhibition itself did not show an appreciable delay in tumour growth. But very interestingly, combining MK2206 with IR showed a significant tumour growth delay and increased survival that clearly exceeded the effect of IR and AKTi monotherapies. This strongly supported our previous findings and signified the importance of using AKT inhibitors in combination with DNA damaging agents in the treatment of p53mut tumours.

Since our observations in the *in vivo* models confirmed our *in vitro* findings, we decided to look at the correlation between the expressions of the main protein players in the AKT-NPM-p53 pathway. We stained Tissue Microarray sections from different cancers that were known to express mutations activating PI3K/AKT for pS48-NPM. Interestingly more than 50% of the Lung, Pancreas, Colon and Breast cancer tissues examined showed increased pS48-NPM staining signifying the importance and prevalence of the phosphorylation in tumours. The tissue sections on the commercially available TMA's were not separately verified by a histo-pathologist and results seen may be confounded by the presence of aberrant tissue samples that do not correlate with the supposed tissue of interest. In spite of this drawback, further analysis of the breast cancer cohort for correlation

between pS48-NPM, the PI3K/AKT activating EGFR/HER2 mutations and p53 (where increased p53 staining is considered a surrogate for p53mut protein) showed a clear association between EGFR/HER2 expression and pS48-NPM as well as increased association between pS48-NPM expression and p53. This was characteristically significant in the cases of late stage disease and corresponded to previous reports where p53 status had been shown to play an important role in defining prognosis (Miller et al., 2005). At this point, we further wanted to clarify a point of contention between our findings and previous reports. As we discussed in previous chapters where we described with our *in vitro* studies, AKT has been shown to negatively regulate p53 by phosphorylating NPM. We wanted to confirm in the patient samples, as with our *in vitro* studies, that the presence of ARF was a crucial deciding factor for the AKT mediated regulation of p53 and that in the presence of ARF, AKT activity stabilises p53 (p53mut). Analysis of invasive breast cancer data retrieved from the TCGA showed an inverse correlation of p53 with increased AKT activity where the CDKN2A activity was below the background levels. This negative correlation was lost with increased CDKN2A expression and persists even in the case of p53mut tumours. This substantiated the importance of AKT as both a positive and negative regulator of p53, but which is dependent on the ARF status. We also looked at the correlation between pS48-NPM and p53 in pancreatic cancer patients (pancreatic cancer derived Tumour Micro Arrays), since pancreatic cancers are associated with almost complete penetrance of KRAS activating mutations (Pylayeva-Gupta et al., 2011) and a high incidence of p53 mutations (Morton et al., 2010; Scarpa et al., 1993). It was interesting to note that there was a high degree of correlation between pS48-NPM and pS473-AKT staining especially in the nucleus of the cells in the tumour sections and there was

a similar increase in the p53 expression in relation to the pS48-NPM staining. Further analysis of the patient derived material showed that as we saw with the breast cancer cohort, there was a significant correlation between p53 expression and pS48-NPM only in the case of tissue expressing ARF.

The *in vivo* animal data clearly substantiated our initial findings about the important role that AKT plays in the regulation of p53 stability in tumours. Having confirmed that AKT was important for NPM oligomerisation and ARF activity, as well as the subsequent p53mut stability in xenograft tumours, the subsequent findings in the patient derived tumour material substantiated the importance of screening for ARF and p53 status in tumours prior to initiating treatment with specific AKT inhibitors. Our data also highlighted the highly effective role that these agents can play in a selected p53 mutant cancer cohort that is competent for ARF function.

Chapter 6

AKT mediated regulation of mutant NPM (NPMc) in AML

Chapter 6 – AKT mediated regulation of mutant NPM (NPMc) in AML

6.1 – Introduction

The Type A mutation of exon 12 of the NPM gene that results in duplication of a TCTG tetranucleotide at position 956 to 959 of the NPM reference sequence (Falini et al., 2007) has been introduced in previous chapters. The resulting loss of either 290 or both 290 and 288 tryptophan residues that cause the helical structure of NPM C-terminal domain to unfold (Grummitt et al., 2008) and the generation of a new nuclear export sequence (NES) motif reinforces the Crm1-dependent nuclear export of NPM that is characteristic of the AML associated NPM mutant (NPMc). This mutant protein has been believed to hetero-dimerise with wild-type NPM protein remnants in the nucleus and promotes its translocation to the cytoplasm. Although the complete biological relevance of this has not been elucidated, the translocation to the cytoplasm is associated with the translocation of the NPM binding/ partner proteins including the tumour suppressor ARF (Colombo et al., 2006), interaction with HEXIM1 and activation of the RNA polymerase II (Gurumurthy et al., 2008) as well as the activation of oncogenic c-Myc (Bonetti et al., 2008). The subsequent destabilization of p53 is also believed to reduce the sensitivity of the AML tumour cells to treatment. Interestingly, the co-occurrence of FLT3 mutations along with NPM is associated with poor prognosis in patients although a clear understanding has been hard to come by.

The relevance of the S48 site on NPM especially in the context of regulating its oligomerisation and sub-cellular localization, led us to hypothesize that the same may play a role in regulating the localization of NPMc in AML cells. Since NPM

mutations are generally heterozygous and the mutations themselves do not affect the S48 site, we decided to determine whether the phosphorylation status of this site would play a role in oligomerisation and sub-cellular localization of the mutant protein. We further wanted to confirm if such a regulation had any value as a therapeutic target, more so in the context of FLT3 mutations which frequently occur along with NPMc and which have been shown to activate both the PI3K/AKT and the MAPK pathways (Dosil et al., 1993; Takahashi, 2011).

6.2 – NPM localisation and oligomerisation is modulated by AKT in OCI- AML 3 cells

In order to identify if the NPMc localisation and oligomerisation was affected by AKT, we used the OCI-AML3 cell-lines that carry the characteristic type A mutation of exon 12 of the NPM gene (Quentmeier et al., 2005). These cells have further been reported to have a heterozygous expression of the NPMc and NPM-wt proteins. Cytospinning the suspension cells onto glass-slides and staining for NPM revealed a heterogeneous pattern on fluorescent microscopy (fig. 6.2.A). While most cells showed diffuse NPM staining, characteristic NPM staining of the nucleoli (co-stained with the nucleolar marker fibrillarin) was also seen in many cells. Treatment of these cells with the AKT inhibitor MK2206 showed a significant increase in the intensity of NPM foci signals with the foci characteristically localised at the nucleoli (fig. 6.2.A) in a manner similar to the staining seen in T24 and other cells as reported in previous chapters. This gave us an indication that AKT mediated regulation was playing a role in the localisation of NPM in these cells. Interestingly, western blot analysis of non-denatured protein lysates from the

cells showed an increase in the oligomeric fraction of the NPM in both the nuclear and cytoplasmic fraction (fig.6.2.B). A corresponding increase in the co-localisation of p14ARF was also seen in the same context. Since this data recapitulated our previous findings, we were encouraged to further study this feature in NPM mutant AML cells.

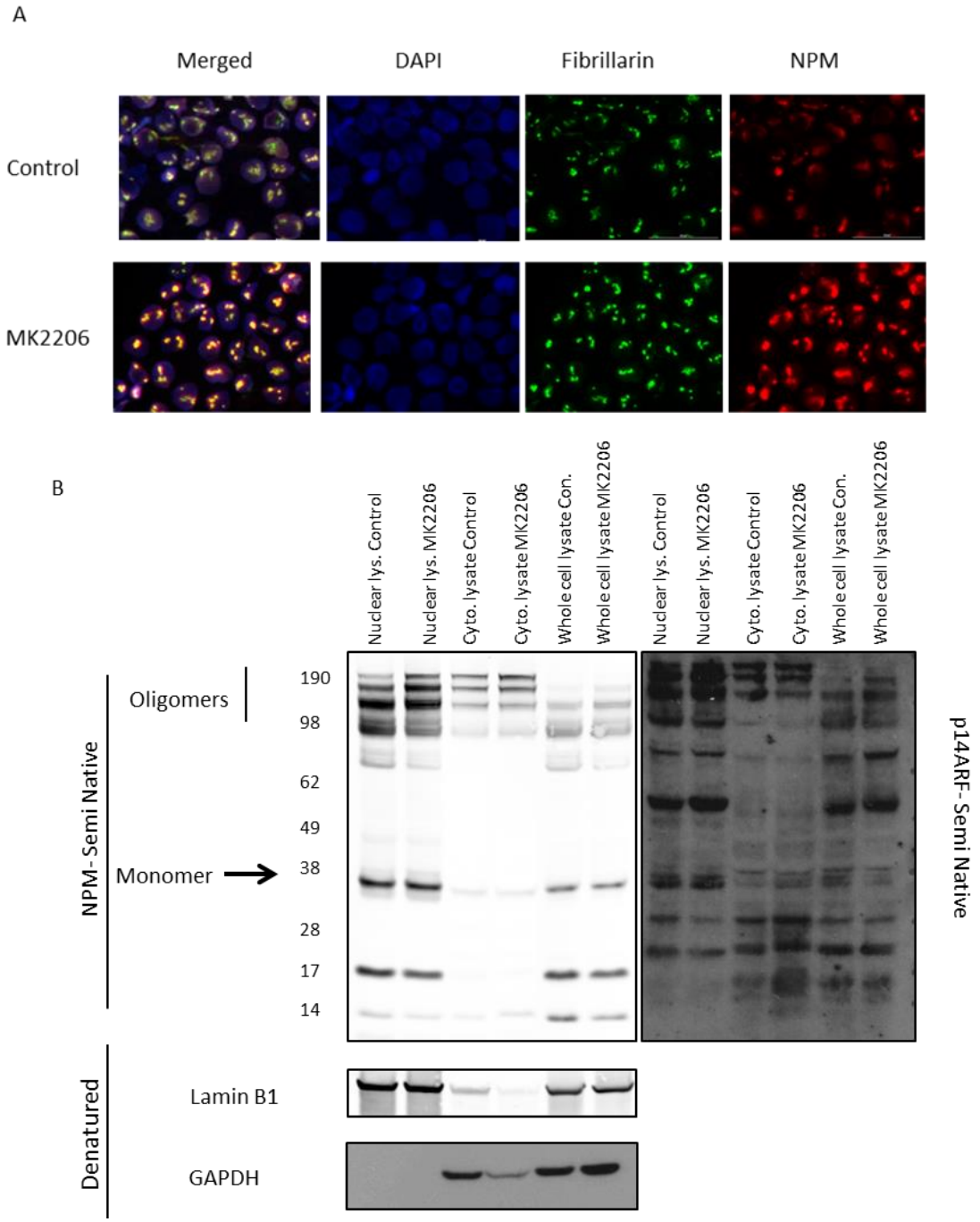


Fig6.2: NPM localisation and oligomerisation is modulated by AKT in OCI- AML 3 cells A) OCI-AML3 cells were cytospun onto glass slides, fixed and stained for

NPM (red) and Fibrillarin (green). B) OCI-AML3 cells treated with DMSO or MK2206 (1μM) for 16 hours and cellular fractionation done. NPM oligomerisation status was determined by semi native gel electrophoresis and denatured lysates, which were probed with the indicated antibodies. Representative Immunofluorescence and western blot images from experimental replicates of n=3

6.3 – S48 regulates oligomerisation of NPMc

The OCI-AML3 cells are heterozygous for NPM mutations (Quentmeier et al., 2005) and since we could not verify if the NPM nucleolar stain was wild-type NPM remnants present in the cells or NPMc, we decided to utilise fluorescent marker tagged variants of NPMc in $NPM^{-/-}$ $p53^{-/-}$ MEFs. Zs Green (ZSG) tagged NPMc-WT, NPMc-S48A and NPMc-S48E variants were transfected using appropriate expression vectors into the $NPM^{-/-}$ $p53^{-/-}$ MEFs. The cells were cultured on coverslips and then treated with DMSO or MK2206 (1μM) before being fixed and imaged using a fluorescence microscope. Interestingly, as seen in our previous studies, the NPMc variants with wild type S48 form oligomers on treatment with the AKT inhibitors, albeit in the different sub-cellular compartments and with no nucleolar localisation.

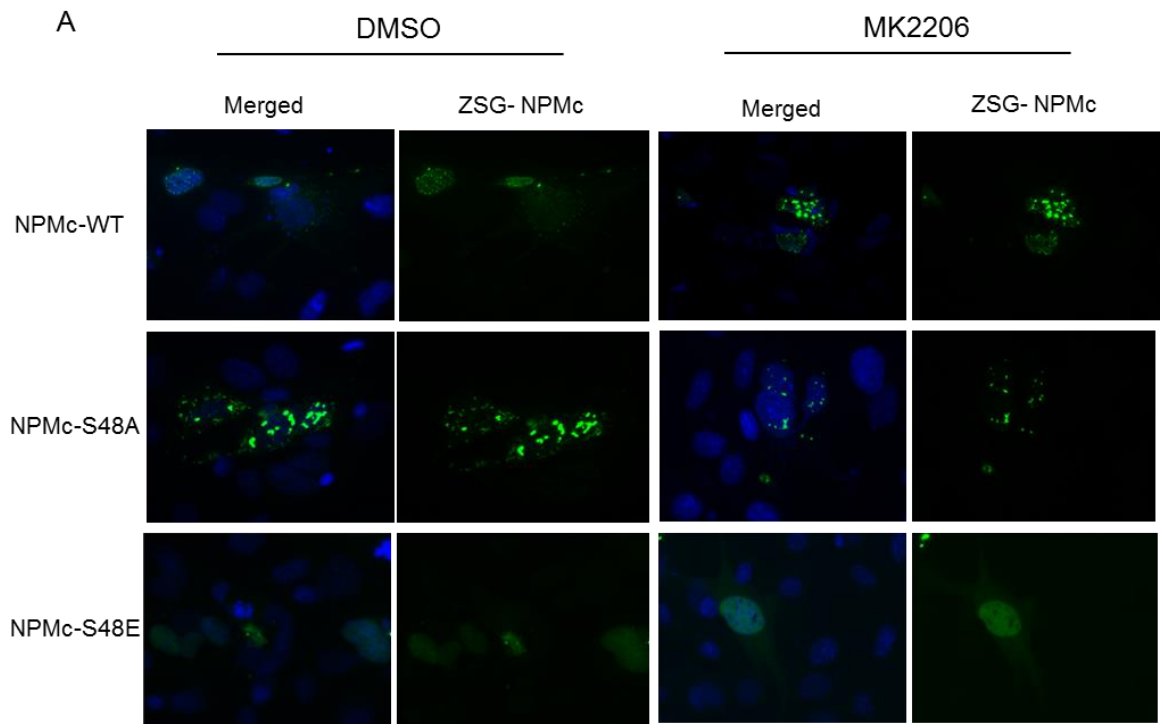


Fig. 6.3.A: S48 plays a role in the oligomerisation of NPMc: $NPM^{-/-}$ $p53^{-/-}$ MEFs reconstituted with ZSG-NPMc-WT, ZSG-NPMc-S48A and ZSG-NPMc-S48E and treated with DMSO or MK2206. Representative Immunofluorescence images from experimental replicates of n=3

The S48A variants show large NPMc oligomeric foci in the different cellular compartments while the phospho-mimetic S48E shows diffuse expression with a predilection to the nucleus. Furthermore, the S48A-NPMc and S48E-NPMc variants do not show any change in localisation on treatment with MK2206 (especially the S48E variant).Thus we could assume that the phosphorylation at S48 was playing a role in the NPMc oligomerisation, but not localisation.

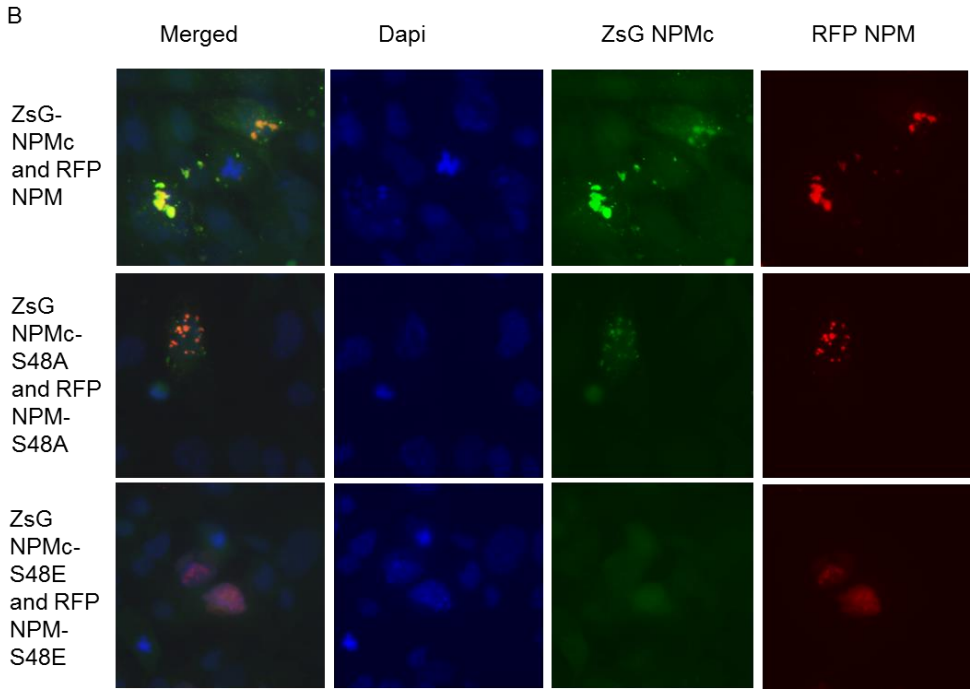


Fig. 6.3.B: $NPM^{-/-}$ $p53^{-/-}$ MEFs reconstituted with ZSG-NPMc-WT and RFP-NPM-WT; ZSG-NPMc-S48A and RFP-NPM-S48A; ZSG-NPMc-S48E and RFP-NPM-S48E and treated with DMSO or MK2206. Representative Immunofluorescence images from experimental replicates of n=3

AML cells generally show heterozygous expression of NPMc and the mutant protein is believed to interact with the wild type NPM remnants and translocate it away from the nucleolus (Falini et al., 2006). We thus wanted to confirm if the interaction between the two NPM variants was dependent on the S48 phosphorylation status. We co-transfected $NPM^{-/-}$ $p53^{-/-}$ MEFs with the fluorescent reporters Zs Green (ZSG) tagged NPMc-WT, NPMc-S48A and NPMc-S48E variants and Red Fluorescent Protein (RFP) tagged NPM-WT, NPM-S48A and NPM-S48E variants and quite interestingly, saw that the proteins do co-localise and follow the same dynamics as the NPMc variants alone in forming olimoers (fig. 6.3.B) depending on the amino acid occupany of 48.

6.4 - NPMc and ARF interaction

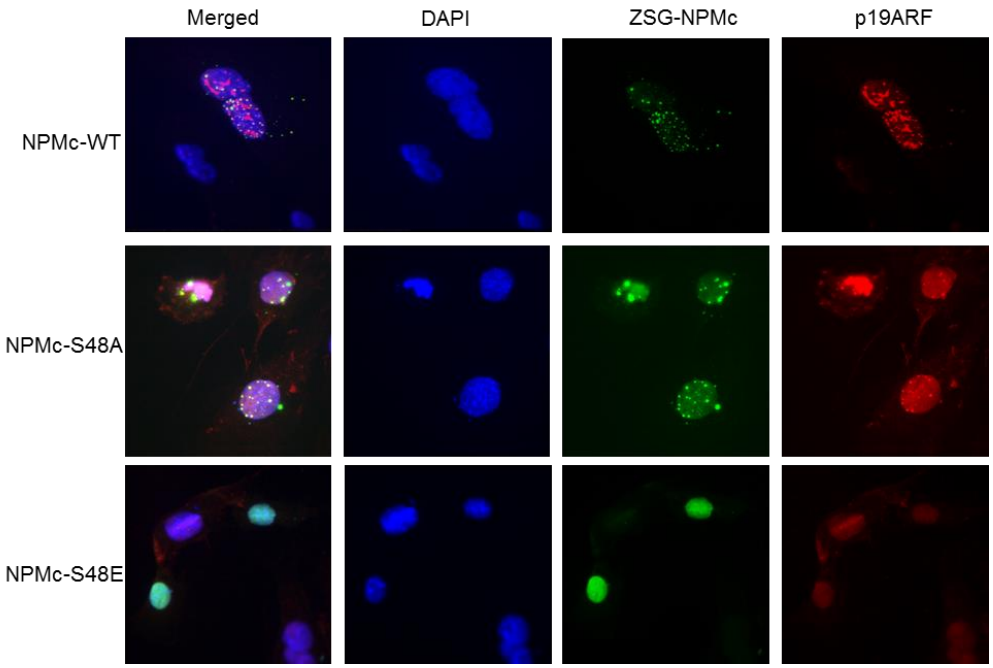


Fig. 6.4: ARF interacts with NPMc: $NPM^{-/-}$ $p53^{-/-}$ MEFs reconstituted with ZSG-NPMc-WT, ZSG-NPMc-S48A and ZSG-NPMc-S48E and co-stained for p19ARF. Representative Immunofluorescence images from experimental replicates of n=3

Having confirmed the S48 mediated regulation of NPM oligomerisation and the interaction between the NPM and NPMc, we next proceeded to look at the regulation of ARF. We used the same $NPM^{-/-}$ $p53^{-/-}$ MEFs described above and transfected them with the NPMc variants. The cells were also stained for p19ARF and imaged. p19ARF co-localises with NPMc, with foci formation in the NPMc-WT and NPMc-S48A variants, and diffuse nuclear staining of ARF which localises with the NPMc in the S48E variant cells.

6.5 – AKT regulates p53 levels in an NPM dependant manner in OCI-AML3 cells

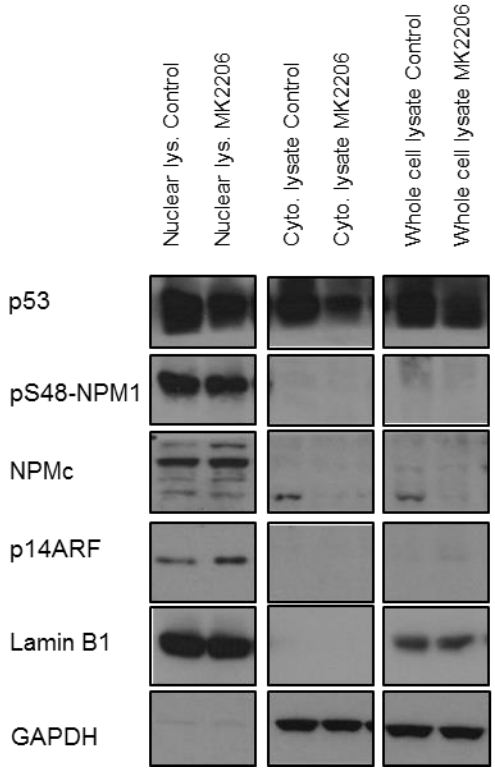


Fig. 6.5: AKT mediates localisation of NPMc and regulation of ARF and p53 stability in OCI-AML3 cells. OCI-AML3 cells were treated with DMSO or MK2206 (1μM) for 16 hours and nuclear-cytoplasmic fractionation done. Lysates were probed by western blot using the indicated antibodies. Representative western blot images from experimental replicates of n=3

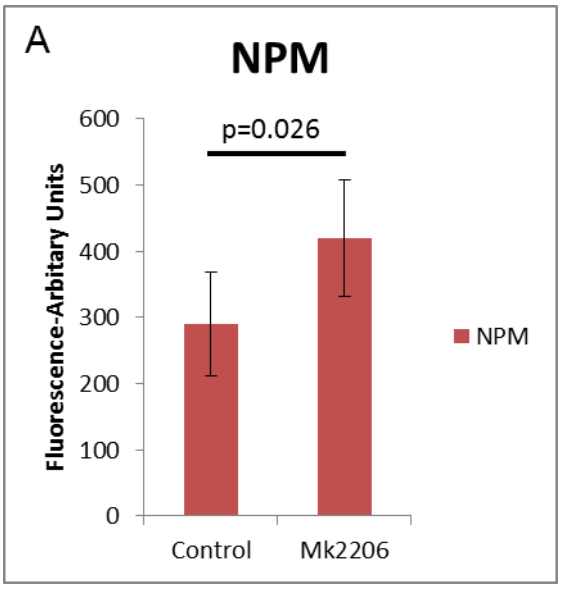
The NPMc-ARF interaction described above led us to look at the OCI-AML3 cells and the effect AKT inhibitors may have on ARF and p53 in these AML cells. Although not surprising based on our initial studies, it was indeed interesting to see that inhibiting AKT in the cells resulted in an increase in nuclear ARF stability and a mild decrease in p53 protein levels by western blot. This was further associated with a moderate increase in the nuclear fraction of NPMc when probed with an antibody specific to the mutant protein. Taken together, along with the

Immuno-Fluorescence data from fig.6.2.A, we can presume that the increased ARF stability and decrease in p53 may be dependent on the NPM wild type remnants rather than just the NPMc. This will be significant in light of the heterozygous nature of the NPM protein expression in most human NPM mutant AML patients.

6.6- AKT inhibition promotes increased nuclear localisation of NPM in the OCI-AML3 cells

In order to further clarify the effect of AKT inhibition on NPM in OCI-AML3 cells, they were treated with MK2206, cytopsun onto glass slides and stained for NPM and p14ARF. The fluorescence intensity of NPM and p14ARF nuclear staining was quantitated on the Nikon NIS Elements software and the values compared between the untreated and the treated cells. Interestingly, we see that the cells treated with the AKT inhibitor showed an increase in the intensity of nuclear NPM staining (Fig.6.6.A), further clarifying our previous observations that the AKT mediated phosphorylation was playing a role in the nuclear localisation of NPM in these cells. Of note is that even though this proves that AKT plays a role in regulating the nuclear localisation of NPM in these cells, since these cells are known to express both wild-type and mutant NPM, the increased localisation of NPM can be attributed to either or both. Taking into consideration the NPMc levels seen in Fig. 6.5, we can assume that part of the increased NPM signal seen in the nucleus can be due to the increased localisation of NPMc to the nucleus. What was also interesting to note was the observation that the increased NPM localisation to the nucleus was associated with a concomitant increase in the

localisation signal of p14ARF to the nucleus. Surprisingly, the increase in ARF localisation was nucleoplasmic rather than nucleolar.



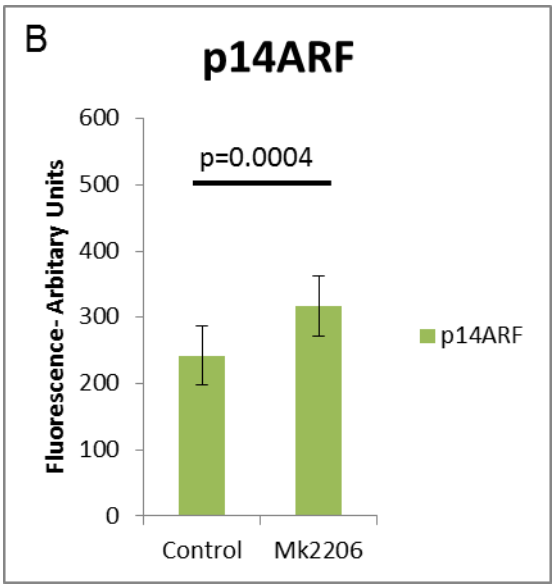


Fig. 6.6: A) Graph showing quantitation (Nikon NIS Elements software) of fluorescence signal in the nucleus of OCI-AML3 cells treated with DMSO or MK2206 (1μM) and stained for NPM B) Graph showing quantitation of fluorescence signal in the nucleus of OCI-AML3 cells treated with DMSO and MK2206 and stained for p14ARF

6.7 - FLT3 signalling disrupts NPM oligomerisation

FLT3 activating mutations are commonly associated with NPM mutations in AML patients with a normal karyotype (Dohner et al., 2005; Schnittger et al., 2005) and they have also been associated with poor prognosis. Since the activating FLT3 mutations can lead to signalling via both the PI3K/AKT and the MAPK pathway, we wanted to asses if these signals would have an effect on the NPM phosphorylation and thus whether our previously described mechanism had any role in this association between NPM mutations and FLT3 mutations.

We looked at both the PI3K/AKT and the MAPK pathway elements focussing on AKT and the MAPK sub-family p38MAPK, which has previously been hypothesised to be activated by FLT3 signalling (Desterke et al., 2011; Odgerel et al., 2010). The p38-MAPK was specifically chosen since previous studies (Mitrea et al., 2014; Mitrea and Kriwacki, 2012) and in-silico analysis (http://www.cbs.dtu.dk/services/NetPhosK/) showed that p38-MAPK could phosphorylate the Threonine-95 moiety of NPM, which in turn was located at an NES as well as playing a role in the oligomerisation of NPM. We first compared the oligomerisation of NPM in OCI-AML3 with the MOLM13 cell-line, which is known to have an activating FLT3-ITD mutation, but carrying wild type NPM. Both the cells were treated for 16 hours with DMSO, MK2206 (1μM), a p38 inhibitor LY2228820 (200nM) and the FLT3 inhibitor CAS 301305-73-7 (200nM). As expected, both the cell lines showed an increase in oligomerisation on treatment with MK2206, while the MOLM13 cell-lines alone showed an increase in NPM oligomerisation following treatment with LY2228820 or CAS 301305-73-7 (fig. 6.7.A). The increased oligomerisation was also associated with an increase in the nuclear localisation of NPM (fig.6.7.B). A phospho T95-NPM antibody (Anti-

Nucleophosmin (phospho T95) antibody [EPNCIR117]) showed a mild decrease in the T95-NPM phosphorylation levels in the cells treated with LY2228820 or CAS 301305-73-7, with p38-MAPK inhibition in MOLM13 cells alone appearing to show a pro-oligomerisation effect, highlighting the possibility of p38-MAPK playing a role in regulating the oligomerisation of NPM in addition to AKT in cells addicted to FLT3 activity.

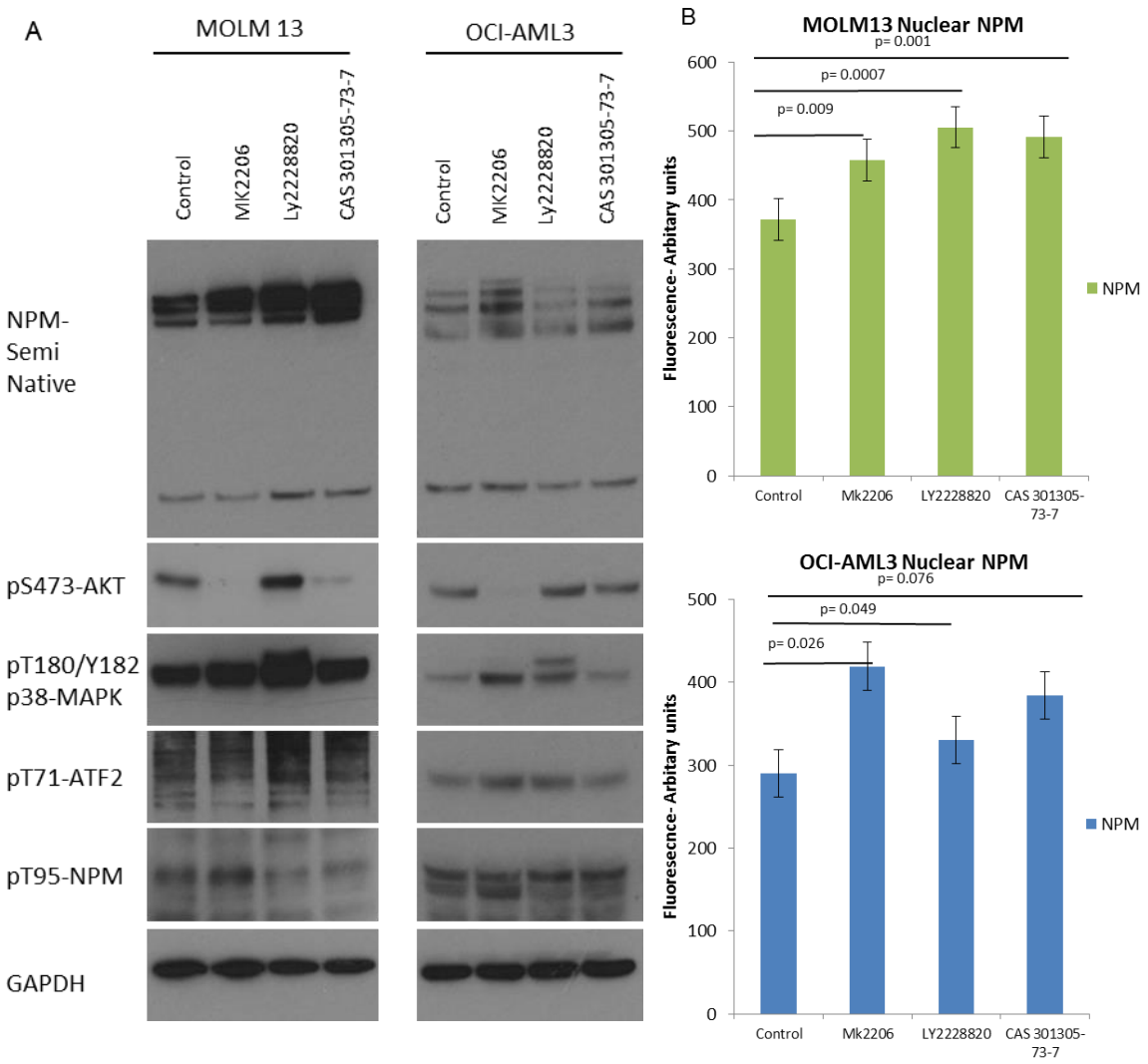


Fig. 6.7.A: FLT3 regulates NPM oligomerisation: MOLM13 and OCI-AML3 cells were treated with DMSO, MK2206 (1μM), LY2228820 (200nM) and CAS 301305-73-7 (200nM) for 16 hours. NPM oligomerisation was determined by semi native gel electrophoresis and denatured lysates, which were probed with the indicated antibodies. B) Graphs on the right shows quantitation of NPM fluorescence signal (Nikon NIS Elements software) in the nucleus of MOLM13 and OCI-AML3 cells

treated with DMSO, MK2206 (1μM) LY2228820 (200nM) and CAS 301305-73-7 (200nM) and stained for NPM. Representative images from experimental replicates of n=3

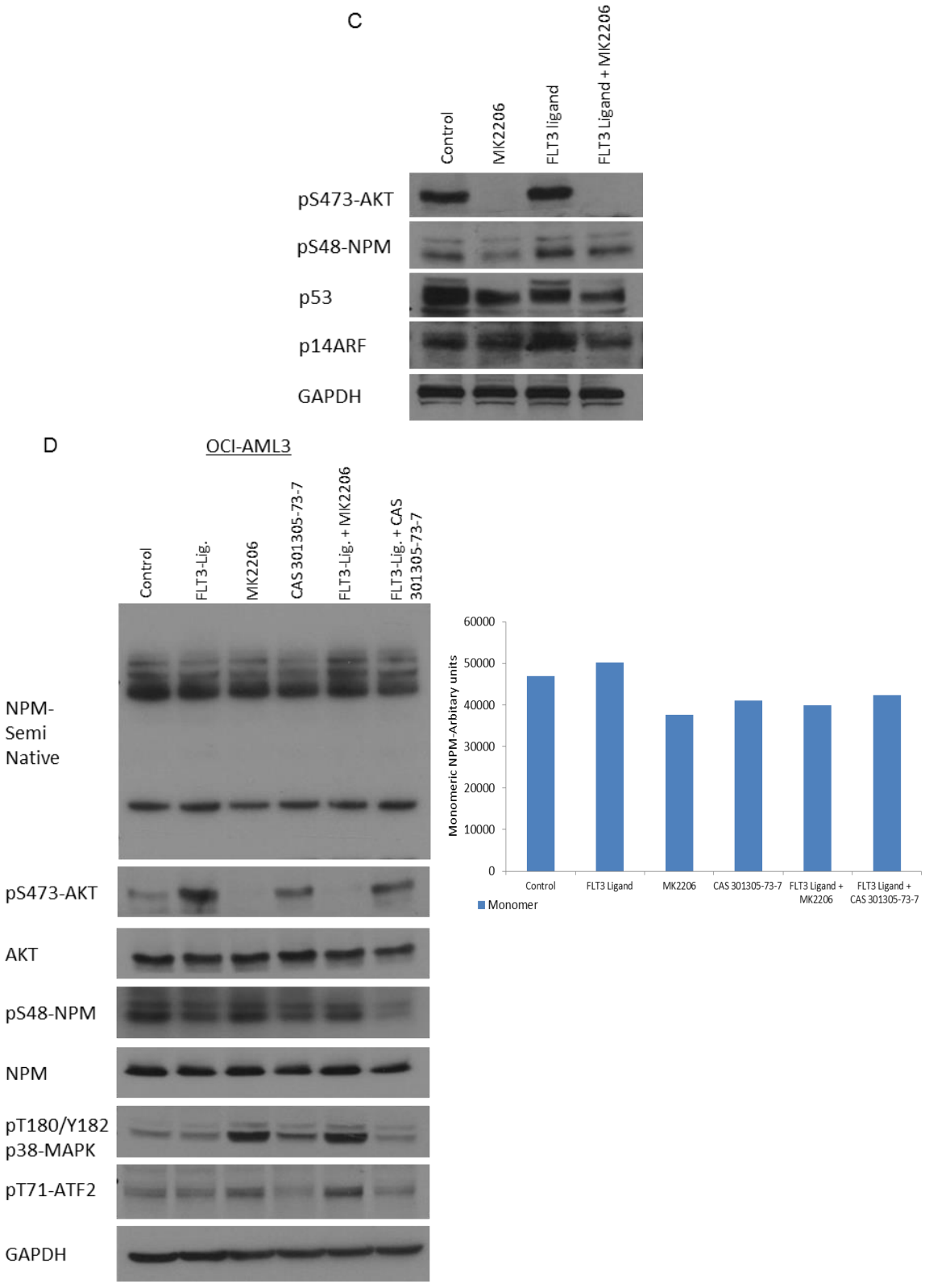


Fig. 6.7: C): OCI-AML3 cells were treated with DMSO, MK2206 (1μM), FLT3 ligand (50nM) and FLT3 ligand combined with MK2206. Cell lysates were

separated by gel electrophoresis and probed with the indicated antibodies D): OCI-AML3 cells were treated with DMSO, MK2206 (1μM), FLT3 ligand (50nM), CAS 301305-73-7 (200nM) singly or in combination for 16 hours. Cells lysates were separated by semi-native gel electrophoresis and probed with the indicated antibodies. Panel on the right shows quantitation of the NPM monomeric (Blue) fractions in the semi-native western blot. Representative images from experimental replicates of n=3

We next treated OCI-AML3 cells with the FLT3 ligand to mimic increased FLT3 signalling, and the protein levels were examined. The increased FLT3 activity led to an increase in the pS473-AKT levels, as well as a decrease in the oligomerisation and a concomitant increase in the monomeric fraction of NPM (fig. 6.7.C and D); strengthening our argument that FLT3 functioned via downstream AKT activity to regulate NPM oligomerisation. Interestingly, we did not see an increase in p38-MAPK activity following treatment with the FLT3 ligand, suggesting that p38-MAPK may play a role in NPM oligomerisation only in the FLT3 addicted cells rather than in the OCI-AML3 cells we studied. Indeed in a set of OCI-AML3 cells that were transfected with FLT3-ITD plasmids which were a kind gift from Prof. Frank Bohmer, University of Jena, we do see an increase in the pAKT-S473 and pP38-MAPK signals (fig.6.7.E). Unfortunately the nucleofection/electroporation that we used to transfect the plasmids was seen to be toxic to the nucleoli as it was seen to disrupt the nucleolar architecture (fig.6.7.F). Consequently, this could affect the oligomerisation of NPM and explain the aberrant NPM oligomerisation that is seen in western blot (fig.6.7.E). Viral based packaging systems for the FLT3-ITD plasmids will be required to eliminate this risk and properly study the interaction between FLT3-ITD and NPMc.

AKT activation has been reported to inhibit p38-MAPK activity (Berra et al., 1998; Gratton et al., 2001). Although reports to the contrary exist (Madrid et al., 2001), we saw in the case of the OCI-AML3 cells that the increased activity of AKT in the cells following treatment with the FLT3 ligand was not associated with an increase in p38-MAPK activity. Interestingly, inhibition of AKT with MK2206 resulted in an increase in the p38-MAPK activity, substantiating the inverse relationship between AKT and p38-MAPK potentially through pathway crosstalk.

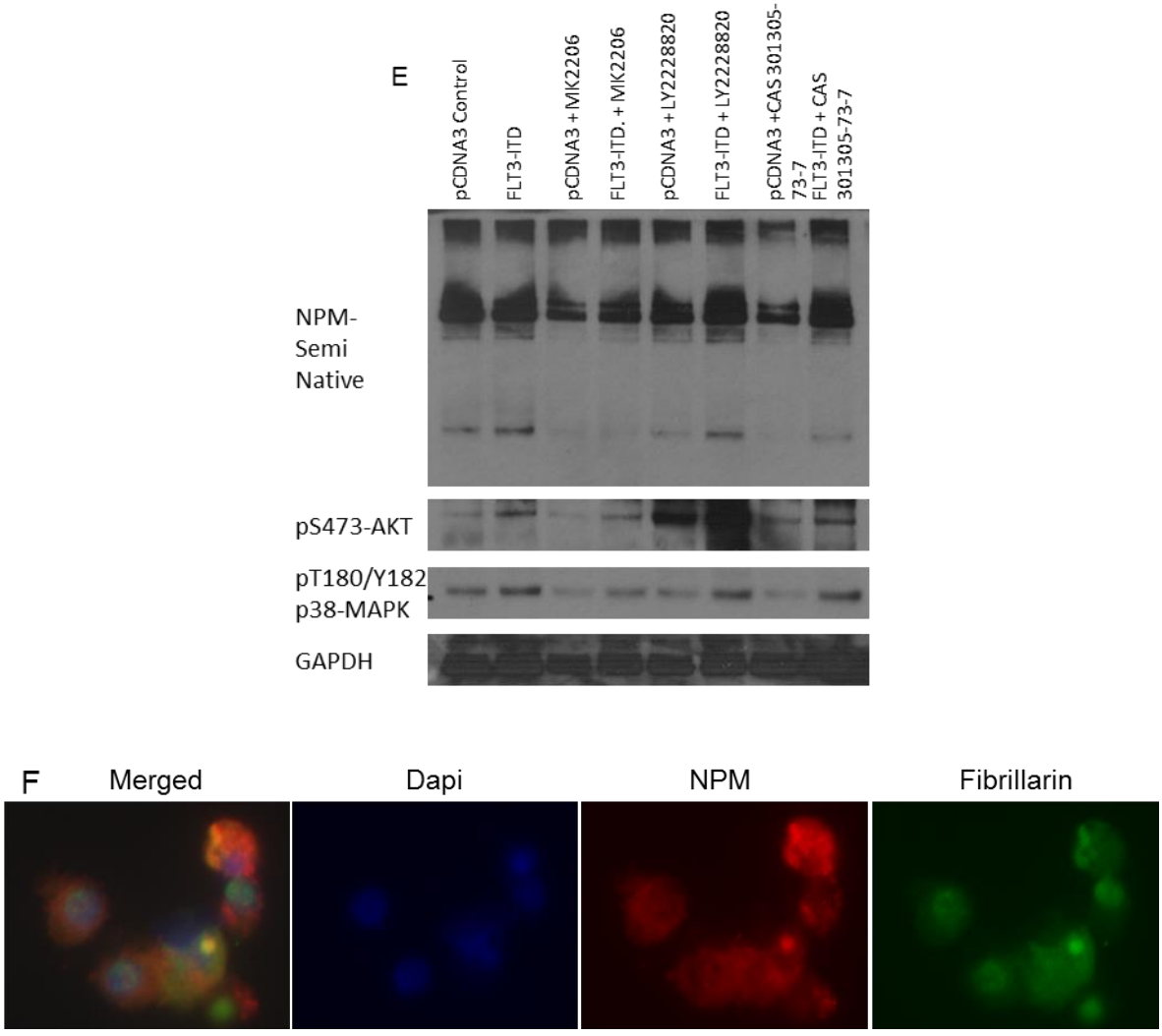
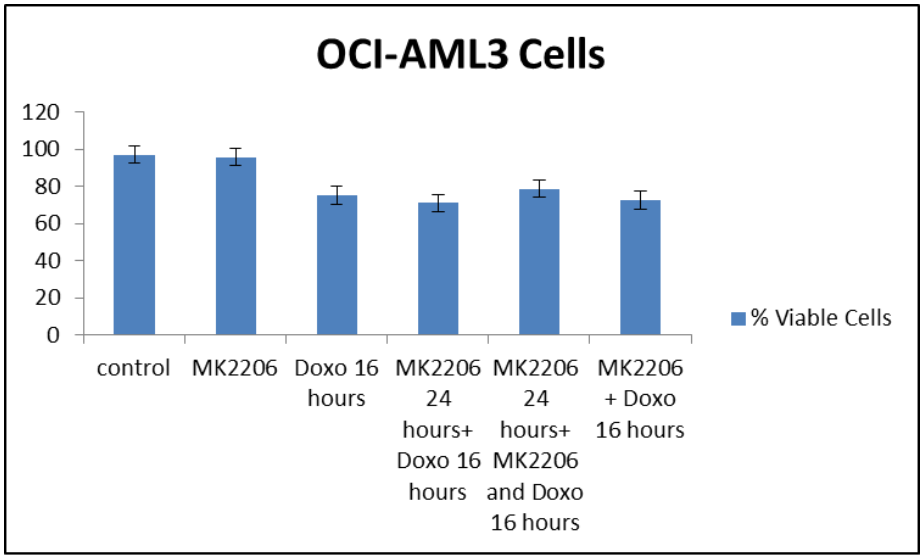


Fig. 6.7.E and F: E) pCDNA3 empty vector or FLT3-ITD expressing plasmid was nucleofected/electroporated into OCI-AML3 cells. 36 hours later, they were treated with DMSO, MK2206 (1μM), LY2228820 (200nM) and CAS 301305-73-7 (200nM) for 16 hours. NPM oligomerisation was determined by semi native gel

electrophoresis and denatured lysates, which were probed with the indicated antibodies F) pCDNA3 empty vector or FLT3-ITD expressing plasmid was nucleofected/electroporated into OCI-AML3 cells. 36 hours later cells were cytospun onto glass slides, fixed and stained for NPM (red) and Fibrillarin (green)

6.8 – AKT inhibition restricts therapeutic benefit of Doxorubicin on AML cells

A recent report about the use of the AKT inhibitor MK2206 in AML, showed no benefit from using the drug in AML patients even though it was effective *in vitro* at higher doses (Konopleva et al., 2014). Interestingly, when we looked at a combination of the DNA intercalating agent Doxorubicin and MK2206 given at different dosing schedules, we observed no added benefit from the use of the AKT inhibitor (fig. 6.8). Furthermore, this was also associated with a decrease in the expression of p53 in cells (fig.6.8) as well as a decrease in the cleaved caspase expression, following treatment with MK2206, which could possibly explain the decreased sensitivity to treatment with combination therapy.



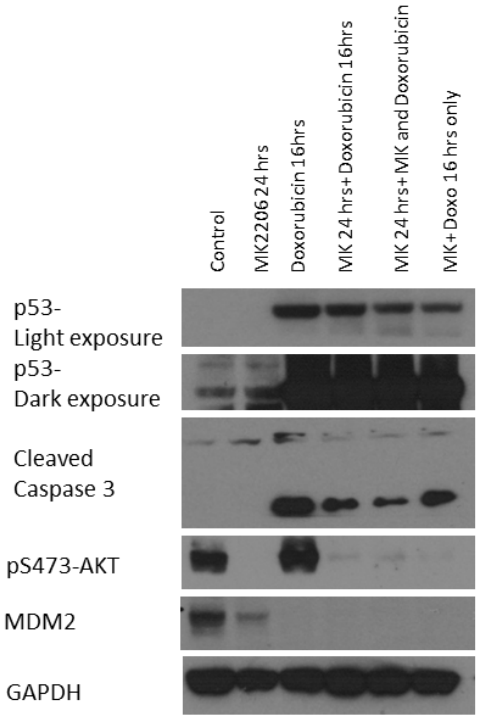


Fig.6.8: OCI-AML3 cells were treated with MK2206 (1μM) and Doxorubicin (1μM) alone (Lane 2); pre-treated with MK2206 for 24 hours followed by Doxorubicin (lane 4); pre-treated with MK2206 for 24 hours followed by MK2206 and Doxorubicin for 16 hours (lane 5) and a combination of MK2206 and Doxorubicin for 16 hours (lane 6). Upper panel shows viability following staining of the treated cells with trypan blue and counting viable cells. Panel below shows protein extracts from the same cells that were separated and probed with the indicated antibodies

6.9 – Chapter 6 discussion

NPM mutations have been observed to occur at high frequency in AML, with Type A mutations seen most commonly. The mutations in the nucleolar localisation (Falini et al., 2008b) signal region of NPM that also results in the formation of an aberrant NES, thereby promoting increased cytoplasmic translocation of the mutant protein has been described by many authors. We investigated whether phosphorylation of the S48 site of NPM that we previously characterised could play a role in the oligomerisation properties of the mutant protein. We were also keen to identify if the co-occurrence of the FLT3 activating mutations with NPMc had a molecular basis that could be explained by our previously described mechanism.

We first examined the OCI-AML3 cell-line (Quentmeier et al., 2005) which were heterozygous for NPM, with the cells expressing both wild type and the mutant type A variant of NPM. Inhibiting AKT using MK2206 showed an increase in localisation of NPM to the nucleolus on immunofluorescence analysis suggesting that NPM in these cells were responding to the AKT inhibition in a manner similar to that demonstrated in the cancer cells although it was unclear if the NPM was wild type or mutant. We next confirmed that the S48 phosphorylation site played a role in the formation of the tertiary structure of the mutant NPM (NPMc). Using fluorescent marker tagged NPMc expression constructs to reconstitute $NPM^{-/-}$ $p53^{-/-}$ MEFs we saw that the S48-NPMc variants do indeed show increased oligomerisation albeit without localisation specifically to the nucleolus while non-phosphorylatable variants formed large foci and the phospho-mimetic variants showed no foci formation. Indeed we also saw that co-expression of the fluorescent tagged NPM variants without the type A mutation, along with the

NPMc variants showed similar staining patterns, confirming our hypothesis that S48 is essential for the interaction with the wild-type as well as the mutant variants and also confirming suggestions by previous authors (Falini et al., 2007) that the that wild type and mutant NPM interact in AML cells. Furthermore, we also showed that ARF closely interacts with NPMc, confirming the existing hypothesis that NPMc plays a role in deregulating the localisation of ARF. Further analysis of the OCI-AML3 cells showed that inhibition of AKT activity using MK2206 resulted in increased NPM and ARF localisation to the nucleus and a decrease in the stability of p53 in these cells. FLT3 activating mutations co-occur with NPM mutations, are associated with poor prognosis and also result in aberrant signalling via downstream PI3K/AKT and MAPK pathways. We observed that FLT3 mutant cells did indeed show increased AKT activity, and inhibiting the same with MK2206 resulted in increased oligomerisation of NPM in the cells. Quite interestingly we also saw that inhibition of FLT3 activity and p38-MAPK activity resulted in an increase in NPM oligomerisation suggesting a FLT3 mediated signalling through p38-MAPK as a possible mechanism of NPM regulation in FLT3 mutant cells, along with the AKT mediated effects. Further site directed mutagenesis studies of the T95 moiety of NPM will be required to confirm the role that p38-MAPK may play in the phosphorylation. We observed that inhibition of AKT activity resulted in an increase in the p38-MAPK activity in the OCI-AML3 cells indicating another AKT mediated activity of FLT3 which can play a role in the AML cells, and suggesting a redundancy mechanism in cells that takes advantage of the constitutively active FLT3 signalling. Surprisingly, inhibition of AKT in OCI-AML3 cells by MK2206 did not increase the therapeutic sensitivity of the cells to the drug, nor did it potentiate the effect of other commonly used DNA damaging

chemotherapeutic agents. This is likely to be due to the characteristic decrease in p53 that we observed on treatment with MK2206 and attains importance in light of using AKT inhibitors in the treatment of AML, as has been recently reported, where treatment with MK2206 as a single agent failed to show a therapeutic response in the patients (Konopleva et al., 2014).

Chapter 7

Circulating cell free DNA for analysis of pancreatic cancer mutations

Chapter 7 – Circulating cell free DNA for analysis of pancreatic cancer mutations

7.1 – Introduction

Having identified a connection between RAS and p53mut in solid tumours, we next wanted to see how best to apply this into the clinical setting, especially for treatment planning, as well as to follow treatment outcome. In this context we decided to investigate tumour derived circulating DNA found in blood as a marker for following the mutational status in the tumours.

Fragmented DNA is found as a normal component of blood and is derived from the turnover of cells in the body. The cell free DNA (cfDNA) can be isolated from the cell free component of blood and has been widely used for evaluation of foetal DNA for germ-line mutations and foetal changes at early stages of pregnancy (Lo et al., 1997; Lo et al., 1998). This early work has led to the development of using cfDNA in cancer patients for mutation identification, tumour follow-up and treatment planning. The circulating cfDNA derived from tumours, otherwise called circulating tumour DNA (ctDNA),has drawbacks in the form of difficulty in discrimination between normal cfDNA and ctDNA as well as variable levels in the cell free component of blood. The detection of ctDNA is also difficult as quite often, the levels can range from 0.01% to more than 90% (Diehl et al., 2005; Diehl et al., 2008) depending on the tumour burden. Low levels make standard sequencing difficult and the tumour burden has to be high enough to show high levels of circulating DNA. In spite of the drawbacks, the advent of digital genomic approaches has made identification of ctDNA and more specifically rare mutations, much more feasible. Single point mutations, amplifications, re-

arrangements and aneuploidy can all be detected by the newer genomic approaches.

In cancer patients, the increasing tumour burden results in increased turnover of cells by apoptosis and necrosis, and the subsequent release of the cellular debris into the circulation (Jahr et al., 2001). Fragments of the cellular DNA form part of this and can be picked up from the cell free component of blood (plasma and serum). Interestingly, much of the ctDNA found in blood measures between 180 to 200 base pairs, suggesting that the ordered cell death associated with apoptosis produces the majority of the circulating DNA. Larger sized DNA fragments (more than 1000 base pairs) are believed to originate from necrosis rather than apoptosis (Mouliere et al., 2011).

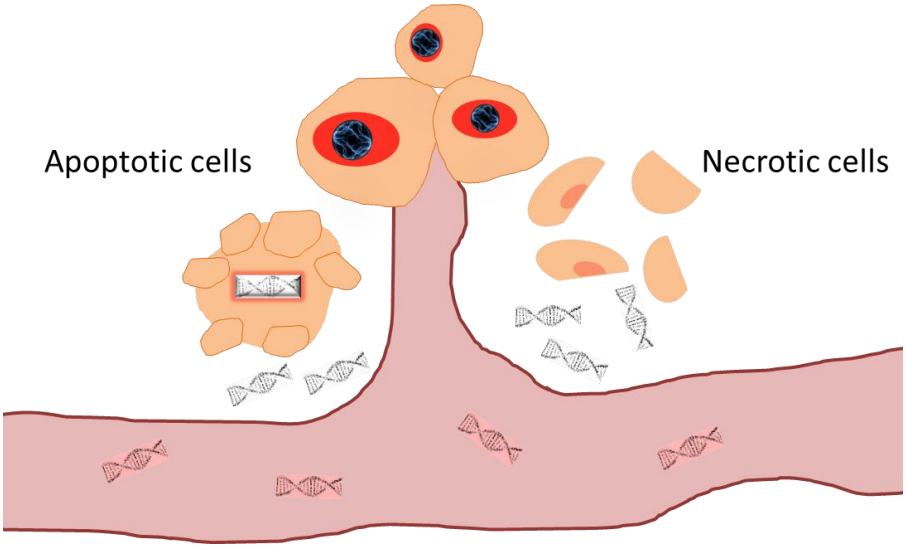


Fig 7.1. A: Tumours show increased turnover of cells leading to release of DNA fragments into the circulation

Most of the existing approaches for retrieving ctDNA require the extraction of DNA from relatively large volumes of plasma. We attempted to take the novel approach of using small volumes of plasma and using Digital PCR for quantifying

the DNA yield as well as identifying the presence of specific mutations, in an attempt to take the technique further and to translate this into using into clinical practise. We used 1ml of plasma from healthy volunteers and pancreatic cancer patients and extracted cfDNA using the Qiagen Circulating Nucleic Acid Extraction kit, according to the manufacturer's instructions. The extracted DNA was quantified using nano-drop as well as pico-green methods. As has previously been done, we obtained absolute copy numbers of the genomic equivalents of DNA by looking for single copy genes in both healthy volunteers and patients, to give us an indirect estimate of the DNA extracted.

KRAS mutations leading to activation of AKT are highly penetrant in pancreatic cancers (Eser et al., 2014) and to study this, we decided to look for the most common KRAS mutations associated with pancreatic cancers (KRAS-G12V and G12D) using mutation specific primers. In order to study the KRAS mutations, we used plasma from patients enrolled in the ARC-2 trial. This trial conducted in Oxford, involved pancreatic cancer patients who were treated with the anti-HIV protease inhibitor Nelfinavir, which has also been shown to have AKT inhibitory activity (Gupta et al., 2007), in conjunction with standard chemo-radiotherapy. The trial plan consisted of daily nelfinavir (1250mg twice daily) in addition to cisplatin and gemcitabine concurrently with 50.4 Gy in 28 fractions to the pancreatic tumour and elective lymph nodes followed by 9 Gy in 5 fractions to the GTV as a sequential boost. We used plasma from patients enrolled in this trial and monitored their KRAS status as well as the cfDNA in plasma samples obtained prior to, during and following treatment. The quantitation as well as mutational analysis was done using the Quantstudio 3D digital PCR system in collaboration

with Life Technologies using off the shelf Taqman genotyping assays as well as proprietary KRAS mutation specific Taqman assays developed by the company.

7.2 – Circulating DNA levels in healthy volunteers

Plasma from healthy volunteers was used for setting up and validating our standard operating protocols as well as for the purpose of establishing reference values to compare with the patient derived material. 1 ml of plasma was used for extracting cfDNA and the DNA obtained was analysed for the single copy gene Haemoglobin β (HBB) on the Quantstudio 3D digital PCR system as a surrogate marker for DNA content. The copy numbers were presented as absolute number of the gene copies and gave an estimate of the whole genomic equivalents of DNA. Healthy volunteers consistently gave 300-1000 gene copies/ml of plasma when analysed for the single copy genes (fig.7.2.A).

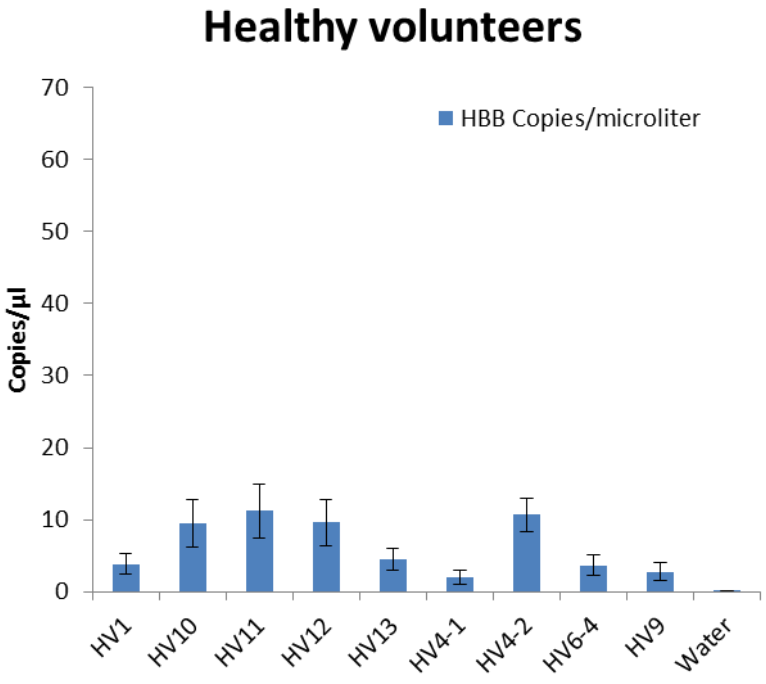


Fig. 7.2: Graph showing the HBB gene copies per µl of DNA elute prepared from plasma when quantitated on the Quantstudio 3D Digital PCR system.

7.3 – Circulating DNA levels in cancer patients

Having validated our techniques on multiple healthy volunteer derived plasma samples, we decided to analyse plasma samples from a cohort of cancer patients for their cfDNA content. As with the healthy volunteers, we proceeded to extract the cfDNA from plasma and analysed it for the single copy gene HBB. cfDNA from two colorectal cancer patients and 4 pancreatic cancer patients enrolled in the ARC 2 trial were analysed. The trial material analysed included matched samples of patients 3 and 4 from days 1 and 9 in order to follow the cfDNA load in the patients over the course of treatment with Nelfinavir. In agreement with previous reports, we see variable but higher levels of total DNA extracted from the patient plasma when compared to the healthy individuals (fig. 7.2) from the quantitation of the surrogate marker HBB (fig. 7.3).

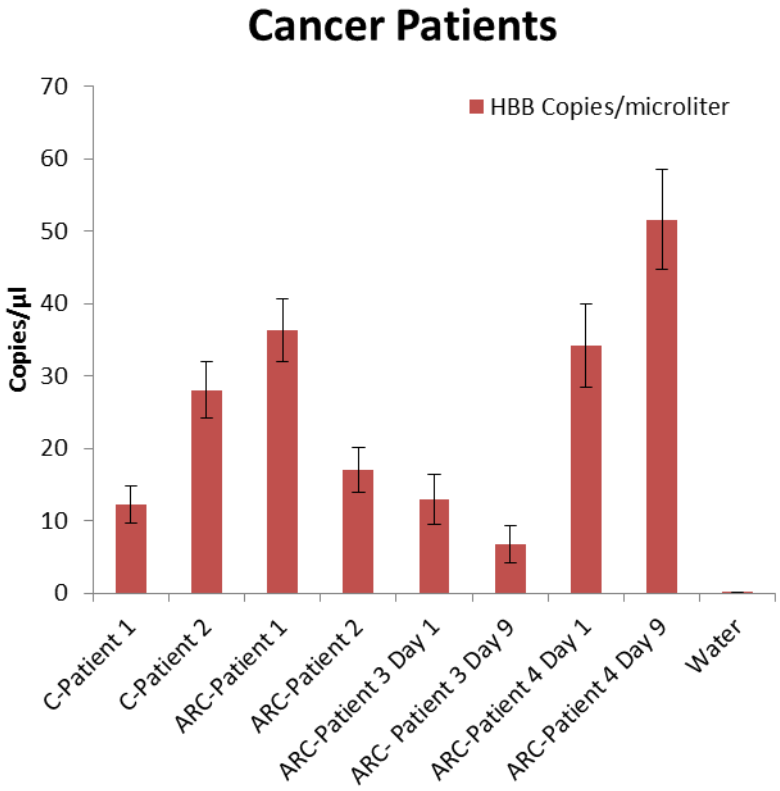


Fig. 7.3: Graph showing the HBB gene copies per µl of DNA elute prepared from plasma when quantitated on the Quantstudio 3D Digital PCR system.

7.4 – Mutation analysis from circulating DNA in pancreatic cancer patients

In order to verify if KRAS mutations could be identified from the small volumes of plasma that we were analysing, we decided to concentrate on the two common KRAS mutations associated with pancreatic cancer- KRAS-G12V and KRAS-G12D (Eser et al., 2014). Proprietary Taqman mutation analysis probes against the two mutant variants were kindly provided by Life technologies. The mutation analysis probes consists of fluorescently labelled (VIC labelled) Taqman primer-probes which identifies the wild type KRAS as well as a primer-probes (FAM labelled) that identifies the mutant KRAS variant. ctDNA extracted from the plasma was analysed on the Quant 3D digital PCR system and the data presented as a percentage of the mutant copies to the total copies of the gene.

Interestingly we see that from the 1 ml of plasma that we used for our cfDNA extraction and subsequent analysis (where we used approximately 50% of the total DNA extracted for mutations analysis), even though we identify KRAS wild-type gene copies, we could not conclusively identify any mutant KRAS variants (Fig.7.4.A and B). While this is in line with previous reports that the copy numbers of the mutant protein can vary from 0.1-90%, it also highlights the difficulty in identifying mutations from the relatively small volume (Aung et al., 2010) of clinical material. In order to identify if this was a technical issue rather than an actual finding where the four patients did not show mutations, we decided to use healthy volunteer plasma spiked with a known amount of mutant DNA to identify if they could be picked up by the assay. Fragmented genomic DNA from 3000 CAPAN-1 cells (KRAS G12V mutant) or PANC-1 cells (KRAS G12D mutant), which roughly translates to just below 20ng of DNA, were added to 1ml each of

the volunteer plasma. The samples were processed as previously described and run on the Quantstudio 3D system for analysis. The samples spiked with the KRAS G12V mutant DNA (fig. 7.4.C) and KRAS G12D mutant DNA (fig. 7.4.D) both shows evidence of mutant KRAS copies following the PCR reaction. Interestingly, when expressed as a percentage of the total KRAS copies, both the assays identify 40-50% of the KRAS genes to be KRAS mutant as would be expected from a heterozygous source for the DNA.

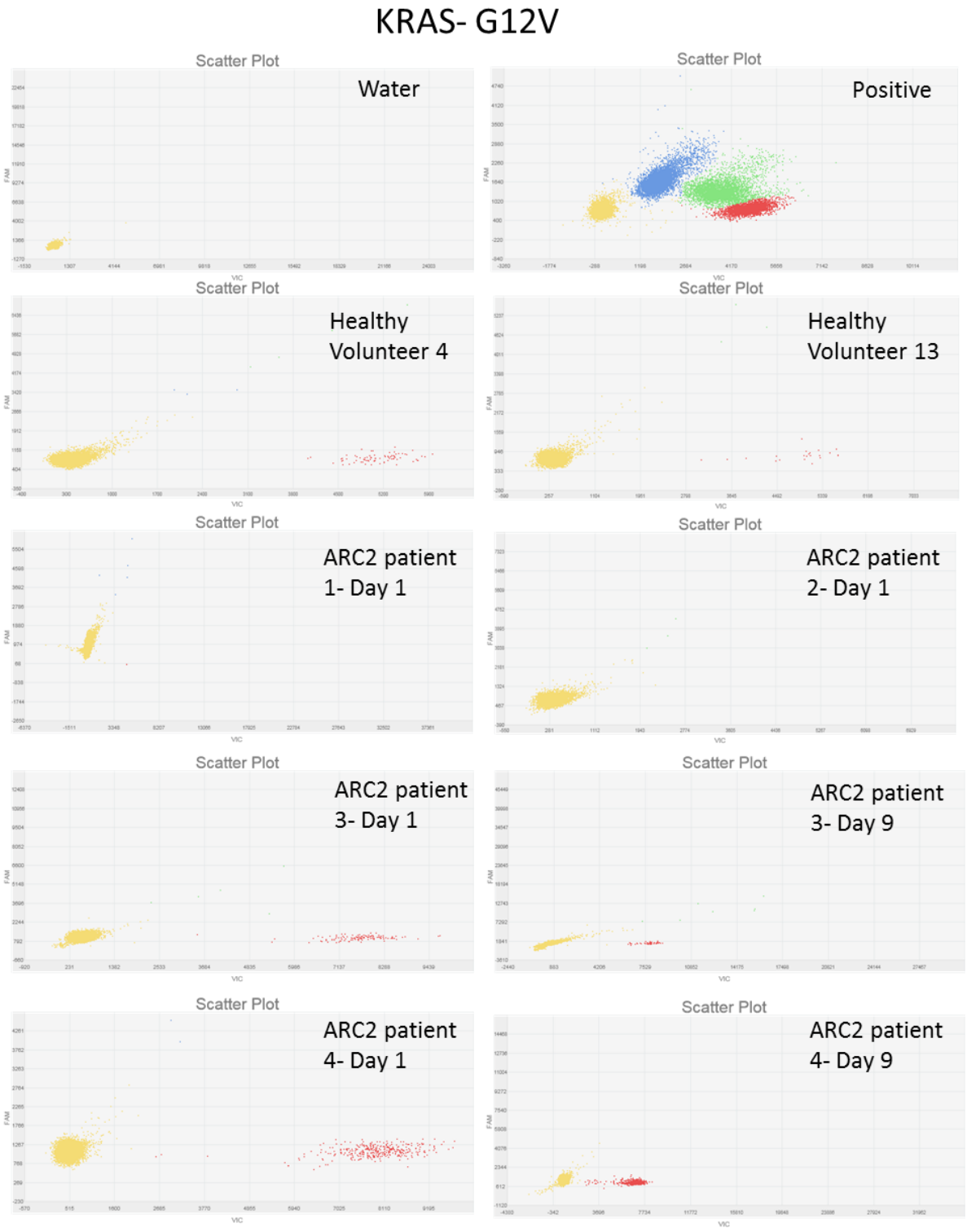


Fig.7.4. A: KRAS G12V mutation assay: Scatter plots from Quantsudio 3D Digital PCR analysis software showing VIC (Red) labelled KRAS-Wt and FAM (Blue) labelled KRAS-G12V. Yellow points refer to wells with no reaction.

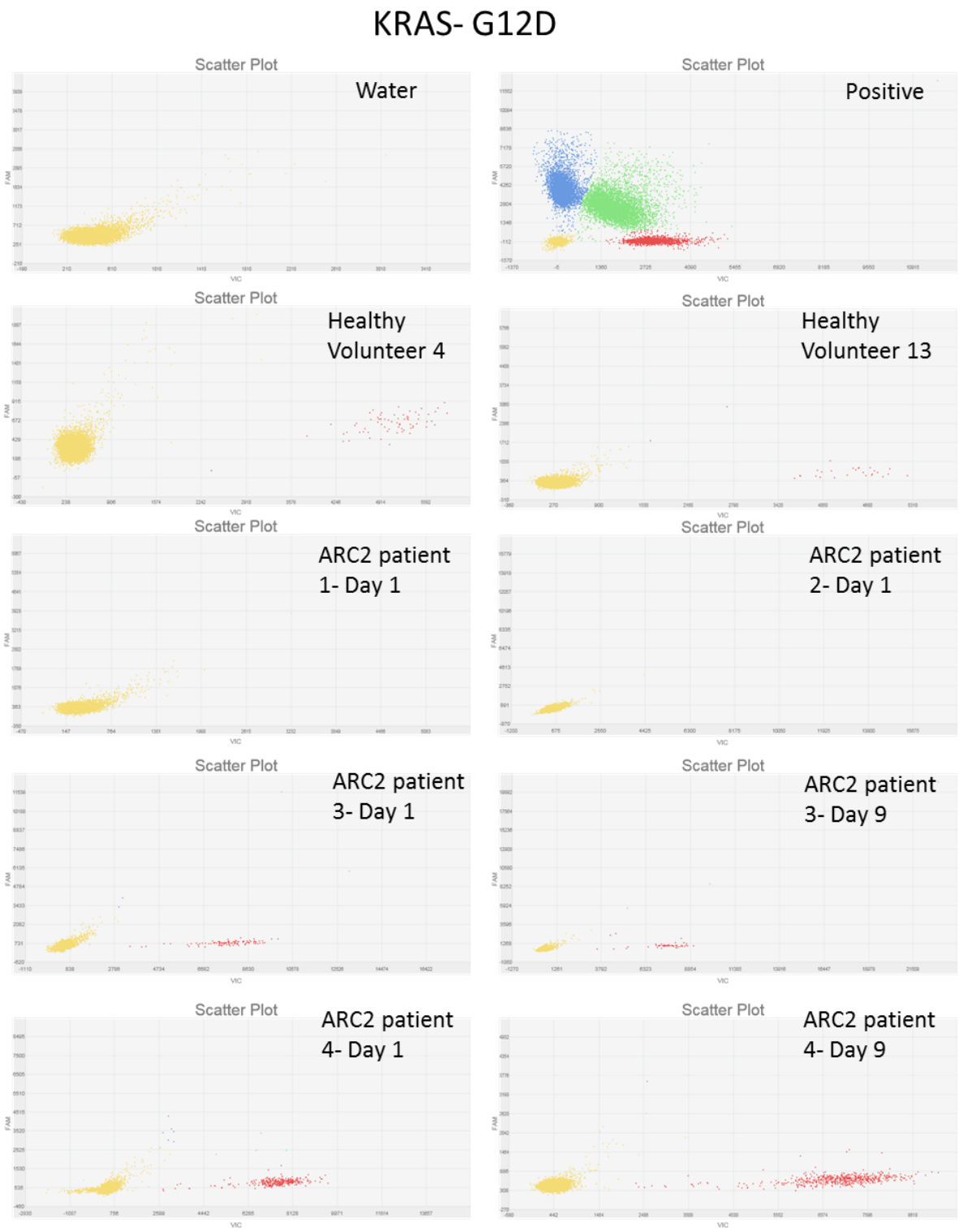


Fig.7.4. B: KRAS G12D mutation assay: Scatter plots from Quantsudio 3D Digital PCR analysis software showing VIC (Red) labelled KRAS-Wt and FAM (Blue) labelled KRAS-G12D. Yellow points refer to wells with no reaction.

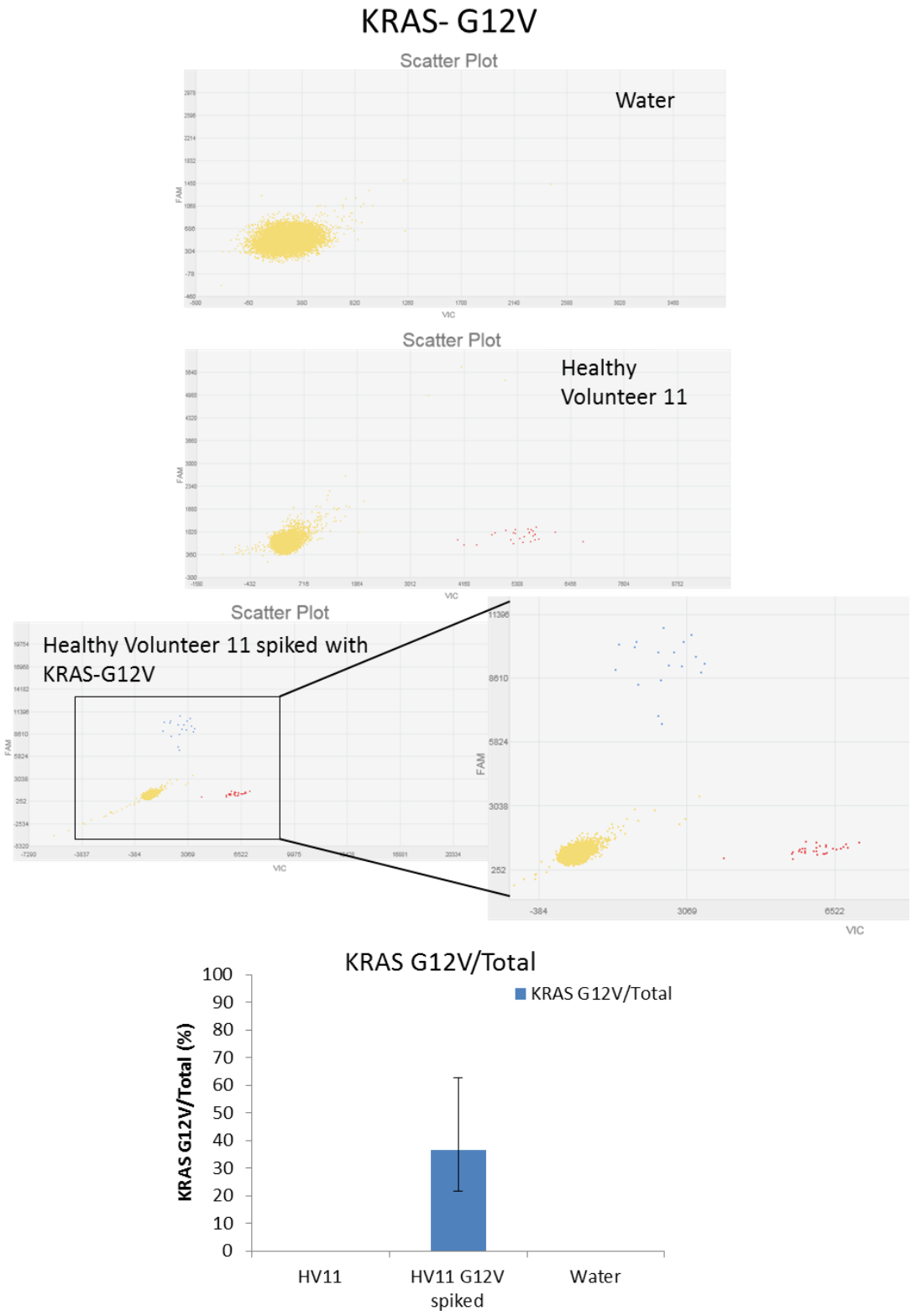


Fig.7.4.C: KRAS G12V mutation assay: 3000 Capan 1 cells were sonicated and added to plasma prior to processing. Scatter plots from Quantsudio 3D Digital PCR analysis software showing VIC (Red) labelled KRAS-Wt and FAM (Blue) labelled KRAS-G12V. Yellow points refer to wells with no reaction. Scatter plot of sample spiked with KRAS-G12V DNA showing FAM labelled KRAS-G12V. Graph at bottom represents the percentage of mutant to the total KRAS copies in the sample.

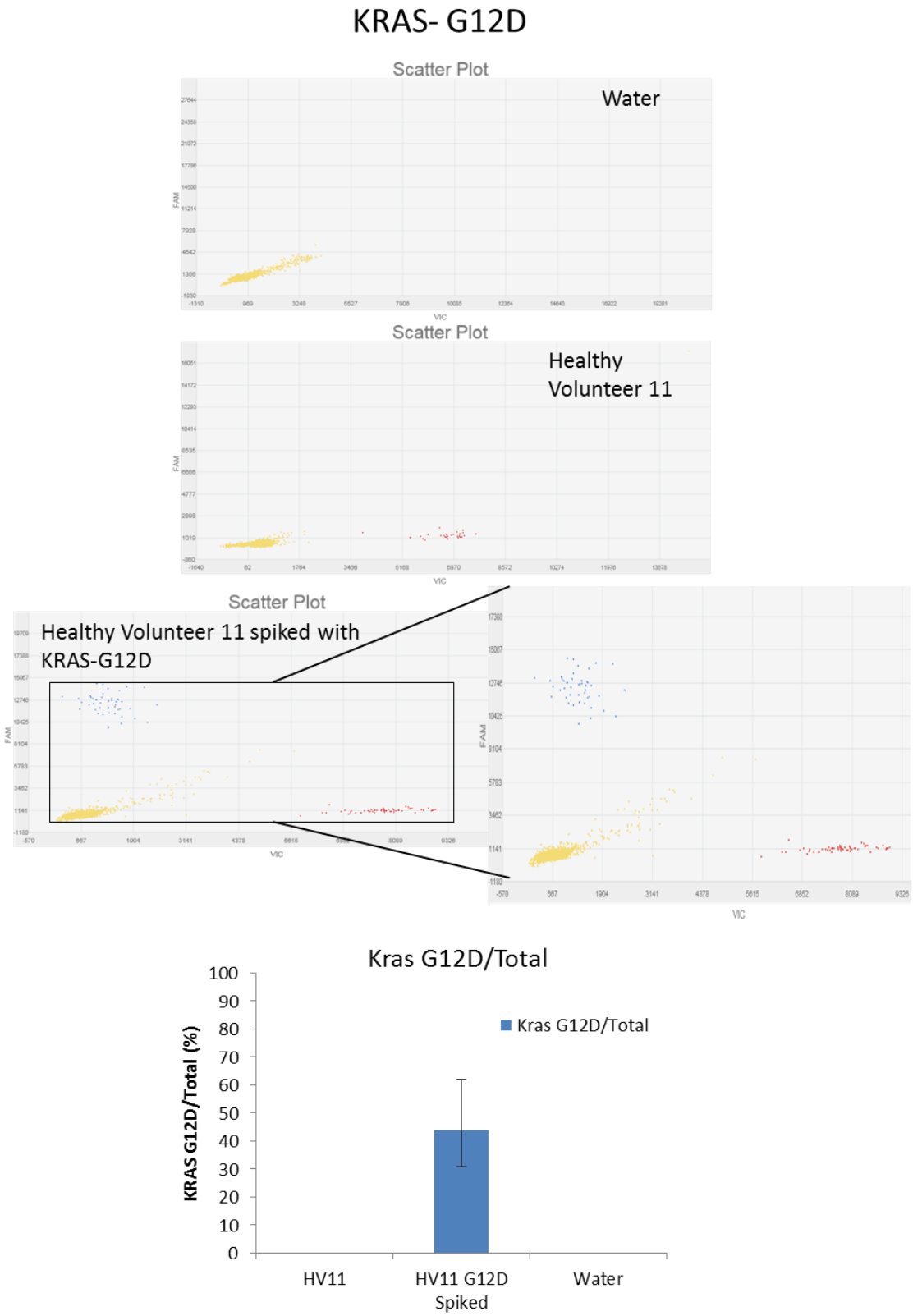


Fig. 7.4.D: KRAS G12D mutation assay: 3000 Panc 1 cells were sonicated and added to plasma prior to processing. Scatter plots from Quantsudio 3D Digital PCR analysis software showing VIC (Red) labelled KRAS-Wt and FAM (Blue) labelled KRAS-G12D. Yellow points refer to wells with no reaction. Scatter plot of sample spiked with KRAS-G12D DNA showing FAM labelled KRAS-G12D. Graph

at bottom represents the percentage of mutant to the total KRAS copies in the sample.

7.5 – Chapter 7 discussion

The presence of high levels of circulating cell free DNA in cancer patients was identified about 30 years ago (Leon et al., 1977; Stroun et al., 1987) and since then has been used to characterise tumour specific somatic mutations in a number of solid tumour types. Although a number of authors have reported on the significance of using cfDNA from “liquid biopsy” for mutation detection, identification of tumour progression and minimal residual disease follow-up, most of the work has been done on prospective studies where relatively large volumes of sample material (plasma) was used. We aimed to identify if it was possible to demonstrate the presence of specific mutations from minimal quantities of plasma especially from retrospective pancreatic cancer trials, where mutation detection could be compared with therapeutic outcome. Our study used cfDNA extracted from 1ml plasma which was assayed for the presence of two of the most common KRAS mutations commonly associated with pancreatic cancers. We also aimed to identify if we could use the information as a tool to follow disease progression through the course of treatment in the different clinical trials.

We first looked at the DNA yield from 1ml of plasma by studying the copy numbers of the single copy gene HBB and comparing then between healthy volunteers and cancer patients (including colorectal cancer patients and pancreatic cancer patients from the ARC 2 trial). As expected, the absolute number of HBB copies picked up from the DNA was 1-5 folds higher in the cancer patients, confirming that the extraction technique worked. We then decided to look

for KRAS mutations from pancreatic cancer patients enrolled in the ARC 2 clinical trial. Plasma from four patients belonging to the cohort were studied. Surprisingly, we found that though the assays were identifying the wild-type KRAS copies in the cfDNA, no mutant variants could be identified. While this could be because of the absence of KRAS mutations, it could also be that the mutations were not detectable in the relatively small volumes of plasma that we were using. We decided to confirm that it was not a technical issue but rather an actual observation by spiking volunteer plasma with KRAS mutant DNA following which we saw evidence of the mutant alleles being picked up on DPCR. This discrepancy could possibly be due to the relatively low copy numbers of the mutant allele in the circulation of patients, which makes detection difficult and could also be due to the fact that the assay probes were not picking up the fragments due to size or sequence constraints. The likelihood of the existing probes being unable to pick up smaller fragments is high as presence of shorter DNA fragments showed varying levels of detection (fig. 9.9). It could also be that the patients analysed carried one of the rarer mutations in pancreatic cancer like KRAS G12R or KRAS Q61H, which would not be detected by the G12V and G12D specific assays we used.

Chapter 8

General Discussion

Chapter 8 - General Discussion

8.1 – Synopsis

The constitutive AKT activation characteristic of many tumours as a result of activating pathway mutations or inactivation of the regulatory upstream components and mutations of the tumour suppressor p53 have been described to co-occur in many *in vivo* models (Blanco-Aparicio et al., 2010; Hanel et al., 2013). A molecular mechanism that can explain this event has not been described previously nor the clinical significance or the therapeutic implications of this interaction dealt-with in detail by previous authors. We attempted to answer this very interesting question which could explain the discrepancies in many previous reports. Our studies into the AKT interacting proteins identified the chaperone protein NPM as a target of AKT kinase activity and a specific amino acid moiety S48 as the AKT phosphorylating site that can regulate NPM oligomerisation and localisation. This regulation was further identified to mediate the localisation of ARF in cells. Constitutive AKT activity was shown to result in delocalisation of NPM and ARF from the nucleolus to the nucleoplasm and a subsequent decrease in MDM2 activity and stability of p53. This was significant in the light of p53mut tumours, as the increased AKT activity and p53mut stability resulted in the dominant negative effect of the p53mut which manifested as increased resistance to treatment. AKT inhibition with targeted agents resulted in a decrease in stability of p53mut and a subsequent p73 mediated cell-cycle arrest/senescence. These original observations from the *in vitro* setting were translated into pre-clinical *in vivo* studies, which showed appreciable effect when combining AKT inhibitors with DNA damaging therapies. We also further confirmed that our pre-clinical findings

were indeed relevant in patients, where we saw significant correlation between AKT activity, pS48-NPM and p53 expression. We also observed that the AKT-p53 relationship was dependent on the ARF status in patients, further validating our findings and clarifying previous reports that looked at the role of AKT mediated regulation of p53 (Hamilton et al., 2014).

The significance of S48 phosphorylation on NPM oligomerisation in the NPMc mutant variants found in AML was also briefly examined. Interestingly, we found that phosphorylation of S48 did indeed play a role in the NPMc-NPMc and NPM-NPMc interaction in AML cells. The downstream signalling interaction between FLT3 and NPM was also briefly analysed, and revealed an AKT and possible p38-MAPK mediated interaction between the two which could potentially be an explanation for the increased co-incidence of the two mutations in AML.

8.2 – AKT and NPM-ARF mediated regulation of MDM2 and p53

In this study, we investigated the AKT mediated regulation of NPM oligomerisation and the subsequent regulation of its localisation in the cell. The ARF chaperoning function of NPM in the context of AKT activity was also investigated, as was the effect of this regulation on downstream effectors, primarily MDM2 and its substrates. The original work from our group on the AKT mediated phosphorylation of the S48 moiety of NPM formed the basis of this study.

Previous authors have reported on the inhibition of NPM oligomerisation using small molecule inhibitors (Qi et al., 2008) and RNA aptamers (Jian et al.,

2009), which resulted in NPM and ARF localisation to the nucleoplasm and a subsequent increase in p53 activity. At the same time, there were also reports of the co-occurrence of AKT activating mutations and p53mut in the *in vivo* setting (Blanco-Aparicio et al., 2010; Hanel et al., 2013), although a physiological connection between all of this was unknown. Our results showed that AKT activity limited NPM oligomerisation and localisation to the nucleolus as well as resulted in a consequent decrease in the ARF localisation. The decrease in the ARF nucleolar localisation and a subsequent re-localisation to the nucleoplasm resulted in increased ARF mediated inhibition of MDM2, which in turn resulted in the stabilisation of the MDM2 substrates, principally p53. Thus in tumours with p53mut and constitutively active AKT signalling, the nucleoplasmic re-localisation of NPM/ARF leads to the inhibition of MDM2 and stabilization of p53mut.

ARF is a recognised tumour suppressor with its function described to be primarily through the inhibition of MDM2 (Kamijo et al., 1997; Pomerantz et al., 1998) and stabilisation of p53. The ARF mediated activity has been reported by some authors to promote p53 stability by sequestering MDM2 to the nucleolus (Lohrum et al., 2000; Weber et al., 1999), while others have reported that it is the nucleoplasmic ARF that inhibits MDM2 activity (Lin and Lowe, 2001; Llanos et al., 2001; Moulin et al., 2008; Rodway et al., 2004) and stabilizes p53. Our observations suggest a model where NPM associated ARF which is localised to the nucleoplasm interacts with MDM2 and inhibits its ubiquitinating activity. This is dependent on the tertiary structure of NPM, with oligomeric NPM being localised to the nucleolus, whereas the monomeric fraction is nucleoplasmic. Since ARF is closely associated with NPM, the oligomerisation of NPM and the subsequent trafficking of NPM to the nucleolus would result in the trafficking of ARF to the

nucleolus as well. On the other hand the monomeric NPM and its associated ARF would be restricted to the nucleoplasm, where the ARF can interact with and inhibit MDM2. Our studies further point out that the phosphorylation of the S48 moiety of NPM by AKT is the essential driving force that regulates the oligomerisation and the trafficking of both NPM and ARF between the nucleolus and the nucleoplasm and that this is utilized by cancer cells addicted to AKT signalling, to stabilize p53mut protein, thereby further enhancing their therapeutic resistance. In agreement with our model, it has been previously shown that overexpression of NPM and its localisation to the nucleolus is a mechanism whereby tumour cells impair the function of ARF as well as increase its stability in the cell (Chen et al., 2010a; Korgaonkar et al., 2005; Moulin et al., 2008; Rodway et al., 2004).

Interestingly, it has also been shown previously that NPM can associate with p53 (Colombo et al., 2002) as well as regulate its stability by binding MDM2 (Kurki et al., 2004). Following cell stress, NPM was reported to translocate to the nucleoplasm from the nucleolus and associate with MDM2 and impair its function, while failure to delocalize to the nucleoplasm prevented its MDM2 inhibitory function (Fukawa et al., 2012). Furthermore, the NPM trafficking between the nucleolus and the nucleoplasm was found to be analogous to the ARF/MDM2 interaction and complex formation following DNA damage or cell stress (Lee et al., 2005). Taking all of this into context, our model argues that the two events are not mutually exclusive, but rather respond to cell stress via regulation of the NPM tertiary structure. Even though the focus of our work has been on p53mut tumours, it also suggests that the same mechanism may play a role in cells carrying wild type p53. This is of significance in the context of oncogene activation in the

absence of DNA damage, where AKT activity as a result of activating upstream mutations can lead to increased p53 stability and induction of senescence (Astle et al., 2012; Chen et al., 2005c; Xue et al., 2007). Indeed we saw that the AKT inhibition resulted in a decrease in both wild type and mutant p53 in a number of cell types (fig.4.4), but not in cells that had deleted CDKN2A or epigenetically suppressed ARF.

AKT has been previously described to phosphorylate the S166 and S186 of MDM2 and promote its nuclear translocation and subsequent p53 ubiquitinating activity (Mayo and Donner, 2001; Zhou et al., 2001b). Interestingly, these studies were done in cells that had INK4A/ARF deletions, and in agreement with these reports, we observe in the ARF null MCF7 cells that inhibition of AKT resulted in an increase in the stability of p53 (fig. 3.10.D). This was also in agreement with previous studies where the S166 and S186 of MDM2 has been shown to be phosphorylated by the oncogenic Protein Kinase Pim-1, but which required ARF activity to regulate the p53 protein levels (Hogan et al., 2008). We saw that expression of ARF cells stabilized p53, but was now dependent on NPM, and inhibition of AKT in this context decreases p53 levels. The ARF localization to the nucleolus following AKT inhibition was also not influenced by the exogenous expression of MDM2. All this is in line with previous reports, where constitutive activation of AKT has been demonstrated to inhibit MDM2 (Astle et al., 2012). Furthermore, an important point to be noted when looking at the above mentioned original studies is that they refer to exogenous expression of MDM2 rather than the endogenous levels of MDM2. This will certainly have an influence on any interpretation of the effect of AKT inhibition on MDM2 activity in these cells. The use of exogenous expression systems to express proteins and study their

interactions may be confounded by the disparity in the protein levels and their regulatory mechanisms compared to physiological systems. This highlights the need to use cell lines as well as knock-in MEFs that do not possess other cancer associated mutations. While NPM oligomerisation and localization was studied in NPM $^{-/-}$ MEFs that were transfected with different NPM variants, our cancer cell model depended on the ARF and p53mut expressing T24 cell lines to address all our questions (which is further substantiated by the panel of cell lines in Fig. 4.4E). In order to clarify the differences that may be present between exogenous expression of proteins in MEFs and the protein regulation in tumour cells, we further addressed our questions using tumour cells derived from genetically modified mouse models of cancer. The KPC pancreatic cancer mouse model consists of a mutant KRasG12D p53R172H expressed in pancreatic tissue by Pdx1-Cre (KPC) mice. KP^{fl}C (KRasG12D floxed *Tp53*) mice were developed to address the contribution of p53 mutant protein to tumorigenesis (Morton et al., 2010). We took advantage of KPC mice crossed with ARF-/- mice which allowed us to address the specific question of AKT and ARF contribution to p53 mutant stability in a genetically clean and comparable background. To directly compare the effect of AKT inhibition on p53mut levels and the effect on therapeutic responses, we took cells derived from KPC (p53R172H); KPC ARF-/- (p53R172H :ARF-/-) and KP^{fl}C (p53Floxed) tumours and cultured them *ex vivo* for further analysis. Substantiating our findings, we observed that the AKT mediated effects were dependent on ARF and p53mut protein using colony survival assays and 3D spheroids of the genetically altered cells.

The INK4A/ARF locus function can be inactivated by methylation or deletion in many cancers (Bardeesy et al., 2006).While deletion of the INK4A

locus can result in loss of both splice variants, quite often, the functional loss of p14ARF may be independent of p16 or vice-versa especially in the presence of inactivating methylation events (Esteller et al., 2000; Freedberg et al., 2008). Taking this into consideration, we analysed the correlation between AKT and p53 in breast and pancreatic cancer patients from The Cancer Genome Atlas (TCGA) portal and this further demonstrated the dependence on CDKN2A expression. This also substantiates our argument that the benefit from AKT inhibitors are likely to be dependent on the p53 and ARF status in patients with solid tumours, highlighting the need for screening the patients prior to initiating treatment.

ARF localisation depends on the oligomeric state of NPM and the signal required to disrupt NPM tertiary state and promote an increase in the monomeric fraction of NPM has to be sufficiently high. This is classically seen following oncogene activation and can act as a sensor for stress in cancer cell. Interestingly, while functional ARF has been shown to be essential for induction of senescence (Christophorou et al., 2006; Efeyan et al., 2006; Kamijo et al., 1997; Pomerantz et al., 1998), our observations as well as reports from other authors (Korgaonkar et al., 2005; Lin and Lowe, 2001; Llanos et al., 2001; Moulin et al., 2008; Rodway et al., 2004) suggest that ARF localisation is also essential for it to exert its senescence promoting function. We have shown that S48-NPM is essential for regulating NPM tertiary structure and the subsequent sub-nuclear localization of NPM and ARF. Other N-terminal post-translational modifications of NPM that are affected by other kinases are also likely to modulate NPM and ARF localization. This has been suggested by in-silico analysis (Mitrea and Kriwacki, 2012) and our own work on NPM mutations in AML where we briefly examined the phosphorylation of T95-NPM as a target of p38-MAPK.

We thus demonstrated a model (fig. 8.2) where AKT inhibition results in increased oligomerisation of NPM and localisation at the nucleolus with the consequent localisation of ARF to the nucleolus. Subsequent increase in the activity of MDM2 promotes increased turnover of p53, which in the context of p53mut cells would result in a decrease in the stability of the mutant protein (Hamilton et al., 2014).

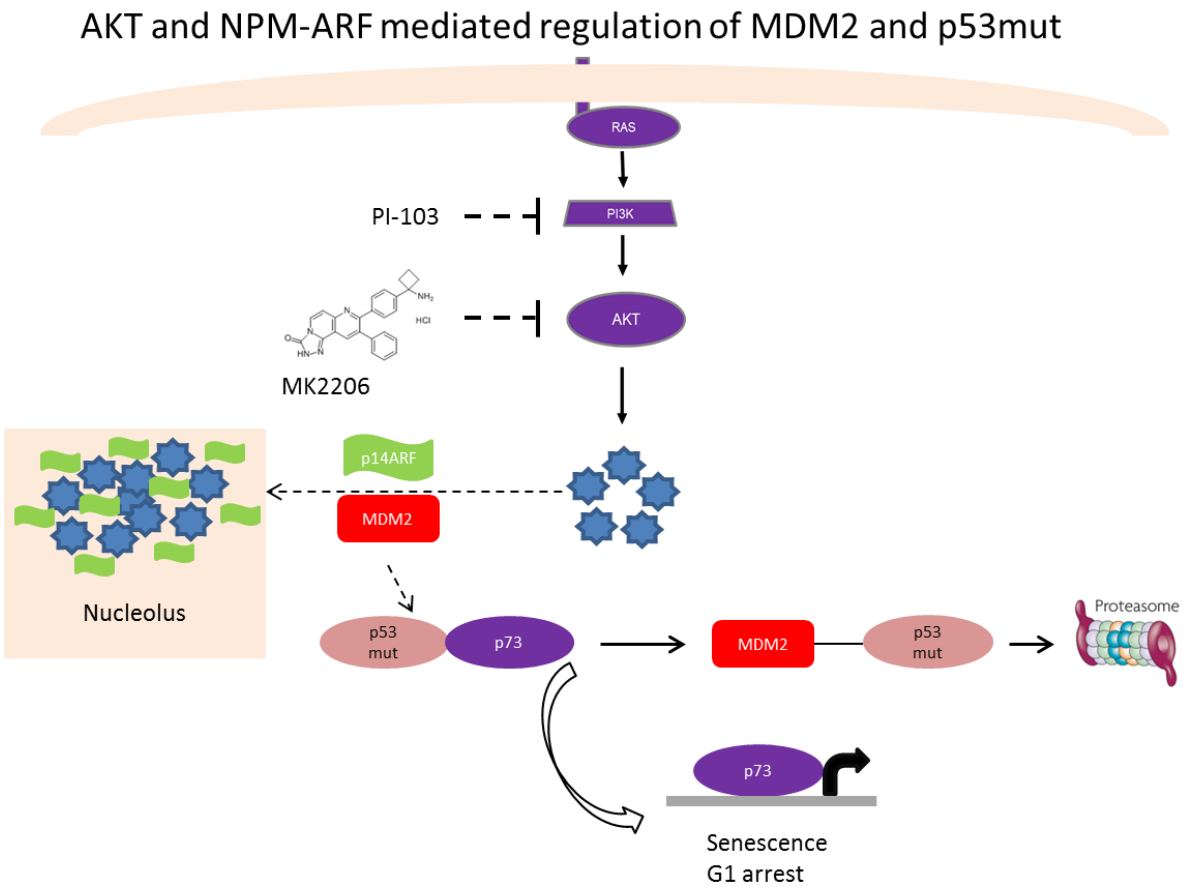


Fig. 8.2: Proposed model of the AKT mediated regulation of NPM and ARF and the subsequent regulation of p53mut protein stability

8.3 – AKT inhibition and sensitisation of p53mut cells and the *in vivo* effects

p53 mutations have been described to confer resistance to tumours and this has been attributed to the increased stability of the p53mut protein (Blandino et al., 1999; Muller and Vousden, 2013; Schmitt et al., 2002b; Vousden and Prives, 2009). While many solid tumours have been associated with missense mutations of p53, the mutations alone do not account for the stability of p53mut protein (Lee et al., 2012). Since MDM2 can mediate degradation of p53mut (Lang et al., 2004; Lee et al., 2012; Olive et al., 2004; Terzian et al., 2008) albeit to varying extends depending on the type of mutation, the stabilization of p53mut should possibly occur when cells transform and attain oncogenic mutations. In such cases, therapies that can promote p53mut turn over have the potential to sensitize tumours to treatment. A previously studied mechanism that can be taken advantage of for therapeutic purposes has been that of the Heat Shock Proteins (HSP) that promote p53mut stability by directly stabilizing p53mut as well as by inhibiting MDM2 and CHIP E3 ubiquitin ligases. Treatment with HSP inhibitors have been shown to decrease the stability of the p53mut and increase the sensitivity to other chemotherapeutic agents (Li et al., 2011b; Nagila et al., 2011). This is in line with our data, where we see that with a decrease in the stability of p53mut, there is an increase in the sensitivity of the cells to other therapeutic agents, especially DNA damaging treatments like IR.

In tumours with constitutively active mitogenic signalling, NPM is unable to oligomerise and remains in the nucleoplasm, where it retains ARF and promotes MDM2 inactivation. A subsequent increase in the stability of p53mut results in increased dominant negative effects with p53mut inhibiting the anti-tumor effects

of transcription factors including p73 (Muller and Vousden, 2013). In such cells, treatment with an AKT inhibitor would result in NPM-ARF localization to the nucleolus, which would in turn promote increased MDM2 activity and p53mut turn-over, releasing the repression of p73 and initiating cell cycle arrest. MDM2 is essential for this regulation of p53mut, as inhibition of AKT in cells treated with MDM2 inhibitors (Nutlin 3a) do not show increased p53mut turnover. At this point, it is also interesting to note that reducing the levels of p53mut protein using AKT inhibitors alone is not an effective treatment strategy, as an additional insult or damage that promotes the activation of other transcription factors is essential to promote activation of the senescence pathways. This was highlighted in the *in vivo* setting, where treatment with the AKT inhibitor alone did not show any significant effect in delaying tumour growth. This was also in line with previous reports where monotherapy with AKT inhibitors were inadequate to produce a significant therapeutic effect (Hirai et al., 2010; Ihle et al., 2009).

ARF has been well characterised as a tumour suppressor due to its role in regulating the levels of wild type p53 that prevents malignant transformation of cells (Bates et al., 1998; Kamijo et al., 1997; Sherr, 1998). Quite interestingly, our findings suggest that in the same manner that ARF stabilizes wild-type p53 following cell stress, it can also stabilize p53mut which in turn contributes to the resistance to IR due to the dominant negative effects of the mutant protein. Other reports have also suggested a similar role for ARF in the p53mut cells. Knockout of the Pml gene in an ARF positive p53 mutant background in mice showed increased stability of the p53mut protein through inhibition of MDM2 (Haupt et al., 2013), whereas inhibiting E2F1 mediated transcription of ARF using TGF-β1 de-stabilised p53mut in lymphoma cells (Chen et al., 2012). Thus there is growing

evidence supporting our data that ARF in the context of p53mut cells can act as an oncogene and promote therapeutic resistance in those cells.

The significance of AKT activity and p53mut in human tumours are still unclear, although a wealth of data exists from *in vitro* and pre-clinical animal studies regarding the relationship between the same. Mutations that activate the PI3K/AKT pathway have been described by various authors, some of the most common of which are the Ras and EGFR/HER2 activating mutations. Pancreatic cancers which are associated with close to 98% KRAS activating mutations (Pylayeva-Gupta et al., 2011) were analysed in our study and we found that in the tumours that retained ARF function, the AKT activity and pS48-NPM expression significantly correlated with p53 levels, indicating that ARF and AKT are contributing to the stability of p53. Likewise, analysis of breast cancer TMA's showed an increased correlation between EGFR/HER2 activity, pS48-NPM and p53, with the highest correlation associated with tumours in the late stage of the disease supporting previous reports where accumulation of p53mut in tumour cells (Davidoff et al., 1991; Miller et al., 2005) occurs in late stage disease and is also associated with increased resistance to treatment with standard lines of therapy. Interestingly, clinical reports of resistance to treatment with EGFR and HER2 targeted agents, has been shown to be reversible with PI3K/AKT inhibition (Gajria and Chandarlapaty, 2011; Rebucci et al., 2011). In a manner that closely resembles this effect, our findings suggest that activation of the PI3K/AKT pathway promotes p53mut stability, which in turn promotes progression of disease in such patients. Treatment with AKT inhibitors would reverse this effect in an NPM and ARF dependent manner leading to a decrease in p53mut stability and sensitise the tumours to other therapeutic agents.

Our findings present a novel explanation for the development and progression of tumours in the presence of oncogenic signalling and impaired p53 function. As has been described previously, the occurrence of mutations rather than deletions is important in the case of p53. The oncogenic signalling via the mitogenic pathways that is commonly seen in tumours promote the stabilisation of the mutant p53 protein, thereby giving added impetus to the cellular machinery that plays a role in overcoming therapeutic sensitivity. Our findings suggest that inhibiting the signalling pathway, especially AKT, plays an important role in re-sensitising tumours to standard treatments. This in turn outlines a patient selection strategy based on the p53mut and ARF status to ensure that the best benefit is achieved from the use of targeted PI3K/AKT inhibitors in the clinic.

8.4 – Regulation of NPMc in AML

NPM mutations that affect the sub-cellular localisation of NPM have been reported to occur frequently (35%) in AML. These mutations are most commonly associated in patients with AML-M3/M4 and a normal karyotype. Due to the important role that NPM plays in regulating ARF and p53 interaction, as well as its chaperoning role in transporting the pre-ribosomal particles in the cell, it has received a lot of attention as a potential initiator of leukaemia. Interestingly it has been shown to be an early initiator mutation that can persist in the malignant clone of cells (Falini et al., 2008a) and persists late in the disease, so much so that they have been frequently used as markers for minimal residual disease (Kronke et al., 2011). Type A mutation of exon 12 of the NPM gene results in duplication of a TCTG tetranucleotide at position 956 to 959 of the NPM reference sequence

(Falini et al., 2007) and loss of either 290 or both 290 and 288 tryptophan residues that causes the helical structure of NPM C-terminal domain to unfold (Grummitt et al., 2008) as well as generate a new nuclear export sequence (NES) motif. The loss of the nucleolar NLS region and the additional NES that is formed subsequently, reinforces nuclear export of NPM that is characteristic of the AML associated NPM mutant (NPMc) (Falini et al., 2006). The increased trafficking of the mutant protein to the cytoplasm is thought to promote translocation of other proteins including NPM and ARF, further promoting the malignant nature of the AML cell.

Our findings on S48-NPM in solid tumours suggested that a similar role may be played by the same phosphorylation site, which could regulate the interaction of NPM with itself and other proteins. Indeed, as described earlier, a principle effect of the mutation has been suggested to be that of aberrant shuttling of remnants of wild-type NPM by NPMc from the nucleus to the cytoplasm as well as impaired localization to the nucleolus (Falini et al., 2006; Falini et al., 2008b). We see that while the NPMc-NPMc interaction requires S48-NPM, the same is the case with interaction between the wild type and mutant proteins. This is interesting in the context of clinical cases where the AML cells are known to be heterozygous for NPM, with both wild-type and mutant proteins being expressed and the mutant NPMc exhibiting dominant-negative effects on the wild type protein (Falini et al., 2006). In such cases the phosphorylation at S48 will play an important role in the interaction between the protein molecules. In AML cells carrying a concomitant FLT3 activating mutation (Dohner et al., 2005; Schnittger et al., 2005), this attains even more significance. Our findings suggest that FLT3 acts via downstream AKT signalling as well as possible p38-MAPK signalling to promote increased

phosphorylation and a consequent increase in the non-oligomeric fraction of NPM. In the heterozygous cells (as exemplified by the OCI-AML3 cells in our study), where increased FLT3 signalling impairs oligomer formation, this can promote increased trafficking of NPM to the cytoplasm. In such cells, there also exists the possibility that the FLT3 signalling maybe affecting the wild type NPM remnants and promoting their translocation to the cytoplasm as evidenced by the increased presence of NPM in the nucleus following AKT inhibition. Indeed AKT inhibition may promote increased localization of NPMc to the nucleus by way of its increased interaction with the wild type NPM. This could also partly explain the interesting correlation between NPMc and FLT3 mutations in many AML patients, where it is associated with poor therapeutic response. In spite of our findings presented previously, quite surprisingly, we see that AKT inhibition has no significant effect on cell survival and worryingly has a negative effect on the p53 protein levels in the cells. Since AML cells are known to carry wild type p53, this is of concern, and could possibly explain why trials using AKT inhibitors as single agents have not been as effective as expected. While our original explanation for the decrease in p53 levels in cells could hold true in this context, we are then restricted to believe that it is the effect of AKT inhibition on the wild type NPM remnants, which promote their localization to the nucleolus that is responsible for the effect. The FLT3 signalling via the p38-MAPK pathway although investigated briefly will require further confirmatory studies, including site directed mutagenesis of the Threonine-95 moiety, before we can conclusively understand the exact role of the mitogenic signalling pathways in these NPM mutant cells. Interestingly, in the <3% of AML patients who are known to carry p53 mutations and are associated with poor prognosis (Wattel et al., 1994), using AKT inhibitors may well

be an ideal targeted therapy to sensitize them to other standard chemotherapeutic agents.

8.5 – Identification of cancer mutations from liquid biopsies

The idea of using plasma derived cfDNA as a sort of “Liquid Biopsy” has been around from the time tumour derived circulating DNA was identified (Stroun et al., 1987) in cancer patients- who were seen to have markedly higher levels of circulating DNA. cfDNA has been found to harbor the genetic or epigenetic alterations, that is seen in the tumour cells (Schwarzenbach et al., 2011) and is also detectable in circulation in higher genomic equivalents even prior to detection of circulating tumour cells. A number of techniques have been used for identifying point mutations in the cfDNA including Next Generation Sequencing, Digital PCR and BEAMing (Diehl et al., 2008; Schwarzenbach et al., 2011) with varying degrees of success.

We sought to analyze cfDNA from pancreatic cancer patients enrolled in the ARC 2 trial in an attempt to identify specific mutations of interest. KRAS mutations have been described to occur in almost 95% of pancreatic cancer patients with 95% of these being restricted to KRAS G12V and KRAS G12D mutations(Eser et al., 2014; Pylayeva-Gupta et al., 2011). We attempted to identify these two most frequent mutations in the cfDNA of pancreatic cancer patient using the Quantstudio 3D Digital PCR platform and proprietary custom Taqman assays developed by Life Technologies. We proposed the use of the digital PCR system to study these mutations due to the higher sensitivity of the system to identify rare mutations from small volumes of material. This was particularly important to us

since our project plan proposed the use of low volumes of plasma (1ml) from retrospectively collected samples. Most studies looking at the circulating DNA in cancers have attempted to use larger sample volumes for extraction of cfDNA (Dawson et al., 2013; Forshew et al., 2012; Murtaza et al., 2013). We on the other hand were specifically looking for techniques that could be utilized to give us maximum information from the least sample material. The KRAS analysis was also undertaken to establish and optimize the techniques for further in depth analysis of the cfDNA for other tumour associated genomic changes including epigenetic changes. We were also keen to correlate the KRAS expression with the disease progression during the course of the trial when samples were collected.

Digital PCR analysis of the cfDNA using the KRAS mutant probes failed to identify KRAS G12V and G12D variants in the patient samples. While this could certainly be attributed to the absence of KRAS mutations or the presence of one of the rarer mutations, it could also mean that the copy numbers of the mutant variant is too low to be detected from the sample that we were using. Interestingly it has been previously reported that circulating DNA does suffer from false negative correlation with the mutation in the tumour in about 25-30% of tumours with KRAS mutations (Theodor et al., 1999). Thus further work using better extraction techniques or prospective trials using larger starting samples will be required to validate the technique as a sensitive and cost effective tool for identification of KRAS mutations. Another interesting area that will benefit from analysing the cfDNA especially in the context of our study is the identification of p53 mutations and the correlations between RAS and p53 mutations and therapeutic sensitivity. Data mined from The Cancer Genome Atlas showed that in pancreatic cancers, unlike other solid tumours like breast cancers, the p53

mutations are not restricted to certain 'hotspots', but is quite evenly spread out in the DNA binding region of p53. This limits the utility of PCR in p53 mutation analysis, due to the sheer number of primer-probes that will require to be synthesised. Targeted sequencing will be an ideal tool in this circumstance, although the present cost may limit its use to speciality centres.

8.6 – Future directions

Our initial work on the regulation of p53mut and p53 by oncogenic signalling via AKT conclusively showed that patients carrying p53mut tumours and functional ARF are likely to benefit from targeted AKT agents. Equally important, it also showed the possible danger of decreased p53 function in tumours carrying wild type p53 and treated with AKT inhibitors. Our data highlights the urgent requirement for ideally controlled clinical trials where the mutation status of the patients will be taken into account when combining AKT inhibitors with other chemotherapeutic agents. Trials planned with our clinical collaborators are hoping to examine these questions in the near future. Our work on cfDNA, analysing trial material, will also play a significant role in the planned trials, as well as help identify other areas of interest like epigenetic changes that may play a significant role in clinical practise.

The findings in AML with NPM mutations confirm the importance that S48 plays in regulating the interaction between wild type NPM and the mutant NPMc variant. The interaction between NPMc and FLT3 activating mutations were also briefly examined, which identified the AKT mediated regulation of NPM as also a possible role for p38-MAPK. Further work looking at the T95-NPM phosphorylation

site in the context of FLT3 mutations as well as the role of FLT3 specific inhibitors will be required to clarify our findings.

Appendix

Chapter 9- Appendix 1

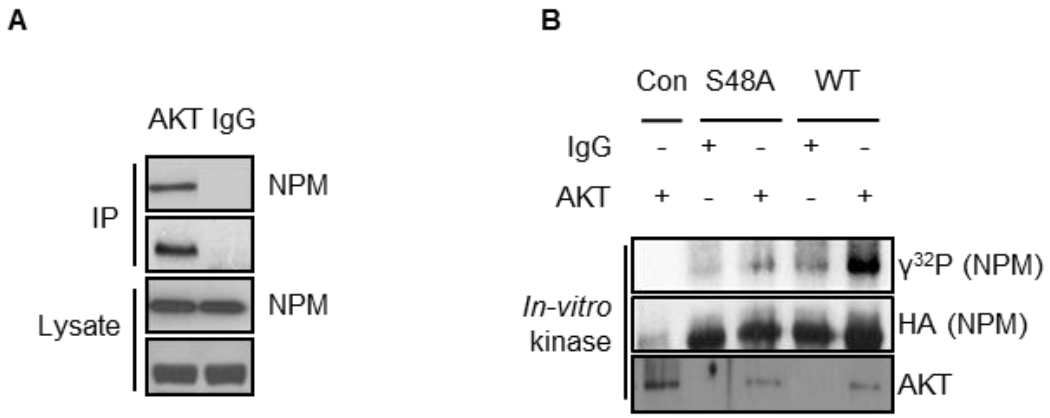


Fig. 9.1 (Figures courtesy of Dr. Garth Hamilton): A) AKT or IgG immunoprecipitates and whole cell lysates from T24 cells were probed with indicated antibodies. B) *In-vitro* kinase assay of immunopurified AKT or IgG control with NPM mutants in the presence of radiolabeled (γ^{32}P) ATP as indicated.

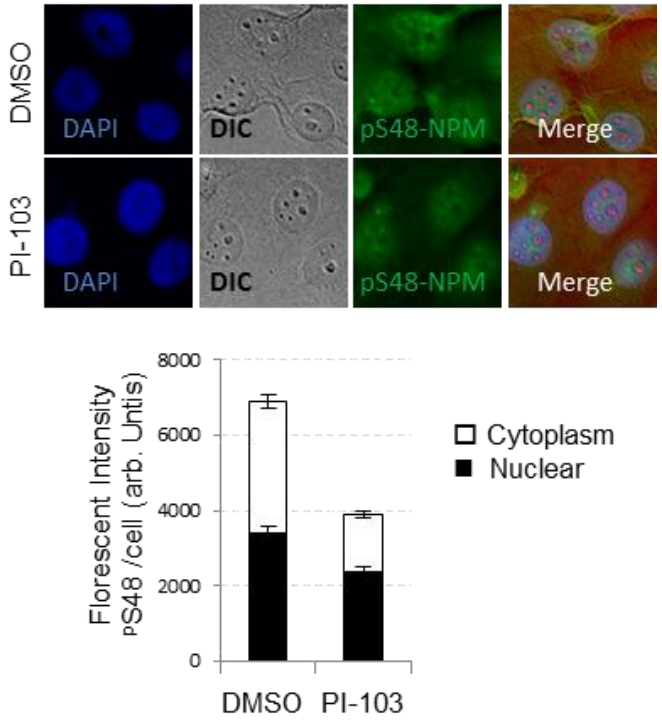


Fig. 9.2 (Figure courtesy of Dr. Oliver Sampson) T24 cells were treated with PI-103 (0.4μM) and stained for pS48-NPM. Phase-contrast microscopy image shows nucleoli and Immunofluorescence image shows pS48-NPM staining (green). Lower panel shows bar graph of fluorescence intensity of pS48-NPM staining in the different compartments of the cell as measured by InCell analyzer 1000 automated florescent microscope

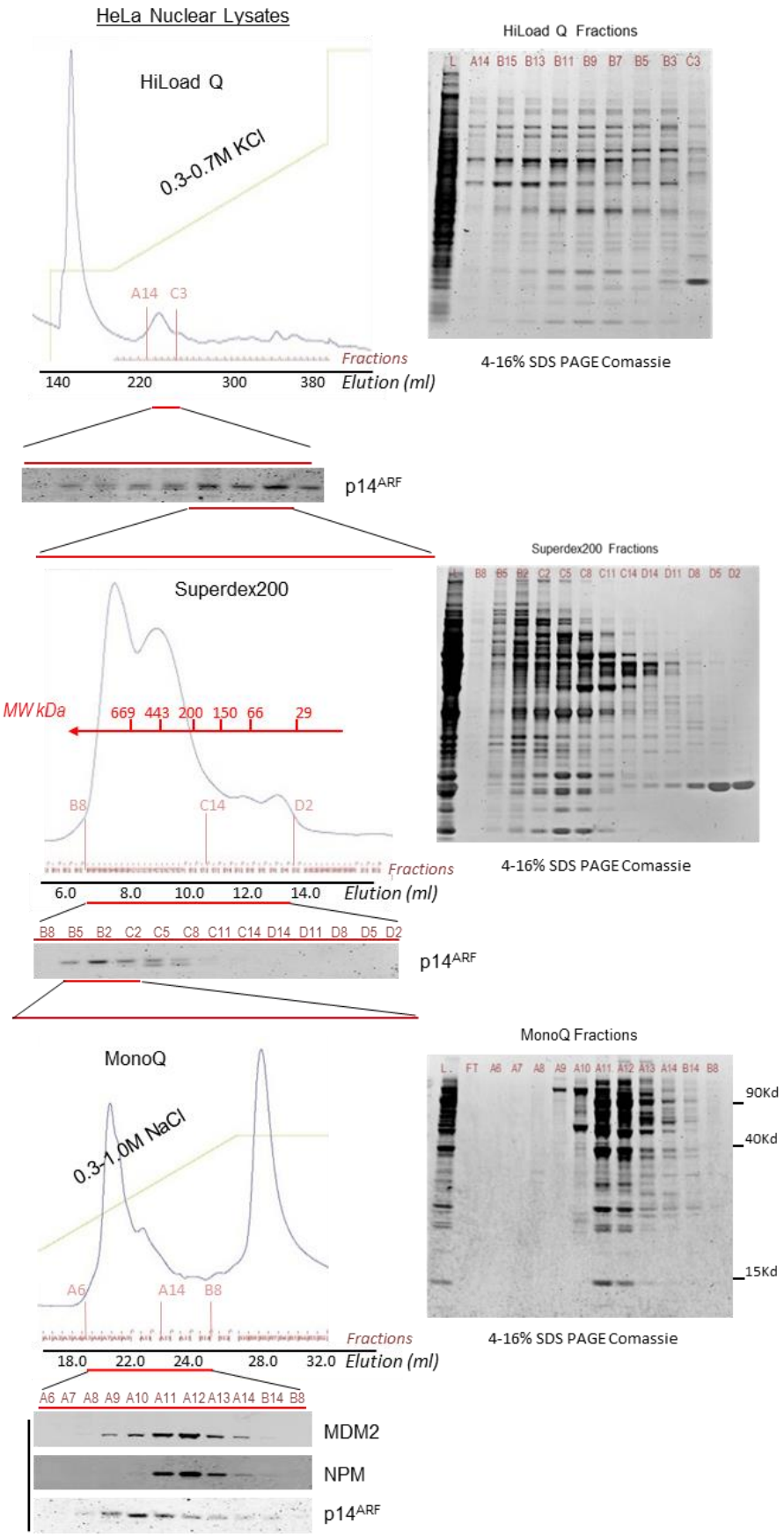


Fig. 9.3 (Figure courtesy of Dr. Svetlana Koronenkova) $p14^{ARF}$ containing complexes were purified from nuclear lysates prepared from a commercial prep of

Hela cells. Lysates were first resolved on a HiLoad Q anion exchange column and proteins eluted with a linear gradient KCl (0.3-0.7 M). p14ARF containing fractions were pooled and further resolved on a Superdex200 gel filtration column. Those fractions containing $p14^{ARF}$ following gel filtration, were pooled and subsequently resolved on a MonoQ anion exchange column. Proteins were eluted with a liner gradient of NaCl (0.3-1.0 M) and fractions containing MDM2, NPM and $p14^{ARF}$ (eluting at approx. 0.5 M NaCl) identified by western blot. Panels on the right show SDS PAGE gels stained with Comassie blue to identify the protein bands

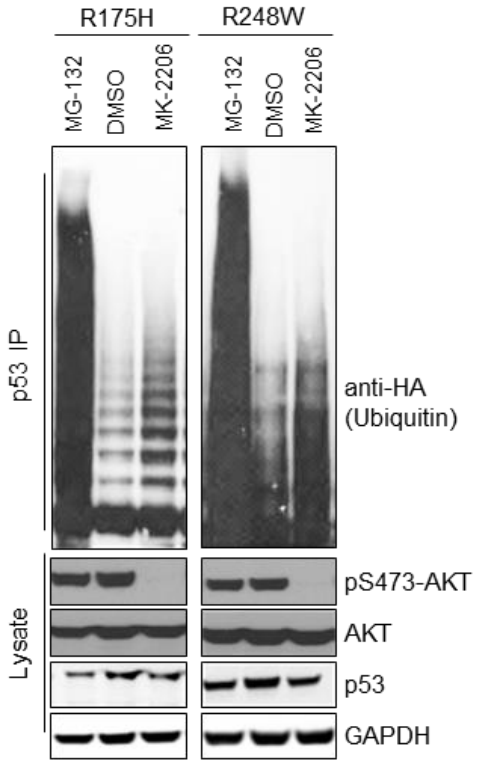


Fig. 9.4 (Figure courtesy of Dr. Dafni Pefani) H1299 cells were transfected with HA-tagged-ubiquitin and mutant p53 (R175H or R248W) as indicated. Transfected cells were treated with DMSO, MK-2206 (5 μM) or MG-132 for 16hrs as indicated. p53 immunoprecipitates and whole cell lysates were probed with the indicated antibodies.

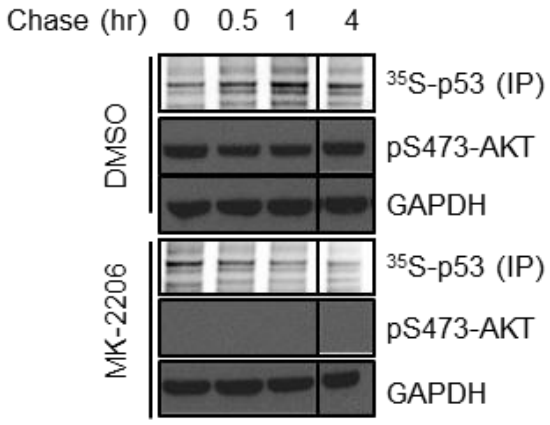


Fig. 9.5 (Figure courtsey of Dr. Garth Hamilton)T24 cells were pre-treated with DMSO or MK-2206 (5 μM,) for 24 hrs before the addition of Met/Cys free media. ^{35}S labeled Met/Cys was then added to the media before being chased for the indicated times with unlabeled Met/Cys. ^{35}S labeled p53 identified by autoradiography of p53 immunoprecipitates. Whole cell lysates were probed with the indicated antibodies

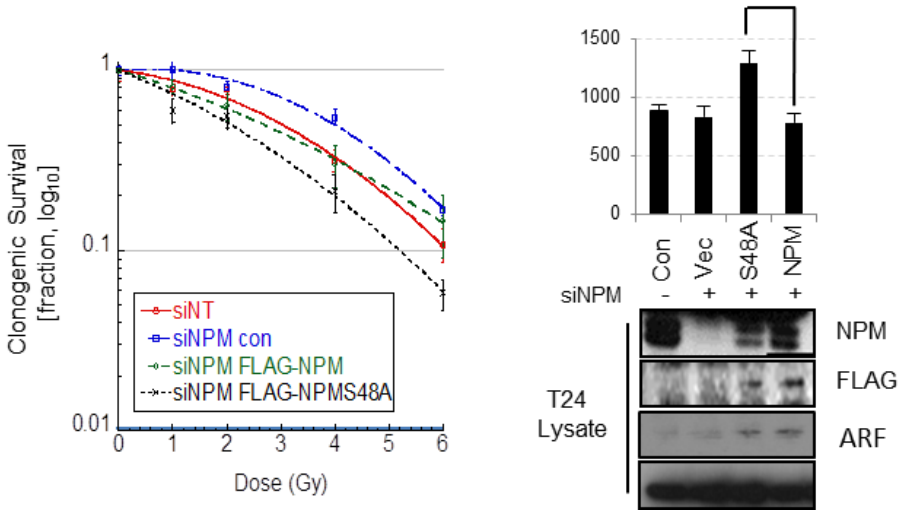
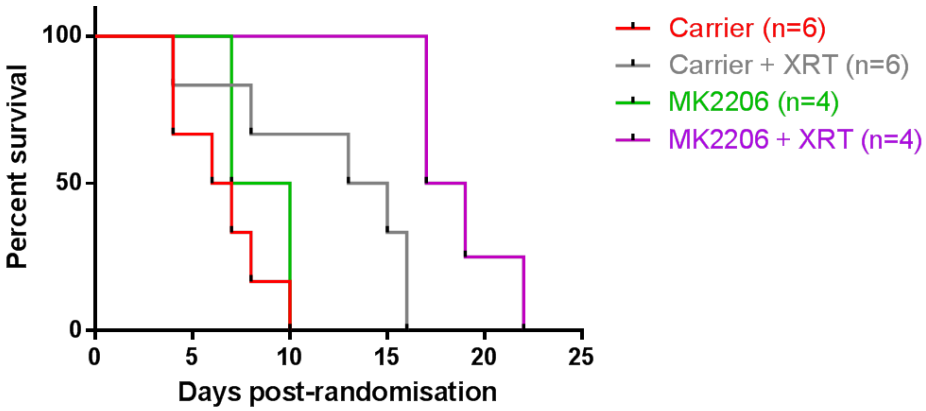


Fig. 9.6 (Figure courtesy of Dr. Oliver Sampson) Clonogenic survival of T24 cells transfected with NT or NPM siRNA and siRNA resistant FLAG-NPMWT or FLAG-NPM-S48A as indicated. Whole cell lysates were blotted with the indicated antibodies. (Right) quantification of p14ARF nuclear fluorescence by In Cell Analyser 1000 automated epifluorescence microscope. Data are represented as mean ± SEM.



Log-rank (Mantel-Cox) test (recommended)	
Chi square	
df	3
P value	< 0.0001
P value summary	****
Are the survival curves sig different?	Yes
Logrank test for trend (recommended)	
Chi square	
df	1
P value	< 0.0001
P value summary	****
Sig. trend?	Yes
Gehan-Breslow-Wilcoxon test	
Chi square	
df	3
P value	< 0.0001
P value summary	****
Are the survival curves sig different?	Yes

Fig. 9.7: Survival statistics of *in vivo* PSN1 xenograft bearing animals

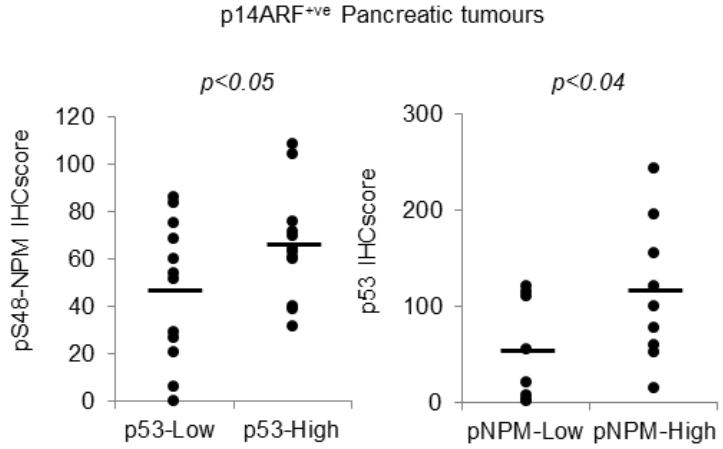


Fig. 9.8 Tumours from 40 patients with pancreatic tumours were subjected to mRNA analysis for ARF expression. 26 out of 40 were positive for $p14^{ARF}$ mRNA. Graph indicates correlation of 25 tumours with $p14^{ARF}$ positive signals, splitting population on median and significance verified by student *t*-test (one tailed)

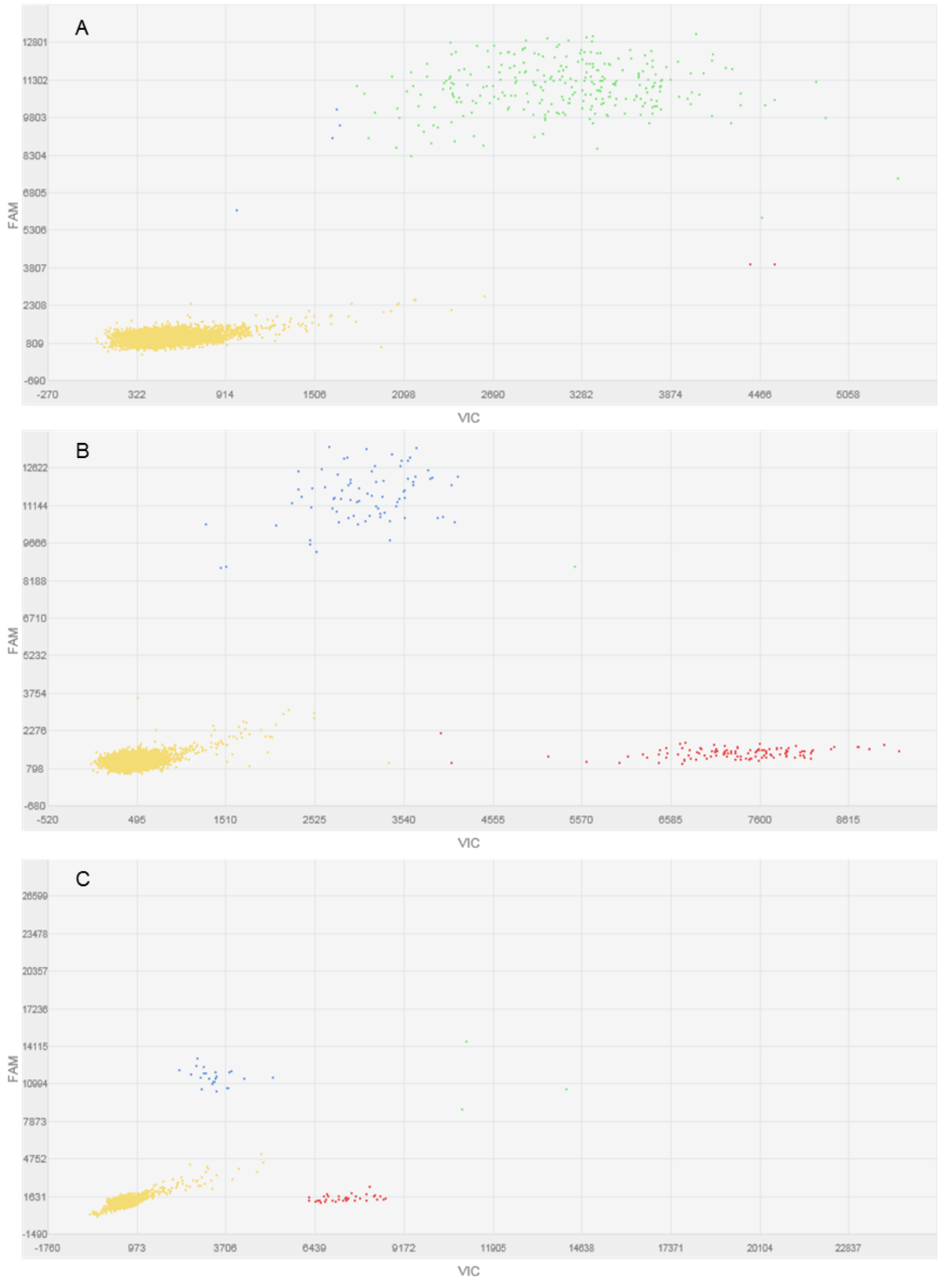
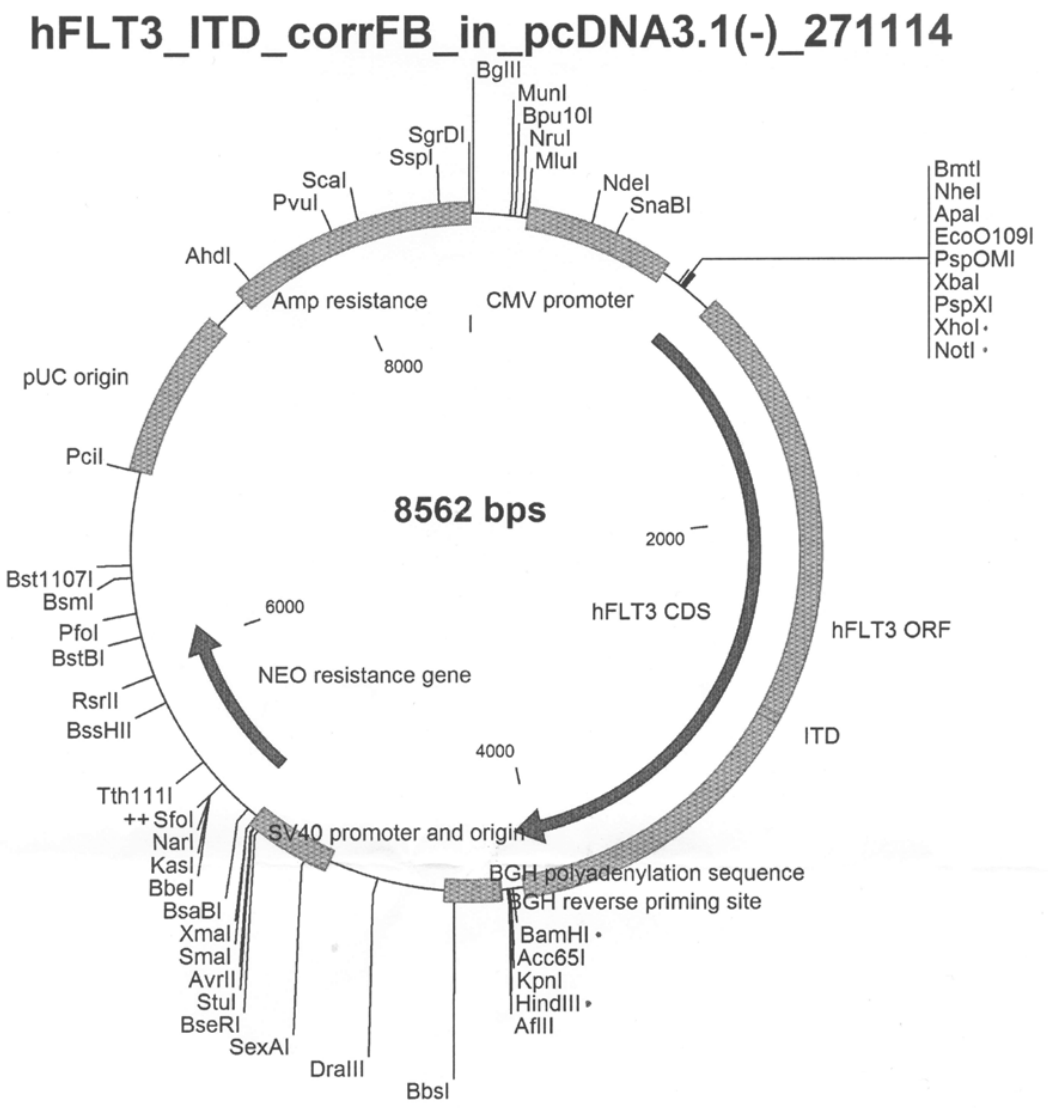


Fig. 9.9: KRAS G12V mutation assay: A) 3000 Capan 1 cells were added to plasma prior to processing and sonicated at high power for 10 secs B) 3000 Capan 1 cells were added to plasma prior to processing and sonicated at high power for 30 secs C) 3000 Capan 1 cells were added to plasma prior to

processing and sonicated at high power for 30 secs for 3 cycles. Scatter plots from Quantsudio 3D Digital PCR analysis software showing VIC (Red) labelled KRAS-Wt and FAM (Blue) labelled KRAS-G12V. Green shows KRAS-Wt and KRAS-G12V double positive. Yellow points refer to wells with no reaction. Scatter plot of sample spiked with KRAS-G12V DNA showing FAM labelled KRAS-G12V.

Appendix 2

Human FLT3-ITD plasmid was a kind gift from Prof. Frank Bohmer, Institute for Molecular Cell Biology, University of Jena, Germany



Appendix 3- Antibodies

Antibody (clone)	dilution media	species derivation	Company	catalogue number
pAkt (T308)	BSA	Rabbit polyclonal	Cell signalling	9275
pAkt (S473)	BSA	Rabbit polyclonal	Cell signalling	9271
p-P70 S6K	BSA	Rabbit polyclonal	Cell Signalling	2211
Akt1 (2H10)	milk	Mouse monoclonal	Cell signalling	2967
Akt2 (AW114)	milk	Rabbit monoclonal	Upstate	05-771
Total Akt	BSA	Rabbit polyclonal	Cell signalling	9272
p14-ARF	milk	Mouse monoclonal	Abcam	ab11048
NPM	milk	Mouse monoclonal	Abcam	ab10530
pS48-NPM	BSA	Rabbit polyclonal	Eurogentech	N/A
pT95-NPM	BSA	Rabbit polyclonal	Abcam	ab133453
NPM-AML mutant	milk	Rabbit polyclonal	Abcam	ab65816
Akt phospho substrate	BSA	Rabbit monoclonal	Cell signalling	9614
FLAG (M2)	milk	Mouse monoclonal	Abcam	ab49763
HA	milk	Mouse monoclonal	Abcam	ab1424
p53 (D01)	milk	Mouse monoclonal	Abcam	ab80645
p53mut (ab240)	milk	Mouse monoclonal	Calbiochem	OP29
MDM2	milk	Mouse monoclonal	Santa Cruz	SC965
GAPDH	milk	Rabbit polyclonal	Cell Signalling	2118
Lamin B1	milk	Rabbit polyclonal	N/A	N/A
Fibrillarin	milk	Rabbit polyclonal	Abcam	ab5821
pP38-MAPK (T180/Y182)	BSA	Rabbit monoclonal	Cell signalling	4511
pATF2 (T71)	BSA	Rabbit monoclonal	Cell signalling	5112

References

References:

Abbas, T., and Dutta, A. (2009a). p21 in cancer: intricate networks and multiple activities. Nature reviews Cancer *9*, 400-414.
Abbas, T., and Dutta, A. (2009b). p21 in cancer: intricate networks and multiple activities. Nature Reviews Cancer *9*, 400-414.
Abraham, A. G., and O'Neill, E. (2014). PI3K/Akt-mediated regulation of p53 in cancer. Biochemical Society transactions *42*, 798-803.
Accili, D., and Arden, K. C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell *117*, 421-426.
Adams, J. M., and Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Current opinion in immunology *19*, 488-496.
Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M. B., Guzzardo, V.*, et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.
Ahn, J. Y., Liu, X., Cheng, D. M., Peng, J. M., Chan, P. K., Wade, P. A., and Ye, K. Q. (2005). Nucleophosmin/B23, a nuclear PI(3,4,5)P-3 receptor, mediates the antiapoptotic actions of NGF by inhibiting CAD. Molecular cell *18*, 435-445.
Aladjem, M. I., Spike, B. T., Rodewald, L. W., Hope, T. J., Klemm, M., Jaenisch, R., and Wahl, G. M. (1998). ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Curr Biol *8*, 145-155.
Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996). Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS letters *399*, 333-338.
Alimonti, A., Nardella, C., Chen, Z. B., Clohessy, J. G., Carracedo, A., Trotman, L. C., Cheng, K., Varmeh, S., Kozma, S. C., Thomas, G.*, et al.* (2010). A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. Journal of Clinical Investigation *120*, 681-693.
Allocati, N., Di Ilio, C., and De Laurenzi, V. (2012). p63/p73 in the control of cell cycle and cell death. Exp Cell Res *318*, 1285-1290.
Alt, J. R., Greiner, T. C., Cleveland, J. L., and Eischen, C. M. (2003). Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis. Embo Journal *22*, 1442-1450.
Altomare, D. A., and Testa, J. R. (2005). Perturbations of the AKT signaling pathway in human cancer. Oncogene *24*, 7455-7464.
An, W., Kim, J., and Roeder, R. G. (2004). Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. Cell *117*, 735-748.
Ando, K., Ozaki, T., Yamamoto, H., Furuya, K., Hosoda, M., Hayashi, S., Fukuzawa, M., and Nakagawara, A. (2004). Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. The Journal of biological chemistry *279*, 25549-25561.
Arranz, E., Robledo, M., Martinez, B., Gallego, J., Roman, A., Rivas, C., and Benitez, J. (1996). Incidence of homogeneously staining regions in non-Hodgkin lymphomas. Cancer genetics and cytogenetics *87*, 1-3.
Astle, M. V., Hannan, K. M., Ng, P. Y., Lee, R. S., George, A. J., Hsu, A. K., Haupt, Y., Hannan, R. D., and Pearson, R. B. (2012). AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy. Oncogene *31*, 1949-1962.
Aung, K. L., Board, R. E., Ellison, G., Donald, E., Ward, T., Clack, G., Ranson, M., Hughes, A., Newman, W., and Dive, C. (2010). Current status and future potential of somatic mutation testing from circulating free DNA in patients with solid tumours. The HUGO journal *4*, 11-21.
Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993). Mdm2 Expression Is Induced by Wild Type-P53 Activity. Embo Journal *12*, 461-468.

Bardeesy, N., Aguirre, A. J., Chu, G. C., Cheng, K. H., Lopez, L. V., Hezel, A. F., Feng, B., Brennan, C., Weissleder, R., Mahmood, U.*, et al.* (2006). Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proceedings of the National Academy of Sciences of the United States of America *103*, 5947-5952.
Bartek, J., and Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol *19*, 238-245.
Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V. F., Kolettas, E., Niforou, K., Zoumpourlis, V. C.*, et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.
Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998). p14ARF links the tumour suppressors RB and p53. Nature *395*, 124-125.
Bayascas, J. R., and Alessi, D. R. (2005). Regulation of Akt/PKB Ser473 phosphorylation. Molecular cell *18*, 143-145.
Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V.*, et al.* (1995). Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. International journal of cancer Journal international du cancer *64*, 280-285.
Bergamaschi, D., Gasco, M., Hiller, L., Sullivan, A., Syed, N., Trigiante, G., Yulug, I., Merlano, M., Numico, G., Comino, A.*, et al.* (2003). p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell *3*, 387-402.
Bergamaschi, D., Samuels, Y., Sullivan, A., Zvelebil, M., Breyssens, H., Bisso, A., Del Sal, G., Syed, N., Smith, P., Gasco, M.*, et al.* (2006). iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. Nature genetics *38*, 1133-1141.
Berra, E., Diaz-Meco, M. T., and Moscat, J. (1998). The activation of p38 and apoptosis by the inhibition of ERK is antagonized by the phosphoinositide S-kinase/Akt pathway. Journal of Biological Chemistry *273*, 10792-10797.
Bertwistle, D., Sugimoto, M., and Sherr, C. J. (2004). Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. Molecular and cellular biology *24*, 985-996.
Bianco, R., Shin, I., Ritter, C. A., Yakes, F. M., Basso, A., Rosen, N., Tsurutani, J., Dennis, P. A., Mills, G. B., and Arteaga, C. L. (2003). Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene *22*, 2812-2822.
Blanco-Aparicio, C., Canamero, M., Cecilia, Y., Pequeno, B., Renner, O., Ferrer, I., and Carnero, A. (2010). Exploring the gain of function contribution of AKT to mammary tumorigenesis in mouse models. PloS one *5*, e9305.
Blandino, G., Levine, A. J., and Oren, M. (1999). Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene *18*, 477-485.
Bode, A. M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nature reviews Cancer *4*, 793-805.
Boehm, J. S., Zhao, J. J., Yao, J., Kim, S. Y., Firestein, R., Dunn, I. F., Sjostrom, S. K., Garraway, L. A., Weremowicz, S., Richardson, A. L.*, et al.* (2007). Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell *129*, 1065-1079.
Boggs, K., and Reisman, D. (2007). C/EBPbeta participates in regulating transcription of the p53 gene in response to mitogen stimulation. The Journal of biological chemistry *282*, 7982-7990.
Bolli, N., De Marco, M. F., Martelli, M. P., Bigerna, B., Pucciarini, A., Rossi, R., Mannucci, R., Manes, N., Pettirossi, V., Pileri, S. A.*, et al.* (2009). A dose-dependent tug of war involving the NPM1 leukaemic mutant, nucleophosmin, and ARF. Leukemia *23*, 501-509.
Bommer, G. T., Gerin, I., Feng, Y., Kaczorowski, A. J., Kuick, R., Love, R. E., Zhai, Y., Giordano, T. J., Qin, Z. S., Moore, B. B.*, et al.* (2007). p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Current biology : CB *17*, 1298-1307.

Bonetti, P., Davoli, T., Sironi, C., Amati, B., Pelicci, P. G., and Colombo, E. (2008). Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7 gamma. The Journal of cell biology *182*, 19-26.
Bottger, V., Bottger, A., Garcia-Echeverria, C., Ramos, Y. F. M., van der Eb, A. J., Jochemsen, A. G., and Lane, D. P. (1999). Comparative study of the p53-mdm2 and p53-MDMX interfaces. Oncogene *18*, 189-199.
Bouffler, S. D., Kemp, C. J., Balmain, A., and Cox, R. (1995). Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. Cancer Res *55*, 3883-3889.
Brady, C. A., Jiang, D., Mello, S. S., Johnson, T. M., Jarvis, L. A., Kozak, M. M., Kenzelmann Broz, D., Basak, S., Park, E. J., McLaughlin, M. E*., et al.* (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell *145*, 571-583.
Brady, S. N., Yu, Y., Maggi, L. B., Jr., and Weber, J. D. (2004a). ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. Molecular and cellular biology *24*, 9327-9338.
Brady, S. N., Yu, Y., Maggi, L. B., and Weber, J. D. (2004b). ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. Molecular and cellular biology *24*, 9327-9338.
Branzei, D., and Foiani, M. (2005). The DNA damage response during DNA replication. Curr Opin Cell Biol *17*, 568-575.
Branzei, D., and Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Bio *9*, 297-308.
Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nature reviews Cancer *9*, 701-713.
Brown, J. M., and Wouters, B. G. (1999). Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer research *59*, 1391-1399.
Brown, K. D., Lataxes, T. A., Shangary, S., Mannino, J. L., Giardina, J. F., Chen, J., and Baskaran, R. (2000). Ionizing radiation exposure results in up-regulation of Ku70 via a p53/ataxia-telangiectasia-mutated protein-dependent mechanism. The Journal of biological chemistry *275*, 6651-6656.
Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995). Radiation-Induced Cell-Cycle Arrest Compromised by P21 Deficiency. Nature *377*, 552-557.
Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.
Bruno, T., Desantis, A., Bossi, G., Di Agostino, S., Sorino, C., De Nicola, F., Iezzi, S., Franchitto, A., Benassi, B., Galanti, S*., et al.* (2010). Che-1 promotes tumor cell survival by sustaining mutant p53 transcription and inhibiting DNA damage response activation. Cancer Cell *18*, 122-134.
Bullock, A. N., Henckel, J., DeDecker, B. S., Johnson, C. M., Nikolova, P. V., Proctor, M. R., Lane, D. P., and Fersht, A. R. (1997). Thermodynamic stability of wild-type and mutant p53 core domain. Proceedings of the National Academy of Sciences of the United States of America *94*, 14338-14342.
Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282*, 1497-1501.
Burd, C. E., Jeck, W. R., Liu, Y., Sanoff, H. K., Wang, Z., and Sharpless, N. E. (2010). Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet *6*, e1001233.
Burns, T. F., Fei, P., Scata, K. A., Dicker, D. T., and El-Deiry, W. S. (2003). Silencing of the novel p53 target gene Snk/Plk2 leads to mitotic catastrophe in paclitaxel (taxol)-exposed cells. Molecular and cellular biology *23*, 5556-5571.
Calera, M. R., Martinez, C., Liu, H., Jack, A. K., Birnbaum, M. J., and Pilch, P. F. (1998). Insulin increases the association of Akt-2 with Glut4-containing vesicles. The Journal of biological chemistry *273*, 7201-7204.

Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998). Increasing complexity of Ras signaling. Oncogene *17*, 1395-1413.
Campisi, J. (2013). Aging, cellular senescence, and cancer. Annual review of physiology *75*, 685-705.
Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nature reviews Molecular cell biology *8*, 729-740.
Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.
Cao, L., Li, W. M., Kim, S., Brodie, S. G., and Deng, C. X. (2003). Senescence aging, and malignant transformation mediated by p53 in mice lacking the Brcal full-length isoform. Genes & development *17*, 201-213.
Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.
Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., Hostetter, G., Boguslawski, S., Moses, T. Y., Savage, S*., et al.* (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439-444.
Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A. T., Thomas, G., Kozma, S. C*., et al.* (2008). Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. The Journal of clinical investigation *118*, 3065-3074.
Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L., Larsson, E*., et al.* (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery *2*, 401-404.
Chan, P. K. (1992). Characterization and Cellular-Localization of Nucleophosmin/B23 in Hela-Cells Treated with Selected Cytotoxic Agents (Studies of B23-Translocation Mechanism). Exp Cell Res *203*, 174-181.
Chan, P. K., and Chan, F. Y. (1995). Nucleophosmin/B23 (Npm) Oligomer Is a Major and Stable Entity in Hela-Cells. Bba-Gene Struct Expr *1262*, 37-42.
Chang, F., Steelman, L. S., Lee, J. T., Shelton, J. G., Navolanic, P. M., Blalock, W. L., Franklin, R. A., and McCubrey, J. A. (2003). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia *17*, 1263-1293.
Chang, J. H., and Olson, M. O. (1990). Structure of the gene for rat nucleolar protein B23. The Journal of biological chemistry *265*, 18227-18233.
Chang, T. C., Wentzel, E. A., Kent, O. A., Ramachandran, K., Mullendore, M., Lee, K. H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C. J*., et al.* (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Molecular cell *26*, 745-752.
Chau, B. N., and Wang, J. Y. J. (2003). Coordinated regulation of life and death by RB. Nature Reviews Cancer *3*, 130-138.
Chen, D., Shan, J., Zhu, W. G., Qin, J., and Gu, W. (2010a). Transcription-independent ARF regulation in oncogenic stress-mediated p53 responses. Nature *464*, 624-627.
Chen, D., Yoon, J. B., and Gu, W. (2010b). Reactivating the ARF-p53 axis in AML cells by targeting ULF. Cell Cycle *9*, 2946-2951.
Chen, G., Ghosh, P., O'Farrell, T., Munk, R., Rezanka, L. J., Sasaki, C. Y., and Longo, D. L. (2012). Transforming growth factor beta1 (TGF-beta1) suppresses growth of B-cell lymphoma cells by p14(ARF)-dependent regulation of mutant p53. The Journal of biological chemistry *287*, 23184-23195.
Chen, L., Gilkes, D. M., Pan, Y., Lane, W. S., and Chen, J. (2005a). ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. The EMBO journal *24*, 3411-3422.

Chen, L., Li, C., Pan, Y., and Chen, J. (2005b). Regulation of p53-MDMX interaction by casein kinase 1 alpha. Molecular and cellular biology *25*, 6509-6520.
Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W*., et al.* (2005c). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.
Cheng, H. L., Mostoslavsky, R., Saito, S., Manis, J. P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F. W., and Chua, K. F. (2003). Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proceedings of the National Academy of Sciences of the United States of America *100*, 10794-10799.
Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. (1992). AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proceedings of the National Academy of Sciences of the United States of America *89*, 9267-9271.
Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. (1996). Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proceedings of the National Academy of Sciences of the United States of America *93*, 3636-3641.
Cheng, Y., Zhang, Y., Zhang, L., Ren, X. C., Huber-Keener, K. J., Liu, X. Y., Zhou, L., Liao, J., Keihack, H., Yan, L*., et al.* (2012). MK-2206, a Novel Allosteric Inhibitor of Akt, Synergizes with Gefitinib against Malignant Glioma via Modulating Both Autophagy and Apoptosis. Molecular cancer therapeutics *11*, 154-164.
Cherrin, C., Haskell, K., Howell, B., Jones, R., Leander, K., Robinson, R., Watkins, A., Bilodeau, M., Hoffman, J., Sanderson, P*., et al.* (2010). An allosteric Akt inhibitor effectively blocks Akt signaling and tumor growth with only transient effects on glucose and insulin levels in vivo. Cancer biology & therapy *9*, 493-503.
Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochemical and biophysical research communications *279*, 383-390.
Chipuk, J. E., and Green, D. R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends in cell biology *18*, 157-164.
Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B., and Evan, G. I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature *443*, 214-217.
Chung, A. S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nature Reviews Cancer *10*, 505-514.
Cirri, P., and Chiarugi, P. (2011). Cancer associated fibroblasts: the dark side of the coin. Am J Cancer Res *1*, 482-497.
Coates, A. S., Millar, E. K., O'Toole, S. A., Molloy, T. J., Viale, G., Goldhirsch, A., Regan, M. M., Gelber, R. D., Sun, Z., Castiglione-Gertsch, M*., et al.* (2012). Prognostic interaction between expression of p53 and estrogen receptor in patients with node-negative breast cancer: results from IBCSG Trials VIII and IX. Breast cancer research : BCR *14*, R143.
Collavin, L., Lunardi, A., and Del Sal, G. (2010). p53-family proteins and their regulators: hubs and spokes in tumor suppression. Cell death and differentiation *17*, 901-911.
Colombo, E., Alcalay, M., and Pelicci, P. G. (2011). Nucleophosmin and its complex network: a possible therapeutic target in hematological diseases. Oncogene *30*, 2595-2609.
Colombo, E., Bonetti, P., Denchi, E. L., Martinelli, P., Zamponi, R., Marine, J. C., Helin, K., Falini, B., and Pelicci, P. G. (2005a). Nucleophosmin is required for DNA integrity and p19(Arf) protein stability. Molecular and cellular biology *25*, 8874-8886.
Colombo, E., Bonetti, P., Lazzerini Denchi, E., Martinelli, P., Zamponi, R., Marine, J. C., Helin, K., Falini, B., and Pelicci, P. G. (2005b). Nucleophosmin is required for DNA integrity and p19Arf protein stability. Molecular and cellular biology *25*, 8874-8886.

Colombo, E., Marine, J. C., Danovi, D., Falini, B., and Pelicci, P. G. (2002). Nucleophosmin regulates the stability and transcriptional activity of p53. Nature cell biology *4*, 529-533.
Colombo, E., Martinelli, P., Zamponi, R., Shing, D. C., Bonetti, P., Luzi, L., Volorio, S., Bernard, L., Pruneri, G., Alcalay, M., and Pelicci, P. G. (2006). Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. Cancer research *66*, 3044-3050.
Concepcion, C. P., Han, Y. C., Mu, P., Bonetti, C., Yao, E., D'Andrea, A., Vidigal, J. A., Maughan, W. P., Ogrodowski, P., and Ventura, A. (2012). Intact p53-dependent responses in miR-34-deficient mice. PLoS Genet *8*, e1002797.
Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.
Cullen, P. J., and Lockyer, P. J. (2002). Integration of calcium and Ras signalling. Nature reviews Molecular cell biology *3*, 339-348.
Das, S., El-Deiry, W. S., and Somasundaram, K. (2003). Regulation of the p53 homolog p73 by adenoviral oncogene E1A. Journal of Biological Chemistry *278*, 18313-18320.
Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.
Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Molecular cell *6*, 41-51.
Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature *379*, 557-560.
Davidoff, A. M., Herndon, J. E., Glover, N. S., Kerns, B. J. M., Pence, J. C., Iglehart, J. D., and Marks, J. R. (1991). Relation between P53 Overexpression and Established Prognostic Factors in Breast-Cancer. Surgery *110*, 259-264.
Davies, M. A. (2011). Regulation, role, and targeting of Akt in cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *29*, 4715-4717.
Davies, M. A., Stemke-Hale, K., Tellez, C., Calderone, T. L., Deng, W., Prieto, V. G., Lazar, A. J., Gershenwald, J. E., and Mills, G. B. (2008). A novel AKT3 mutation in melanoma tumours and cell lines. British journal of cancer *99*, 1265-1268.
Dawson, S. J., Rosenfeld, N., and Caldas, C. (2013). Circulating tumor DNA to monitor metastatic breast cancer. The New England journal of medicine *369*, 93-94.
DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., and Thompson, C. B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell metabolism *7*, 11-20.
Dell'Orso, S., Fontemaggi, G., Stambolsky, P., Goeman, F., Voellenkle, C., Levrero, M., Strano, S., Rotter, V., Oren, M., and Blandino, G. (2011). ChIP-on-chip analysis of in vivo mutant p53 binding to selected gene promoters. Omics : a journal of integrative biology *15*, 305-312.
DeNardo, D., Andreu, P., and Coussens, L. M. (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metast Rev *29*, 309-316.
Deng, C. X., Zhang, P. M., Harper, J. W., Elledge, S. J., and Leder, P. (1995). Mice Lacking P21(C/P1/Waf1) Undergo Normal Development, but Are Defective in G1 Checkpoint Control. Cell *82*, 675-684.
Dergunova, N., Bulycheva, T. I., Artemenko, E. G., Shpakova, A. P., Pegova, A. N., Gemjian, E. G., Dudnik, O. A., Zatsepina, O. V., and Malashenko, O. S. (2002). A major nucleolar protein B23 as a marker of proliferation activity of human peripheral lymphocytes. Immunology letters *83*, 67-72.
Desterke, C., Bilhou-Nabera, C., Guerton, B., Martinaud, C., Tonetti, C., Clay, D., Guglielmelli, P., Vannucchi, A., Bordessoule, D., Hasselbalch, H.*, et al.* (2011). FLT3-mediated p38-MAPK activation participates in the control of megakaryopoiesis in primary myelofibrosis. Cancer research *71*, 2901-2915.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191-202.
Di Como, C. J., Gaiddon, C., and Prives, C. (1999a). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Molecular and cellular biology *19*, 1438-1449.
Di Como, C. J., Gaiddon, C., and Prives, C. (1999b). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Molecular and cellular biology *19*, 1438-1449.
di Fagagna, F. D., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.
Diehl, F., Li, M., Dressman, D., He, Y., Shen, D., Szabo, S., Diaz, L. A., Jr., Goodman, S. N., David, K. A., Juhl, H.*, et al.* (2005). Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proceedings of the National Academy of Sciences of the United States of America *102*, 16368-16373.
Diehl, F., Schmidt, K., Choti, M. A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S. A.*, et al.* (2008). Circulating mutant DNA to assess tumor dynamics. Nature medicine *14*, 985-990.
Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes & development *12*, 3499-3511.
Dijkers, P. F., Birkenkamp, K. U., Lam, E. W., Thomas, N. S., Lammers, J. W., Koenderman, L., and Coffer, P. J. (2002). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. The Journal of cell biology *156*, 531-542.
Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature *399*, 601-605.
Ding, K., Lu, Y., Nikolovska-Coleska, Z., Qiu, S., Ding, Y., Gao, W., Stuckey, J., Krajewski, K., Roller, P. P., Tomita, Y.*, et al.* (2005). Structure-based design of potent non-peptide MDM2 inhibitors. Journal of the American Chemical Society *127*, 10130-10131.
Do, P. M., Varanasi, L., Fan, S., Li, C., Kubacka, I., Newman, V., Chauhan, K., Daniels, S. R., Boccetta, M., Garrett, M. R.*, et al.* (2012). Mutant p53 cooperates with ETS2 to promote etoposide resistance. Genes & development *26*, 830-845.
Dohner, K., Schlenk, R. F., Habdank, M., Scholl, C., Rucker, F. G., Corbacioglu, A., Bullinger, L., Frohling, S., and Dohner, H. (2005). Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood *106*, 3740-3746.
Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.
Dong, P., Karaayvaz, M., Jia, N., Kaneuchi, M., Hamada, J., Watari, H., Sudo, S., Ju, J., and Sakuragi, N. (2013). Mutant p53 gain-of-function induces epithelial-mesenchymal transition through modulation of the miR-130b-ZEB1 axis. Oncogene *32*, 3286-3295.
Donovan, S., Shannon, K. M., and Bollag, G. (2002). GTPase activating proteins: critical regulators of intracellular signaling. Biochimica et biophysica acta *1602*, 23-45.
Donzelli, S., Biagioni, F., Fausti, F., Strano, S., Fontemaggi, G., and Blandino, G. (2008). Oncogenomic Approaches in Exploring Gain of Function of Mutant p53. Current genomics *9*, 200-207.
Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., O' Rourke, K., Koeppen, H., and Dixit, V. M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature *429*, 86-92.

Dosil, M., Wang, S. L., and Lemischka, I. R. (1993). Mitogenic Signaling and Substrate-Specificity of the Flk2/Flt3 Receptor Tyrosine Kinase in Fibroblasts and Interleukin 3-Dependent Hematopoietic-Cells. Molecular and cellular biology *13*, 6572-6585.
Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. Nature reviews Cancer *3*, 11-22.
Dulloo, I., and Sabapathy, K. (2005). Transactivation-dependent and -independent regulation of p73 stability. Journal of Biological Chemistry *280*, 28203-28214.
Duro, D., Bernard, O., Della Valle, V., Berger, R., and Larsen, C. J. (1995). A new type of p16INK4/MTS1 gene transcript expressed in B-cell malignancies. Oncogene *11*, 21-29.
Dutta, S., Akey, I. V., Dingwall, C., Hartman, K. L., Laue, T., Nolte, R. T., Head, J. F., and Akey, C. W. (2001). The crystal structure of nucleoplasmin-core: implications for histone binding and nucleosome assembly. Molecular cell *8*, 841-853.
Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998). RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science *280*, 1082-1086.
Efeyan, A., Garcia-Cao, I., Herranz, D., Velasco-Miguel, S., and Serrano, M. (2006). Tumour biology: Policing of oncogene activity by p53. Nature *443*, 159.
Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E., and McGraw, T. E. (2005). Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. Cell metabolism *2*, 263-272.
Elstrom, R. L., Bauer, D. E., Buzzai, M., Karnauskas, R., Harris, M. H., Plas, D. R., Zhuang, H., Cinalli, R. M., Alavi, A., Rudin, C. M., and Thompson, C. B. (2004). Akt stimulates aerobic glycolysis in cancer cells. Cancer research *64*, 3892-3899.
Enge, M., Bao, W., Hedstrom, E., Jackson, S. P., Moumen, A., and Selivanova, G. (2009). MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. Cancer Cell *15*, 171-183.
Engelman, J. A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nature reviews Cancer *9*, 550-562.
Engelman, J. A., Luo, J., and Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nature reviews Genetics *7*, 606-619.
Enomoto, T., Lindstrom, M. S., Jin, A., Ke, H., and Zhang, Y. (2006). Essential role of the B23/NPM core domain in regulating ARF binding and B23 stability. The Journal of biological chemistry *281*, 18463-18472.
Eser, S., Schnieke, A., Schneider, G., and Saur, D. (2014). Oncogenic KRAS signalling in pancreatic cancer. British journal of cancer *111*, 817-822.
Esser, C., Scheffner, M., and Hohfeld, J. (2005). The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. Journal of Biological Chemistry *280*, 27443-27448.
Esteller, M., Tortola, S., Toyota, M., Capella, G., Peinado, M. A., Baylin, S. B., and Herman, J. G. (2000). Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer research *60*, 129-133.
Falini, B., Bolli, N., Shan, J., Martelli, M. P., Liso, A., Pucciarini, A., Bigerna, B., Pasqualucci, L., Mannucci, R., Rosati, R.*, et al.* (2006). Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc(+) AML. Blood *107*, 4514-4523.
Falini, B., Martelli, M. P., Mecucci, C., Liso, A., Bolli, N., Bigerna, B., Pucciarini, A., Pileri, S., Meloni, G., Martelli, M. F.*, et al.* (2008a). Cytoplasmic mutated nucleophosmin is stable in primary leukemic cells and in a xenotransplant model of NPMc(+) acute myeloid leukemia in SCID mice. Haematol-Hematol J *93*, 775-779.
Falini, B., Martelli, M. P., Mecucci, C., Liso, A., Bolli, N., Bigerna, B., Pucciarini, A., Pileri, S., Meloni, G., Martelli, M. F.*, et al.* (2008b). Cytoplasmic mutated nucleophosmin is stable in primary

leukemic cells and in a xenotransplant model of NPMc+ acute myeloid leukemia in SCID mice. Haematologica *93*, 775-779.
Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A.*, et al.* (2005). Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. The New England journal of medicine *352*, 254-266.
Falini, B., Nicoletti, I., Martelli, M. F., and Mecucci, C. (2007). Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. Blood *109*, 874-885.
Falini, B., Sportoletti, P., and Martelli, M. P. (2009). Acute myeloid leukemia with mutated NPM1: diagnosis, prognosis and therapeutic perspectives. Current opinion in oncology *21*, 573-581.
Fan, Q. W., Knight, Z. A., Goldenberg, D. D., Yu, W., Mostov, K. E., Stokoe, D., Shokat, K. M., and Weiss, W. A. (2006). A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. Cancer Cell *9*, 341-349.
Fang, S. Y., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. Journal of Biological Chemistry *275*, 8945-8951.
Feldman, M. E., Apsel, B., Uotila, A., Loewith, R., Knight, Z. A., Ruggero, D., and Shokat, K. M. (2009). Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS biology *7*, e38.
Feng, Z., Zhang, C., Wu, R., and Hu, W. (2011). Tumor suppressor p53 meets microRNAs. Journal of molecular cell biology *3*, 44-50.
Feron, O. (2009). Pyruvate into lactate and back: From the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol *92*, 329-333.
Finkel, T., Serrano, M., and Blasco, M. A. (2007). The common biology of cancer and ageing. Nature *448*, 767-774.
Flinn, I. W., Byrd, J. C., Furman, R. R., Brown, J. R., Lin, T. S., Bello, C., Giese, N. A., and Yu, A. S. (2009a). Preliminary evidence of clinical activity in a phase I study of CAL-101, a selective inhibitor of the p1108 isoform of phosphatidylinositol 3-kinase (P13K), in patients with select hematologic malignancies. Journal of Clinical Oncology *27*.
Flinn, W., Byrd, J., Furman, R., Brown, J., Lin, T., Bello, C., Giese, N., and Yu, A. (2009b). Preliminary Evidence of Clinical Activity in a Phase 1 Study of Cal-101, a Potent Selective Inhibitor of the P110delta Isoform of Phosphatidylinositol 3-Kinase, in Patient with B-Cell Malignancies. Haematol-Hematol J *94*, 303-303.
Flores, E. R., Sengupta, S., Miller, J. B., Newman, J. J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: Evidence for broader tumor suppressor functions for the p53 family. Cancer Cell *7*, 363-373.
Forshew, T., Murtaza, M., Parkinson, C., Gale, D., Tsui, D. W., Kaper, F., Dawson, S. J., Piskorz, A. M., Jimenez-Linan, M., Bentley, D.*, et al.* (2012). Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science translational medicine *4*, 136ra168.
Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J. C. (2006). Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proceedings of the National Academy of Sciences of the United States of America *103*, 3232-3237.
Freed-Pastor, W. A., and Prives, C. (2012). Mutant p53: one name, many proteins. Genes & development *26*, 1268-1286.
Freedberg, D. E., Rigas, S. H., Russak, J., Gai, W., Kaplow, M., Osman, I., Turner, F., Randerson-Moor, J. A., Houghton, A., Busam, K.*, et al.* (2008). Frequent p16-independent inactivation of p14ARF in human melanoma. Journal of the National Cancer Institute *100*, 784-795.

Frehlick, L. J., Eirin-Lopez, J. M., and Ausio, J. (2007). New insights into the nucleophosmin/nucleoplasmin family of nuclear chaperones. BioEssays : news and reviews in molecular, cellular and developmental biology *29*, 49-59.
Fridman, J. S., and Lowe, S. W. (2003). Control of apoptosis by p53. Oncogene *22*, 9030-9040.
Friedler, A., Veprintsev, D. B., Freund, S. M., von Glos, K. I., and Fersht, A. R. (2005). Modulation of binding of DNA to the C-terminal domain of p53 by acetylation. Structure *13*, 629-636.
Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998). Phosphoinositide kinases. Annual review of biochemistry *67*, 481-507.
Fukawa, T., Ono, M., Matsuo, T., Uehara, H., Miki, T., Nakamura, Y., Kanayama, H. O., and Katagiri, T. (2012). DDX31 regulates the p53-HDM2 pathway and rRNA gene transcription through its interaction with NPM1 in renal cell carcinomas. Cancer research *72*, 5867-5877.
Fumagalli, M., Rosiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J. M., Bucci, G., Dobreva, M., Matti, V., Beausejour, C. M.*, et al.* (2012). Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nature cell biology *14*, 355-+.
Gaidarov, I., Smith, M. E., Domin, J., and Keen, J. H. (2001). The class II phosphoinositide 3-kinase C2alpha is activated by clathrin and regulates clathrin-mediated membrane trafficking. Molecular cell *7*, 443-449.
Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Molecular and cellular biology *21*, 1874-1887.
Gajria, D., and Chandarlapaty, S. (2011). HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. Expert review of anticancer therapy *11*, 263-275.
Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993). Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature *363*, 88-92.
Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., and Kroemer, G. (2007). Cell death modalities: classification and pathophysiological implications. Cell Death Differ *14*, 1237-1243.
Gamper, A. M., and Roeder, R. G. (2008). Multivalent binding of p53 to the STAGA complex mediates coactivator recruitment after UV damage. Molecular and cellular biology *28*, 2517-2527.
Gao, J. J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., Sun, Y. C., Jacobsen, A., Sinha, R., Larsson, E.*, et al.* (2013). Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Sci Signal *6*.
Gao, T., Furnari, F., and Newton, A. C. (2005). PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Molecular cell *18*, 13-24.
Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes & development *15*, 1383-1392.
Garcia-Cao, I., Garcia-Cao, M., Martin-Caballero, J., Criado, L. M., Klatt, P., Flores, J. M., Weill, J. C., Blasco, M. A., and Serrano, M. (2002). "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. The EMBO journal *21*, 6225-6235.
Garcia-Echeverria, C., and Sellers, W. R. (2008). Drug discovery approaches targeting the PI3K/Akt pathway in cancer. Oncogene *27*, 5511-5526.
Gilliland, D. G., and Griffin, J. D. (2002). Role of FLT3 in leukemia. Current opinion in hematology *9*, 274-281.
Girardini, J. E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A.*, et al.* (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell *20*, 79-91.
Gjerset, R. A. (2006). DNA damage, p14ARF, nucleophosmin (NPM/B23), and cancer. Journal of molecular histology *37*, 239-251.

Gjerset, R. A., and Bandyopadhyay, K. (2006). Regulation of p14ARF through subnuclear compartmentalization. Cell Cycle *5*, 686-690.
Goh, A. M., Coffill, C. R., and Lane, D. P. (2011). The role of mutant p53 in human cancer. The Journal of pathology *223*, 116-126.
Gohler, T., Jager, S., Warnecke, G., Yasuda, H., Kim, E., and Deppert, W. (2005). Mutant p53 proteins bind DNA in a DNA structure-selective mode. Nucleic acids research *33*, 1087-1100.
Golstein, P., and Kroemer, G. (2007). Cell death by necrosis: towards a molecular definition. Trends in biochemical sciences *32*, 37-43.
Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F., and Serrano, M. (2006). Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. Nature *440*, 702-706.
Gorlick, R., Maris, J. M., Houghton, P. J., Lock, R., Carol, H., Kurmasheva, R. T., Kolb, E. A., Keir, S. T., Reynolds, C. P., Kang, M. H.*, et al.* (2012). Testing of the Akt/PKB inhibitor MK-2206 by the Pediatric Preclinical Testing Program. Pediatric blood & cancer *59*, 518-524.
Gratton, J. P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., and Sessa, W. C. (2001). Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. Journal of Biological Chemistry *276*, 30359-30365.
Graupera, M., Guillermet-Guibert, J., Foukas, L. C., Phng, L. K., Cain, R. J., Salpekar, A., Pearce, W., Meek, S., Millan, J., Cutillas, P. R.*, et al.* (2008). Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. Nature *453*, 662-666.
Green, D. R. (2005). Apoptotic pathways: ten minutes to dead. Cell *121*, 671-674.
Grisendi, S., Bernardi, R., Rossi, M., Cheng, K., Khandker, L., Manova, K., and Pandolfi, P. P. (2005). Role of nucleophosmin in embryonic development and tumorigenesis. Nature *437*, 147-153.
Grisendi, S., Mecucci, C., Falini, B., and Pandolfi, P. P. (2006). Nucleophosmin and cancer. Nature reviews Cancer *6*, 493-505.
Grivennikov, S. I., Greten, F. R., and Karin, M. (2010). Immunity, Inflammation, and Cancer. Cell *140*, 883-899.
Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999a). BCL-2 family members and the mitochondria in apoptosis. Genes & development *13*, 1899-1911.
Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999b). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem *274*, 1156-1163.
Grossman, S. R. (2001). p300/CBP/p53 interaction and regulation of the p53 response. Eur J Biochem *268*, 2773-2778.
Grummitt, C. G., Townsley, F. M., Johnson, C. M., Warren, A. J., and Bycroft, M. (2008). Structural consequences of nucleophosmin mutations in acute myeloid leukemia. The Journal of biological chemistry *283*, 23326-23332.
Gu, W., Shi, X. L., and Roeder, R. G. (1997). Synergistic activation of transcription by CBP and p53. Nature *387*, 819-823.
Gudmundsdottir, K., and Ashworth, A. (2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene *25*, 5864-5874.
Gupta, A. K., Li, B., Cerniglia, G. J., Ahmed, M. S., Hahn, S. M., and Maity, A. (2007). The HIV protease inhibitor nelfinavir downregulates Akt phosphorylation by inhibiting proteasomal activity and inducing the unfolded protein response. Neoplasia *9*, 271-278.
Gurumurthy, M., Tan, C. H., Ng, R., Zeiger, L., Lau, J., Lee, J., Dey, A., Philp, R., Li, Q., Lim, T. M.*, et al.* (2008). Nucleophosmin interacts with HEXIM1 and regulates RNA polymerase II transcription. Journal of molecular biology *378*, 302-317.

Hafsi, S., Pezzino, F. M., Candido, S., Ligresti, G., Spandidos, D. A., Soua, Z., McCubrey, J. A., Travali, S., and Libra, M. (2012). Gene alterations in the PI3K/PTEN/AKT pathway as a mechanism of drug-resistance (review). International journal of oncology *40*, 639-644.
Hamilton, G., Abraham, A. G., Morton, J., Sampson, O., Pefani, D. E., Khoronenkova, S., Grawenda, A., Papaspyropoulos, A., Jamieson, N., McKay, C.*, et al.* (2014). AKT regulates NPM dependent ARF localization and p53mut stability in tumors. Oncotarget *5*, 6142-6167.
Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989). All Ras Proteins Are Polyisoprenylated but Only Some Are Palmitoylated. Cell *57*, 1167-1177.
Hancock, J. F., Paterson, H., and Marshall, C. J. (1990). A Polybasic Domain or Palmitoylation Is Required in Addition to the Caax Motif to Localize P21ras to the Plasma-Membrane. Cell *63*, 133-139.
Hanel, W., Marchenko, N., Xu, S., Yu, S. X., Weng, W., and Moll, U. (2013). Two hot spot mutant p53 mouse models display differential gain of function in tumorigenesis. Cell death and differentiation *20*, 898-909.
Harms, K., Nozell, S., and Chen, X. (2004). The common and distinct target genes of the p53 family transcription factors. Cellular and molecular life sciences : CMLS *61*, 822-842.
Haupt, S., Mitchell, C., Corneille, V., Shortt, J., Fox, S., Pandolfi, P. P., Castillo-Martin, M., Bonal, D. M., Cordon-Cardo, C., Lozano, G., and Haupt, Y. (2013). Loss of PML cooperates with mutant p53 to drive more aggressive cancers in a gender-dependent manner. Cell Cycle *12*, 1722-1731.
Hemann, M. T., and Lowe, S. W. (2006). The p53-Bcl-2 connection. Cell Death Differ *13*, 1256-1259.
Hennessy, B. T., Smith, D. L., Ram, P. T., Lu, Y., and Mills, G. B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. Nature reviews Drug discovery *4*, 988-1004.
Hingorani, K., Szebeni, A., and Olson, M. O. (2000). Mapping the functional domains of nucleolar protein B23. The Journal of biological chemistry *275*, 24451-24457.
Hingorani, S. R., Petricoin, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A.*, et al.* (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer cell *4*, 437-450.
Hirai, H., Sootome, H., Nakatsuru, Y., Miyama, K., Taguchi, S., Tsujioka, K., Ueno, Y., Hatch, H., Majumder, P. K., Pan, B. S., and Kotani, H. (2010). MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Molecular cancer therapeutics *9*, 1956-1967.
Hochegger, H., Dejsuphong, D., Fukushima, T., Morrison, C., Sonoda, E., Schreiber, V., Zhao, G. Y., Saberi, A., Masutani, M., Adachi, N.*, et al.* (2006). Parp-1 protects homologous recombination from interference by Ku and Ligase IV in vertebrate cells. The EMBO journal *25*, 1305-1314.
Hogan, C., Hutchison, C., Marcar, L., Milne, D., Saville, M., Goodlad, J., Kernohan, N., and Meek, D. (2008). Elevated levels of oncogenic protein kinase Pim-1 induce the p53 pathway in cultured cells and correlate with increased Mdm2 in mantle cell lymphoma. The Journal of biological chemistry *283*, 18012-18023.
Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature *460*, 1132-1135.
Hotchkiss, R. S., Strasser, A., McDunn, J. E., and Swanson, P. E. (2009). Cell death. The New England journal of medicine *361*, 1570-1583.
Hsu, C. Y., and Yung, B. Y. (2000). Over-expression of nucleophosmin/B23 decreases the susceptibility of human leukemia HL-60 cells to retinoic acid-induced differentiation and apoptosis. International journal of cancer Journal international du cancer *88*, 392-400.

Huang, C. H., Mandelker, D., Schmidt-Kittler, O., Samuels, Y., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., Gabelli, S. B., and Amzel, L. M. (2007). The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. Science *318*, 1744-1748.
Ihle, N. T., Lemos, R., Jr., Wipf, P., Yacoub, A., Mitchell, C., Siwak, D., Mills, G. B., Dent, P., Kirkpatrick, D. L., and Powis, G. (2009). Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance. Cancer research *69*, 143-150.
Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nature cell biology *4*, 648-657.
Irie, H. Y., Pearline, R. V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J. S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. The Journal of cell biology *171*, 1023-1034.
Irwin, M. S., Kondo, K., Marin, M. C., Cheng, L. S., Hahn, W. C., and Kaelin, W. G., Jr. (2003). Chemosensitivity linked to p73 function. Cancer Cell *3*, 403-410.
Itahana, K., Bhat, K. P., Jin, A., Itahana, Y., Hawke, D., Kobayashi, R., and Zhang, Y. (2003). Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. Molecular cell *12*, 1151-1164.
Ito, A., Kawaguchi, Y., Lai, C. H., Kovacs, J. J., Higashimoto, Y., Appella, E., and Yao, T. P. (2002). MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. The EMBO journal *21*, 6236-6245.
Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. The EMBO journal *20*, 1331-1340.
Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell *127*, 125-137.
Jackson, E. L., Willis, N., Mercer, K., Bronson, R. T., Crowley, D., Montoya, R., Jacks, T., and Tuveson, D. A. (2001). Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes & development *15*, 3243-3248.
Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071-1078.
Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., and Knippers, R. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer research *61*, 1659-1665.
Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S. H., Zhang, J., Signoretti, S., Loda, M., Roberts, T. M., and Zhao, J. J. (2008). Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature *454*, 776-779.
Jian, Y., Gao, Z., Sun, J., Shen, Q., Feng, F., Jing, Y., and Yang, C. (2009). RNA aptamers interfering with nucleophosmin oligomerization induce apoptosis of cancer cells. Oncogene *28*, 4201-4211.
Jiricny, J. (2006). The multifaceted mismatch-repair system. Nat Rev Mol Cell Bio *7*, 335-346.
Joerger, A. C., Ang, H. C., and Fersht, A. R. (2006). Structural basis for understanding oncogenic p53 mutations and designing rescue drugs. Proceedings of the National Academy of Sciences of the United States of America *103*, 15056-15061.
Joerger, A. C., and Fersht, A. R. (2007). Structure-function-rescue: the diverse nature of common p53 cancer mutants. Oncogene *26*, 2226-2242.
Johannessen, C. M., Reczek, E. E., James, M. F., Brems, H., Legius, E., and Cichowski, K. (2005). The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proceedings of the National Academy of Sciences of the United States of America *102*, 8573-8578.
Jones, R. G., and Thompson, C. B. (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes & development *23*, 537-548.

Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nature genetics *29*, 418-425.
Jost, C. A., Marin, M. C., and Kaelin, W. G. (1997). p73 is a human p53-related protein that can induce apoptosis. Nature *389*, 191-194.
Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X*., et al.* (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell *90*, 809-819.
Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-659.
Kawamura, T., Suzuki, J., Wang, Y. V., Menendez, S., Morera, L. B., Raya, A., Wahl, G. M., and Izpisua Belmonte, J. C. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature *460*, 1140-1144.
Kennedy, A. L., Morton, J. P., Manoharan, I., Nelson, D. M., Jamieson, N. B., Pawlikowski, J. S., McBryan, T., Doyle, B., McKay, C., Oien, K. A*., et al.* (2011). Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis. Molecular cell *42*, 36-49.
Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001). Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Molecular and cellular biology *21*, 893-901.
Kim, H. J., Cho, J. H., and Kim, J. R. (2013). Downregulation of Polo-Like Kinase 1 Induces Cellular Senescence in Human Primary Cells Through a p53-Dependent Pathway. J Gerontol a-Biol *68*, 1145-1156.
Kim, J. S., Krasieva, T. B., Kurumizaka, H., Chen, D. J., Taylor, A. M., and Yokomori, K. (2005). Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. The Journal of cell biology *170*, 341-347.
Kim, M. S., Jeong, E. G., Yoo, N. J., and Lee, S. H. (2008). Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. British journal of cancer *98*, 1533-1535.
King, F. W., Skeen, J., Hay, N., and Shtivelman, E. (2004). Inhibition of Chk1 by activated PKB/Akt. Cell Cycle *3*, 634-637.
Klionsky, D. J. (2007). Autophagy: from phenomenology to molecular understanding in less than a decade. Nature reviews Molecular cell biology *8*, 931-937.
Kodaki, T., Woscholski, R., Hallberg, B., Rodriguez-Viciana, P., Downward, J., and Parker, P. J. (1994). The activation of phosphatidylinositol 3-kinase by Ras. Current biology : CB *4*, 798-806.
Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996). Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. The Journal of biological chemistry *271*, 21920-21926.
Kondo, T., Minamino, N., NagamuraInoue, T., Matsumoto, M., Taniguchi, T., and Tanaka, N. (1997). Identification and characterization of nucleophosmin/B23/numatrin which binds the anti-oncogenic transcription factor IRF-1 and manifests oncogenic activity. Oncogene *15*, 1275-1281.
Konopleva, M. Y., Walter, R. B., Faderl, S. H., Jabbour, E. J., Zeng, Z., Borthakur, G., Huang, X., Kadia, T. M., Ruvolo, P. P., Feliu, J. B*., et al.* (2014). Preclinical and early clinical evaluation of the oral AKT inhibitor, MK-2206, for the treatment of acute myelogenous leukemia. Clinical cancer research : an official journal of the American Association for Cancer Research *20*, 2226-2235.
Korgaonkar, C., Hagen, J., Tompkins, V., Frazier, A. A., Allamargot, C., Quelle, F. W., and Quelle, D. E. (2005). Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function. Molecular and cellular biology *25*, 1258-1271.
Kraus, W. L. (2008). Transcriptional control by PARP-1: chromatin modulation, enhancer-binding, coregulation, and insulation. Curr Opin Cell Biol *20*, 294-302.

Kravchenko, J. E., Ilyinskaya, G. V., Komarov, P. G., Agapova, L. S., Kochetkov, D. V., Strom, E., Frolova, E. I., Kovriga, I., Gudkov, A. V., Feinstein, E., and Chumakov, P. M. (2008). Small-molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway. Proceedings of the National Academy of Sciences of the United States of America *105*, 6302-6307.
Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K. H. (2000). Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene *19*, 5435-5443.
Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R.*, et al.* (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ *16*, 3-11.
Kroemer, G., and Jaattela, M. (2005). Lysosomes and autophagy in cell death control. Nature reviews Cancer *5*, 886-897.
Kroemer, G., and Reed, J. C. (2000). Mitochondrial control of cell death. Nature medicine *6*, 513-519.
Kronke, J., Schlenk, R. F., Jensen, K. O., Tschurtz, F., Corbacioglu, A., Gaidzik, V. I., Paschka, P., Onken, S., Eiwen, K., Habdank, M.*, et al.* (2011). Monitoring of Minimal Residual Disease in NPM1-Mutated Acute Myeloid Leukemia: A Study From the German-Austrian Acute Myeloid Leukemia Study Group. Journal of Clinical Oncology *29*, 2709-2716.
Kuo, M. L., den Besten, W., Bertwistle, D., Roussel, M. F., and Sherr, C. J. (2004). N-terminal polyubiquitination and degradation of the Arf tumor suppressor. Genes Dev *18*, 1862-1874.
Kurki, S., Peltonen, K., Latonen, L., Kiviharju, T. M., Ojala, P. M., Meek, D., and Laiho, M. (2004). Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell *5*, 465-475.
Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.
Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C.*, et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861-872.
Laster, S. M., Wood, J. G., and Gooding, L. R. (1988). Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol *141*, 2629-2634.
Lee, C., Smith, B. A., Bandyopadhyay, K., and Gjerset, R. A. (2005). DNA damage disrupts the p14ARF-B23(nucleophosmin) interaction and triggers a transient subnuclear redistribution of p14ARF. Cancer research *65*, 9834-9842.
Lee, H. H., Kim, H. S., Kang, J. Y., Lee, B. I., Ha, J. Y., Yoon, H. J., Lim, S. O., Jung, G., and Suh, S. W. (2007). Crystal structure of human nucleophosmin-core reveals plasticity of the pentamer-pentamer interface. Proteins *69*, 672-678.
Lee, H. O., Lee, J. H., Choi, E., Seol, J. Y., Yun, Y., and Lee, H. (2006). A dominant negative form of p63 inhibits apoptosis in a p53-independent manner. Biochemical and biophysical research communications *344*, 166-172.
Lee, J. M., and Bernstein, A. (1993). p53 mutations increase resistance to ionizing radiation. Proceedings of the National Academy of Sciences of the United States of America *90*, 5742-5746.
Lee, M. K., and Sabapathy, K. (2008). The R246S hot-spot p53 mutant exerts dominant-negative effects in embryonic stem cells in vitro and in vivo. Journal of cell science *121*, 1899-1906.
Lee, M. K., Teoh, W. W., Phang, B. H., Tong, W. M., Wang, Z. Q., and Sabapathy, K. (2012). Cell-type, dose, and mutation-type specificity dictate mutant p53 functions in vivo. Cancer Cell *22*, 751-764.
Lee, S. B., Xuan Nguyen, T. L., Choi, J. W., Lee, K. H., Cho, S. W., Liu, Z., Ye, K., Bae, S. S., and Ahn, J. Y. (2008). Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival. Proceedings of the National Academy of Sciences of the United States of America *105*, 16584-16589.

Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature *369*, 411-414.
Leon, S. A., Shapiro, B., Sklaroff, D. M., and Yaros, M. J. (1977). Free DNA in the serum of cancer patients and the effect of therapy. Cancer research *37*, 646-650.
Levine, A. J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nature reviews Cancer *9*, 749-758.
Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J. Y., and Melino, G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. Journal of cell science *113 (Pt 10)*, 1661-1670.
Li, D., Marchenko, N. D., and Moll, U. M. (2011a). SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. Cell death and differentiation *18*, 1904-1913.
Li, D., Marchenko, N. D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., and Moll, U. M. (2011b). Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells. Molecular cancer research : MCR *9*, 577-588.
Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M. A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136-1139.
Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R.*, et al.* (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.
Li, J., Zhang, X. L., Sejas, D. P., Bagby, G. C., and Pang, Q. S. (2004). Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. Journal of Biological Chemistry *279*, 41275-41279.
Li, J., Zhang, X. L., Sejas, D. P., and Pang, Q. S. (2005). Negative regulation of p53 by nucleophosmin antagonizes stress-induced apoptosis in human normal and malignant hematopoietic cells. Leukemia Res *29*, 1415-1423.
Li, Y., and Prives, C. (2007). Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene *26*, 2220-2225.
Li, Z., and Hann, S. R. (2009). The Myc-nucleophosmin-ARF network: a complex web unveiled. Cell Cycle *8*, 2703-2707.
Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M.*, et al.* (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature genetics *16*, 64-67.
Lin, A. W., and Lowe, S. W. (2001). Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. Proceedings of the National Academy of Sciences of the United States of America *98*, 5025-5030.
Linares, L. K., Hengstermann, A., Ciechanover, A., Muller, S., and Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proceedings of the National Academy of Sciences of the United States of America *100*, 12009-12014.
Liu, K., Ling, S., and Lin, W. C. (2011). TopBP1 mediates mutant p53 gain of function through NF-Y and p63/p73. Molecular and cellular biology *31*, 4464-4481.
Liu, X., Liu, Z. X., Jang, S. W., Ma, Z. Y., Shinmura, K., Kang, S. M., Dong, S. Z., Chen, J., Fukasawa, K., and Ye, K. Q. (2007). Sumoylation of nucleophosmin/B23 regulates its subcellular localization, mediating cell proliferation and survival. Proceedings of the National Academy of Sciences of the United States of America *104*, 9679-9684.
Llanos, S., Clark, P. A., Rowe, J., and Peters, G. (2001). Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. Nature cell biology *3*, 445-452.
Lo, Y. M., Corbetta, N., Chamberlain, P. F., Rai, V., Sargent, I. L., Redman, C. W., and Wainscoat, J. S. (1997). Presence of fetal DNA in maternal plasma and serum. Lancet *350*, 485-487.

Lo, Y. M., Hjelm, N. M., Fidler, C., Sargent, I. L., Murphy, M. F., Chamberlain, P. F., Poon, P. M., Redman, C. W., and Wainscoat, J. S. (1998). Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. The New England journal of medicine *339*, 1734-1738.
Lohrum, M. A., Ashcroft, M., Kubbutat, M. H., and Vousden, K. H. (2000). Identification of a cryptic nucleolar-localization signal in MDM2. Nature cell biology *2*, 179-181.
Lopez-Pajares, V., Kim, M. M., and Yuan, Z. M. (2008). Phosphorylation of MDMX mediated by Akt leads to stabilization and induces 14-3-3 binding. The Journal of biological chemistry *283*, 13707-13713.
Lowe, S. W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. Nature *432*, 307-315.
Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature *362*, 847-849.
Lowy, D. R., and Willumsen, B. M. (1993). Function and regulation of ras. Annual review of biochemistry *62*, 851-891.
Ludwig, R. L., Bates, S., and Vousden, K. H. (1996). Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. Molecular and cellular biology *16*, 4952-4960.
Luke, C. J., Pak, S. C., Askew, Y. S., Naviglia, T. L., Askew, D. J., Nobar, S. M., Vetica, A. C., Long, O. S., Watkins, S. C., Stolz, D. B.*, et al.* (2007). An intracellular serpin regulates necrosis by inhibiting the induction and sequelae of lysosomal injury. Cell *130*, 1108-1119.
Maddika, S., Ande, S. R., Wiechec, E., Hansen, L. L., Wesselborg, S., and Los, M. (2008). Akt-mediated phosphorylation of CDK2 regulates its dual role in cell cycle progression and apoptosis. Journal of cell science *121*, 979-988.
Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. The Journal of biological chemistry *276*, 18934-18940.
Maecker, H. L., Koumenis, C., and Giaccia, A. J. (2000). p53 promotes selection for Fas-mediated apoptotic resistance. Cancer Res *60*, 4638-4644.
Maeda, T., Hobbs, R. M., Merghoub, T., Guernah, I., Zelent, A., Cordon-Cardo, C., Teruya-Feldstein, J., and Pandolfi, P. P. (2005). Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. Nature *433*, 278-285.
Maehama, T., and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. The Journal of biological chemistry *273*, 13375-13378.
Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A., Thorner, M., and Scrable, H. (2004). Modulation of mammalian life span by the short isoform of p53. Genes & development *18*, 306-319.
Maiuri, M. C., Tasdemir, E., Criollo, A., Morselli, E., Vicencio, J. M., Carnuccio, R., and Kroemer, G. (2009). Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ *16*, 87-93.
Malanga, D., Scrima, M., De Marco, C., Fabiani, F., De Rosa, N., De Gisi, S., Malara, N., Savino, R., Rocco, G., Chiappetta, G.*, et al.* (2008). Activating E17K mutation in the gene encoding the protein kinase AKT1 in a subset of squamous cell carcinoma of the lung. Cell Cycle *7*, 665-669.
Mallardo, M., Caronno, A., Pruneri, G., Raviele, P. R., Viale, A., Pelicci, P. G., and Colombo, E. (2013). NPMc+ and FLT3_ITD mutations cooperate in inducing acute leukaemia in a novel mouse model. Leukemia *27*, 2248-2251.
Mandke, P., Wyatt, N., Fraser, J., Bates, B., Berberich, S. J., and Markey, M. P. (2012). MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame. PloS one *7*, e42034.
Manning, B. D., and Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Molecular cell *10*, 151-162.
Mantoni, T. S., Lunardi, S., Al-Assar, O., Masamune, A., and Brunner, T. B. (2011). Pancreatic stellate cells radioprotect pancreatic cancer cells through beta1-integrin signaling. Cancer research *71*, 3453-3458.
Mao, L., Merlo, A., Bedi, G., Shapiro, G. I., Edwards, C. D., Rollins, B. J., and Sidransky, D. (1995). A novel p16INK4A transcript. Cancer Res *55*, 2995-2997.
Marine, J. C., Francoz, S., Maetens, M., Wahl, G., Toledo, F., and Lozano, G. (2006). Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ *13*, 927-934.
Marion, R. M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M. A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature *460*, 1149-1153.
Martello, G., Rosato, A., Ferrari, F., Manfrin, A., Cordenonsi, M., Dupont, S., Enzo, E., Guzzardo, V., Rondina, M., Spruce, T*., et al.* (2010). A MicroRNA targeting dicer for metastasis control. Cell *141*, 1195-1207.
Matheu, A., Maraver, A., Klatt, P., Flores, I., Garcia-Cao, I., Borras, C., Flores, J. M., Vina, J., Blasco, M. A., and Serrano, M. (2007). Delayed ageing through damage protection by the Arf/p53 pathway. Nature *448*, 375-U314.
Matheu, A., Pantoja, C., Efeyan, A., Criado, L. M., Martin-Caballero, J., Flores, J. M., Klatt, P., and Serrano, M. (2004). Increased gene dosage of Ink4a/Arf results in cancer resistance and normal aging. Genes & development *18*, 2736-2746.
Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E., and Green, D. R. (2006). Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Molecular cell *21*, 749-760.
Mayo, L. D., and Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proceedings of the National Academy of Sciences of the United States of America *98*, 11598-11603.
Mayo, L. D., Turchi, J. J., and Berberich, S. J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. Cancer research *57*, 5013-5016.
McCloskey, R., Menges, C., Friedman, A., Patel, D., and McCance, D. J. (2010). Human papillomavirus type 16 E6/E7 upregulation of nucleophosmin is important for proliferation and inhibition of differentiation. Journal of virology *84*, 5131-5139.
McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A*., et al.* (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochimica et biophysica acta *1773*, 1263-1284.
Meek, D. W., and Hupp, T. R. (2010). The regulation of MDM2 by multisite phosphorylation--opportunities for molecular-based intervention to target tumours? Seminars in cancer biology *20*, 19-28.
Melino, G., Lu, X., Gasco, M., Crook, T., and Knight, R. A. (2003). Functional regulation of p73 and p63: development and cancer. Trends in biochemical sciences *28*, 663-670.
Mello, S. S., and Attardi, L. D. (2013). Not all p53 gain-of-function mutants are created equal. Cell death and differentiation *20*, 855-857.
Mendrysa, S. M., O'Leary, K. A., McElwee, M. K., Michalowski, J., Eisenman, R. N., Powell, D. A., and Perry, M. E. (2006). Tumor suppression and normal aging in mice with constitutively high p53 activity. Genes & development *20*, 16-21.
Miller, L. D., Smeds, J., George, J., Vega, V. B., Vergara, L., Ploner, A., Pawitan, Y., Hall, P., Klaar, S., Liu, E. T., and Bergh, J. (2005). An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proceedings of the National Academy of Sciences of the United States of America *102*, 13550-13555.

Mitrea, D. M., Grace, C. R., Buljan, M., Yun, M. K., Pytel, N. J., Satumba, J., Nourse, A., Park, C. G., Madan Babu, M., White, S. W., and Kriwacki, R. W. (2014). Structural polymorphism in the N-terminal oligomerization domain of NPM1. Proceedings of the National Academy of Sciences of the United States of America *111*, 4466-4471.
Mitrea, D. M., and Kriwacki, R. W. (2012). Cryptic disorder: an order-disorder transformation regulates the function of nucleophosmin. Pacific Symposium on Biocomputing Pacific Symposium on Biocomputing, 152-163.
Mizuki, M., Schwable, J., Steur, C., Choudhary, C., Agrawal, S., Sargin, B., Steffen, B., Matsumura, I., Kanakura, Y., Bohmer, F. D*., et al.* (2003). Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood *101*, 3164-3173.
Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992). The Mdm-2 Oncogene Product Forms a Complex with the P53 Protein and Inhibits P53-Mediated Transactivation. Cell *69*, 1237-1245.
Morbidelli, L., Donnini, S., and Ziche, M. (2003). Role of nitric oxide in the modulation of angiogenesis. Current pharmaceutical design *9*, 521-530.
Moroni, M. C., Hickman, E. S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. (2001). Apaf-1 is a transcriptional target for E2F and p53. Nature cell biology *3*, 552-558.
Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L., and Look, A. T. (1994). Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science *263*, 1281-1284.
Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G*., et al.* (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proceedings of the National Academy of Sciences of the United States of America *107*, 246-251.
Mouliere, F., Robert, B., Arnau Peyrotte, E., Del Rio, M., Ychou, M., Molina, F., Gongora, C., and Thierry, A. R. (2011). High fragmentation characterizes tumour-derived circulating DNA. PloS one *6*, e23418.
Moulin, S., Llanos, S., Kim, S. H., and Peters, G. (2008). Binding to nucleophosmin determines the localization of human and chicken ARF but not its impact on p53. Oncogene *27*, 2382-2389.
Muller, P. A., Caswell, P. T., Doyle, B., Iwanicki, M. P., Tan, E. H., Karim, S., Lukashchuk, N., Gillespie, D. A., Ludwig, R. L., Gosselin, P*., et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell *139*, 1327-1341.
Muller, P. A., and Vousden, K. H. (2013). p53 mutations in cancer. Nature cell biology *15*, 2-8.
Murtaza, M., Dawson, S. J., Tsui, D. W., Gale, D., Forshew, T., Piskorz, A. M., Parkinson, C., Chin, S. F., Kingsbury, Z., Wong, A. S*., et al.* (2013). Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature *497*, 108-112.
Nagila, A., Netsawang, J., Srisawat, C., Noisakran, S., Morchang, A., Yasamut, U., Puttikhunt, C., Kasinrerk, W., Malasit, P., Yenchitsomanus, P. T., and Limjindaporn, T. (2011). Role of CD137 signaling in dengue virus-mediated apoptosis. Biochemical and biophysical research communications *410*, 428-433.
Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999). Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. The Journal of biological chemistry *274*, 21528-21532.
Negrini, S., Gorgoulis, V. G., and Halazonetis, T. D. (2010). Genomic instability--an evolving hallmark of cancer. Nature reviews Molecular cell biology *11*, 220-228.
Neilsen, P. M., Noll, J. E., Mattiske, S., Bracken, C. P., Gregory, P. A., Schulz, R. B., Lim, S. P., Kumar, R., Suetani, R. J., Goodall, G. J., and Callen, D. F. (2013). Mutant p53 drives invasion in breast tumors through up-regulation of miR-155. Oncogene *32*, 2992-3000.
Neilsen, P. M., Noll, J. E., Suetani, R. J., Schulz, R. B., Al-Ejeh, F., Evdokiou, A., Lane, D. P., and Callen, D. F. (2011). Mutant p53 uses p63 as a molecular chaperone to alter gene expression and induce a pro-invasive secretome. Oncotarget *2*, 1203-1217.

Nishimura, Y., Ohkubo, T., Furuichi, Y., and Umekawa, H. (2002). Tryptophans 286 and 288 in the C-terminal region of protein B23.1 are important for its nucleolar localization. Bioscience, biotechnology, and biochemistry *66*, 2239-2242.
Nobukuni, T., Joaquin, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L.*, et al.* (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proceedings of the National Academy of Sciences of the United States of America *102*, 14238-14243.
Nojima, K., Hochegger, H., Saberi, A., Fukushima, T., Kikuchi, K., Yoshimura, M., Orelli, B. J., Bishop, D. K., Hirano, S., Ohzeki, M.*, et al.* (2005). Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. Cancer research *65*, 11704-11711.
Noll, J. E., Jeffery, J., Al-Ejeh, F., Kumar, R., Khanna, K. K., Callen, D. F., and Neilsen, P. M. (2012). Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11. Oncogene *31*, 2836-2848.
Nozawa, Y., Van Belzen, N., Van der Made, A. C., Dinjens, W. N., and Bosman, F. T. (1996). Expression of nucleophosmin/B23 in normal and neoplastic colorectal mucosa. The Journal of pathology *178*, 48-52.
Odgerel, T., Kikuchi, J., Wada, T., Shimizu, R., Kano, Y., and Furukawa, Y. (2010). MSK1 activation in acute myeloid leukemia cells with FLT3 mutations. Leukemia *24*, 1087-1090.
Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes & development *10*, 2462-2477.
Ogawara, Y., Kishishita, S., Obata, T., Isazawa, Y., Suzuki, T., Tanaka, K., Masuyama, N., and Gotoh, Y. (2002). Akt enhances Mdm2-mediated ubiquitination and degradation of p53. The Journal of biological chemistry *277*, 21843-21850.
Okuwaki, M., Tsujimoto, M., and Nagata, K. (2002). The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype. Mol Biol Cell *13*, 2016-2030.
Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847-860.
Olivier, M., Langerod, A., Carrieri, P., Bergh, J., Klaar, S., Eyfjord, J., Theillet, C., Rodriguez, C., Lidereau, R., Bieche, I.*, et al.* (2006). The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 1157-1167.
Olson, M. O., Wallace, M. O., Herrera, A. H., Marshall-Carlson, L., and Hunt, R. C. (1986). Preribosomal ribonucleoprotein particles are a major component of a nucleolar matrix fraction. Biochemistry *25*, 484-491.
Osaki, M., Oshimura, M., and Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis : an international journal on programmed cell death *9*, 667-676.
Ozenne, P., Eymin, B., Brambilla, E., and Gazzeri, S. (2010). The ARF tumor suppressor: structure, functions and status in cancer. International Journal of Cancer *127*, 2239-2247.
Pal, S. K., Reckamp, K., Yu, H., and Figlin, R. A. (2010). Akt inhibitors in clinical development for the treatment of cancer. Expert opinion on investigational drugs *19*, 1355-1366.
Peter, M. E., and Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ *10*, 26-35.
Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., and 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. Human mutation *28*, 622-629.
Phillips, A., Teunisse, A., Lam, S., Lodder, K., Darley, M., Emaduddin, M., Wolf, A., Richter, J., de Lange, J., Verlaan-de Vries, M.*, et al.* (2010). HDMX-L Is Expressed from a Functional p53-

responsive Promoter in the First Intron of the HDMX Gene and Participates in an Autoregulatory Feedback Loop to Control p53 Activity. Journal of Biological Chemistry *285*, 29111-29127.
Pichiorri, F., Suh, S. S., Rocci, A., De Luca, L., Taccioli, C., Santhanam, R., Zhou, W., Benson, D. M., Jr., Hofmainster, C., Alder, H.*, et al.* (2010). Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. Cancer Cell *18*, 367-381.
Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. The EMBO journal *15*, 2452-2467.
Plattner, R., Anderson, M. J., Sato, K. Y., Fasching, C. L., Der, C. J., and Stanbridge, E. J. (1996). Loss of oncogenic ras expression does not correlate with loss of tumorigenicity in human cells. Proceedings of the National Academy of Sciences of the United States of America *93*, 6665-6670.
Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proceedings of the National Academy of Sciences of the United States of America *96*, 1563-1568.
Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W.*, et al.* (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell *92*, 713-723.
Prevo, R., Deutsch, E., Sampson, O., Diplexcito, J., Cengel, K., Harper, J., O'Neill, P., McKenna, W. G., Patel, S., and Bernhard, E. J. (2008). Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity. Cancer Res *68*, 5915-5923.
Prost, S., Bellamy, C. O., Clarke, A. R., Wyllie, A. H., and Harrison, D. J. (1998). p53-independent DNA repair and cell cycle arrest in embryonic stem cells. FEBS letters *425*, 499-504.
Puc, J., Keniry, M., Li, H. S., Pandita, T. K., Choudhury, A. D., Memeo, L., Mansukhani, M., Murty, V. V., Gaciong, Z., Meek, S. E.*, et al.* (2005). Lack of PTEN sequesters CHK1 and initiates genetic instability. Cancer Cell *7*, 193-204.
Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011). RAS oncogenes: weaving a tumorigenic web. Nature reviews Cancer *11*, 761-774.
Qi, W., Shakalya, K., Stejskal, A., Goldman, A., Beeck, S., Cooke, L., and Mahadevan, D. (2008). NSC348884, a nucleophosmin inhibitor disrupts oligomer formation and induces apoptosis in human cancer cells. Oncogene *27*, 4210-4220.
Quentmeier, H., Martelli, M. P., Dirks, W. G., Bolli, N., Liso, A., Macleod, R. A., Nicoletti, I., Mannucci, R., Pucciarini, A., Bigerna, B.*, et al.* (2005). Cell line OCI/AML3 bears exon-12 NPM gene mutation-A and cytoplasmic expression of nucleophosmin. Leukemia *19*, 1760-1767.
Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T., and Kerbel, R. S. (1995). Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer research *55*, 4575-4580.
Raynaud, F. I., Eccles, S., Clarke, P. A., Hayes, A., Nutley, B., Alix, S., Henley, A., Di-Stefano, F., Ahmad, Z., Guillard, S.*, et al.* (2007). Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositide 3-kinases. Cancer research *67*, 5840-5850.
Rebucci, M., Peixoto, P., Dewitte, A., Wattez, N., De Nuncques, M. A., Rezvoy, N., Vautravers-Dewas, C., Buisine, M. P., Guerin, E., Peyrat, J. P.*, et al.* (2011). Mechanisms underlying resistance to cetuximab in the HNSCC cell line: role of AKT inhibition in bypassing this resistance. International journal of oncology *38*, 189-200.
Redner, R. L., Rush, E. A., Faas, S., Rudert, W. A., and Corey, S. J. (1996). The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood *87*, 882-886.

Restle, A., Farber, M., Baumann, C., Bohringer, M., Scheidtmann, K. H., Muller-Tidow, C., and Wiesmuller, L. (2008). Dissecting the role of p53 phosphorylation in homologous recombination provides new clues for gain-of-function mutants. Nucleic acids research *36*, 5362-5375.
Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., McMahon, M., Oren, M., and McCormick, F. (2000). Opposing effects of Ras on p53: Transcriptional activation of mdm2 and induction of p19(ARF). Cell *103*, 321-330.
Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nature reviews Molecular cell biology *9*, 402-412.
Robles, A. I., and Harris, C. C. (2010). Clinical outcomes and correlates of TP53 mutations and cancer. Cold Spring Harbor perspectives in biology *2*, a001016.
Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature *370*, 527-532.
Rodway, H., Llanos, S., Rowe, J., and Peters, G. (2004). Stability of nucleolar versus non-nucleolar forms of human p14(ARF). Oncogene *23*, 6186-6192.
Rozakisadcock, M., Mcglade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G*., et al.* (1992). Association of the Shc and Grb2/Sem5 Sh2-Containing Proteins Is Implicated in Activation of the Ras Pathway by Tyrosine Kinases. Nature *360*, 689-692.
Ruggero, D., Montanaro, L., Ma, L., Xu, W., Londei, P., Cordon-Cardo, C., and Pandolfi, P. P. (2004). The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. Nature medicine *10*, 484-486.
Ruggero, D., and Pandolfi, P. P. (2003). Does the ribosome translate cancer? Nature Reviews Cancer *3*, 179-192.
Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J*., et al.* (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.
Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual review of biochemistry *73*, 39-85.
Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509-514.
Sato, K., Hayami, R., Wu, W. W., Nishikawa, T., Nishikawa, H., Okuda, Y., Ogata, H., Fukuda, M., and Ohta, T. (2004). Nucleophosmin/B23 is a candidate substrate for the BRCA1-BARD1 ubiquitin ligase. Journal of Biological Chemistry *279*, 30919-30922.
Sax, J. K., Fei, P., Murphy, M. E., Bernhard, E., Korsmeyer, S. J., and El-Deiry, W. S. (2002). BID regulation by p53 contributes to chemosensitivity. Nature cell biology *4*, 842-849.
Scarpa, A., Capelli, P., Mukai, K., Zamboni, G., Oda, T., Iacono, C., and Hirohashi, S. (1993). Pancreatic Adenocarcinomas Frequently Show P53 Gene-Mutations. Am J Pathol *142*, 1534-1543.
Schmitt, C. A., Fridman, J. S., Yang, M., Lee, S., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002a). A senescence program controlled by p53 and p16(INK4a) contributes to the outcome of cancer therapy. Cell *109*, 335-346.
Schmitt, C. A., Fridman, J. S., Yang, M., Lee, S., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002b). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell *109*, 335-346.
Schnittger, S., Kern, W., Tschulik, C., Weiss, T., Dicker, F., Falini, B., Haferlach, C., and Haferlach, T. (2009). Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. Blood *114*, 2220-2231.
Schnittger, S., Schoch, C., Kern, W., Mecucci, C., Tschulik, C., Martelli, M. F., Haferlach, T., Hiddemann, W., and Falini, B. (2005). Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood *106*, 3733-3739.

Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic acids research *17*, 6419.
Schumm, K., Rocha, S., Caamano, J., and Perkins, N. D. (2006). Regulation of p53 tumour suppressor target gene expression by the p52 NF-kappaB subunit. The EMBO journal *25*, 4820-4832.
Schwarzenbach, H., Hoon, D. S., and Pantel, K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. Nature reviews Cancer *11*, 426-437.
Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. Nature reviews Cancer *3*, 721-732.
Semenza, G. L. (2010). Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene *29*, 625-634.
Serra, V., Markman, B., Scaltriti, M., Eichhorn, P. J. A., Valero, V., Guzman, M., Botero, M. L., Llonch, E., Atzori, F., Di Cosimo, S*., et al.* (2008). NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer research *68*, 8022-8030.
Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). Cell *88*, 593-602.
Sharpless, N. E. (2005). INK4a/ARF: a multifunctional tumor suppressor locus. Mutation research *576*, 22-38.
Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004). The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell *6*, 91-99.
Shaw, R. J., and Cantley, L. C. (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature *441*, 424-430.
She, Q. B., Chandarlapaty, S., Ye, Q., Lobo, J., Haskell, K. M., Leander, K. R., DeFeo-Jones, D., Huber, H. E., and Rosen, N. (2008a). Breast Tumor Cells with PI3K Mutation or HER2 Amplification Are Selectively Addicted to Akt Signaling. PloS one *3*.
She, Q. B., Chandarlapaty, S., Ye, Q., Lobo, J., Haskell, K. M., Leander, K. R., DeFeo-Jones, D., Huber, H. E., and Rosen, N. (2008b). Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. PloS one *3*, e3065.
Sherr, C. J. (1998). Tumor surveillance via the ARF-p53 pathway. Genes & development *12*, 2984-2991.
Sherr, C. J. (2006). Divorcing ARF and p53: an unsettled case. Nature reviews Cancer *6*, 663-673.
Sherr, C. J., Bertwistle, D., Den Besten, W., Kuo, M. L., Sugimoto, M., Tago, K., Williams, R. T., Zindy, F., and Roussel, M. F. (2005). p53-dependent and -independent functions of the Arf tumor suppressor. Cold Sh Q B *70*, 129-137.
Shields, J. M., Pruitt, K., McFall, A., Shaub, A., and Der, C. J. (2000). Understanding Ras: 'it ain't over 'til it's over'. Trends in cell biology *10*, 147-154.
Shields, L. B., Gercel-Taylor, C., Yashar, C. M., Wan, T. C., Katsanis, W. A., Spinnato, J. A., and Taylor, D. D. (1997). Induction of immune responses to ovarian tumor antigens by multiparity. Journal of the Society for Gynecologic Investigation *4*, 298-304.
Shin, I., Yakes, F. M., Rojo, F., Shin, N. Y., Bakin, A. V., Baselga, J., and Arteaga, C. L. (2002). PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nature medicine *8*, 1145-1152.
Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer research *60*, 6788-6793.
Silva, R. L., Thornton, J. D., Martin, A. C., Rehg, J. E., Bertwistle, D., Zindy, F., and Skapek, S. X. (2005). Arf-dependent regulation of Pdgf signaling in perivascular cells in the developing mouse eye. The EMBO journal *24*, 2803-2814.
Skeen, J. E., Bhaskar, P. T., Chen, C. C., Chen, W. S., Peng, X. D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and

suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell *10*, 269-280.
So, L., Yea, S. S., Oak, J. S., Lu, M., Manmadhan, A., Ke, Q. H., Janes, M. R., Kessler, L. V., Kucharski, J. M., Li, L. S.*, et al.* (2013). Selective inhibition of phosphoinositide 3-kinase p110alpha preserves lymphocyte function. The Journal of biological chemistry *288*, 5718-5731.
Soengas, M. S., Alarcon, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999). Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science *284*, 156-159.
Solomon, H., Buganim, Y., Kogan-Sakin, I., Pomeraniec, L., Assia, Y., Madar, S., Goldstein, I., Brosh, R., Kalo, E., Beatus, T.*, et al.* (2012). Various p53 mutant proteins differently regulate the Ras circuit to induce a cancer-related gene signature. Journal of cell science *125*, 3144-3152.
Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nature cell biology *9*, 573-580.
Song, M. S., Salmena, L., and Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. Nature reviews Molecular cell biology *13*, 283-296.
Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.
Sonoda, E., Hochegger, H., Saberi, A., Taniguchi, Y., and Takeda, S. (2006). Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. DNA Repair *5*, 1021-1029.
Stal, O., Perez-Tenorio, G., Akerberg, L., Olsson, B., Nordenskjold, B., Skoog, L., and Rutqvist, L. E. (2003). Akt kinases in breast cancer and the results of adjuvant therapy. Breast cancer research : BCR *5*, R37-44.
Steelman, L. S., Pohnert, S. C., Shelton, J. G., Franklin, R. A., Bertrand, F. E., and McCubrey, J. A. (2004). JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia *18*, 189-218.
Stommel, J. M., Kimmelman, A. C., Ying, H., Nabioullin, R., Ponugoti, A. H., Wiedemeyer, R., Stegh, A. H., Bradner, J. E., Ligon, K. L., Brennan, C.*, et al.* (2007). Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science *318*, 287-290.
Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. The EMBO journal *17*, 5001-5014.
Strano, S., and Blandino, G. (2003). p73-mediated chemosensitivity: a preferential target of oncogenic mutant p53. Cell Cycle *2*, 348-349.
Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M. G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M., and Blandino, G. (2002). Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. The Journal of biological chemistry *277*, 18817-18826.
Stroun, M., Anker, P., Lyautey, J., Lederrey, C., and Maurice, P. A. (1987). Isolation and characterization of DNA from the plasma of cancer patients. European journal of cancer & clinical oncology *23*, 707-712.
Su, X. H., Chakravarti, D., Cho, M. S., Liu, L. Z., Gi, Y. J., Lin, Y. L., Leung, M. L., El-Naggar, A., Creighton, C. J., Suraokar, M. B.*, et al.* (2010). TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature *467*, 986-U168.
Subong, E. N., Shue, M. J., Epstein, J. I., Briggman, J. V., Chan, P. K., and Partin, A. W. (1999). Monoclonal antibody to prostate cancer nuclear matrix protein (PRO:4-216) recognizes nucleophosmin/B23. The Prostate *39*, 298-304.
Sugimoto, M., Kuo, M. L., Roussel, M. F., and Sherr, C. J. (2003). Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. Molecular cell *11*, 415-424.

Suh, Y. A., Post, S. M., Elizondo-Fraire, A. C., Maccio, D. R., Jackson, J. G., El-Naggar, A. K., Van Pelt, C., Terzian, T., and Lozano, G. (2011). Multiple stress signals activate mutant p53 in vivo. Cancer Res *71*, 7168-7175.
Sun, S. Y., Rosenberg, L. M., Wang, X., Zhou, Z., Yue, P., Fu, H., and Khuri, F. R. (2005). Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer research *65*, 7052-7058.
Sundqvist, A., Bengoechea-Alonso, M. T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J. W., and Ericsson, J. (2005). Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7). Cell metabolism *1*, 379-391.
Sung, P., and Klein, H. (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nature reviews Molecular cell biology *7*, 739-750.
Sur, S., Pagliarini, R., Bunz, F., Rago, C., Diaz, L. A., Kinzler, K. W., Vogelstein, B., and Papadopoulos, N. (2009). A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. Proceedings of the National Academy of Sciences of the United States of America *106*, 3964-3969.
Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell *78*, 703-711.
Tago, K., Chiocca, S., and Sherr, C. J. (2005). Sumoylation induced by the Arf tumor suppressor: a p53-independent function. Proceedings of the National Academy of Sciences of the United States of America *102*, 7689-7694.
Taha, C., Liu, Z., Jin, J., Al-Hasani, H., Sonenberg, N., and Klip, A. (1999). Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. Role of mammalian target of rapamycin, protein kinase b, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation. The Journal of biological chemistry *274*, 33085-33091.
Takahashi, S. (2011). Downstream molecular pathways of FLT3 in the pathogenesis of acute myeloid leukemia: biology and therapeutic implications. Journal of hematology & oncology *4*, 13.
Tanaka, M., Sasaki, H., Kino, I., Sugimura, T., and Terada, M. (1992). Genes preferentially expressed in embryo stomach are predominantly expressed in gastric cancer. Cancer research *52*, 3372-3377.
Tazawa, H., Tsuchiya, N., Izumiya, M., and Nakagama, H. (2007). Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proceedings of the National Academy of Sciences of the United States of America *104*, 15472-15477.
Tepper, C. G., Gregg, J. P., Shi, X. B., Vinall, R. L., Baron, C. A., Ryan, P. E., Desprez, P. Y., Kung, H. J., and deVere White, R. W. (2005). Profiling of gene expression changes caused by p53 gain-of-function mutant alleles in prostate cancer cells. The Prostate *65*, 375-389.
Terzian, T., Suh, Y. A., Iwakuma, T., Post, S. M., Neumann, M., Lang, G. A., Van Pelt, C. S., and Lozano, G. (2008). The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes & development *22*, 1337-1344.
Theodor, L., Melzer, E., Sologov, M., Idelman, G., Friedman, E., and Bar-Meir, S. (1999). Detection of pancreatic carcinoma: diagnostic value of K-ras mutations in circulating DNA from serum. Digestive diseases and sciences *44*, 2014-2019.
Thompson, F. H., Nelson, M. A., Trent, J. M., Guan, X. Y., Liu, Y., Yang, J. M., Emerson, J., Adair, L., Wymer, J., Balfour, C*., et al.* (1996). Amplification of 19q13.1-q13.2 sequences in ovarian cancer. G-band, FISH, and molecular studies. Cancer genetics and cytogenetics *87*, 55-62.
Tomasini, R., Tsuchihara, K., Wilhelm, M., Fujitani, M., Rufini, A., Cheung, C. C., Khan, F., Itie-Youten, A., Wakeham, A., Tsao, M. S*., et al.* (2008). TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. Genes & development *22*, 2677-2691.
Torbett, N. E., Luna-Moran, A., Knight, Z. A., Houk, A., Moasser, M., Weiss, W., Shokat, K. M., and Stokoe, D. (2008). A chemical screen in diverse breast cancer cell lines reveals genetic enhancers

and suppressors of sensitivity to PI3K isoform-selective inhibition. The Biochemical journal *415*, 97-110.
Tran, H., Brunet, A., Griffith, E. C., and Greenberg, M. E. (2003). The many forks in FOXO's road. Science's STKE : signal transduction knowledge environment *2003*, RE5.
Trost, T. M., Lausch, E. U., Fees, S. A., Schmitt, S., Enklaar, T., Reutzel, D., Brixel, L. R., Schmidtke, P., Maringer, M., Schiffer, I. B*., et al.* (2005). Premature senescence is a primary fail-safe mechanism of ERBB2-driven tumorigenesis in breast carcinoma cells. Cancer research *65*, 840-849.
Tsui, K. H., Cheng, A. J., Chang, P., Pan, T. L., and Yung, B. Y. (2004). Association of nucleophosmin/B23 mRNA expression with clinical outcome in patients with bladder carcinoma. Urology *64*, 839-844.
Tucci, P., Agostini, M., Grespi, F., Markert, E. K., Terrinoni, A., Vousden, K. H., Muller, P. A., Dotsch, V., Kehrloesser, S., Sayan, B. S*., et al.* (2012). Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. Proceedings of the National Academy of Sciences of the United States of America *109*, 15312-15317.
Tucker, M., Goldstein, A., Dean, M., and Knudson, A. (2000). National Cancer Institute Workshop Report: the phakomatoses revisited. Journal of the National Cancer Institute *92*, 530-533.
Tyner, S. D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X. B., Soron, G., Cooper, B., Brayton, C*., et al.* (2002). p53 mutant mice that display early ageing-associated phenotypes. Nature *415*, 45-53.
Uldrijan, S., Pannekoek, W. J., and Vousden, K. H. (2007). An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. Embo Journal *26*, 102-112.
Utikal, J., Polo, J. M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R. M., Khalil, A., Rheinwald, J. G., and Hochedlinger, K. (2009). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature *460*, 1145-1148.
Valenti, F., Fausti, F., Biagioni, F., Shay, T., Fontemaggi, G., Domany, E., Yaffe, M. B., Strano, S., Blandino, G., and Di Agostino, S. (2011). Mutant p53 oncogenic functions are sustained by Plk2 kinase through an autoregulatory feedback loop. Cell Cycle *10*, 4330-4340.
Vaseva, A. V., and Moll, U. M. (2009). The mitochondrial p53 pathway. Biochimica et biophysica acta *1787*, 414-420.
Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C*., et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.
Velimezi, G., Liontos, M., Vougas, K., Roumeliotis, T., Bartkova, J., Sideridou, M., Dereli-Oz, A., Kocylowski, M., Pateras, I. S., Evangelou, K*., et al.* (2013). Functional interplay between the DNA-damage-response kinase ATM and ARF tumour suppressor protein in human cancer. Nature cell biology *15*, 967-977.
Viglietto, G., Motti, M. L., Bruni, P., Melillo, R. M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A*., et al.* (2002). Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nature medicine *8*, 1136-1144.
Vousden, K. H., and Lane, D. P. (2007). p53 in health and disease. Nature reviews Molecular cell biology *8*, 275-283.
Vousden, K. H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.
Wade, M., Li, Y. C., and Wahl, G. M. (2013a). MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nature reviews Cancer *13*, 83-96.
Wade, M., Li, Y. C., and Wahl, G. M. (2013b). MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nature Reviews Cancer *13*, 83-96.
Walerych, D., Napoli, M., Collavin, L., and Del Sal, G. (2012). The rebel angel: mutant p53 as the driving oncogene in breast cancer. Carcinogenesis *33*, 2007-2017.

Walker, K. K., and Levine, A. J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proceedings of the National Academy of Sciences of the United States of America *93*, 15335-15340.
Wang, C., Cigliano, A., Delogu, S., Armbruster, J., Dombrowski, F., Evert, M., Chen, X., and Calvisi, D. F. (2013). Functional crosstalk between AKT/mTOR and Ras/MAPK pathways in hepatocarcinogenesis: implications for the treatment of human liver cancer. Cell Cycle *12*, 1999-2010.
Wang, C., Ivanov, A., Chen, L., Fredericks, W. J., Seto, E., Rauscher, F. J., 3rd, and Chen, J. (2005a). MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. The EMBO journal *24*, 3279-3290.
Wang, D., Umekawa, H., and Olson, M. O. (1993). Expression and subcellular locations of two forms of nucleolar protein B23 in rat tissues and cells. Cellular & molecular biology research *39*, 33-42.
Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G. V., Li, G., Roy-Burman, P., Nelson, P. S*., et al.* (2003). Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell *4*, 209-221.
Wang, W., Budhu, A., Forgues, M., and Wang, X. W. (2005b). Temporal and spatial control of nucleophosmin by the Ran-Crm1 complex in centrosome duplication. Nature cell biology *7*, 823-830.
Ward, A., and Hudson, J. W. (2014). p53-Dependent and Cell Specific Epigenetic Regulation of the Polo-like kinases under Oxidative Stress. PloS one *9*.
Wattel, E., Preudhomme, C., Hecquet, B., Vanrumbeke, M., Quesnel, B., Dervite, I., Morel, P., and Fenaux, P. (1994). p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood *84*, 3148-3157.
Weber, J. D., Jeffers, J. R., Rehg, J. E., Randle, D. H., Lozano, G., Roussel, M. F., Sherr, C. J., and Zambetti, G. P. (2000). p53-independent functions of the p19(ARF) tumor suppressor. Genes & development *14*, 2358-2365.
Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nature cell biology *1*, 20-26.
Wee, S., Wiederschain, D., Maira, S. M., Loo, A., Miller, C., deBeaumont, R., Stegmeier, F., Yao, Y. M., and Lengauer, C. (2008). PTEN-deficient cancers depend on PIK3CB. Proceedings of the National Academy of Sciences of the United States of America *105*, 13057-13062.
Wei, W., Jin, J., Schlisio, S., Harper, J. W., and Kaelin, W. G., Jr. (2005). The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell *8*, 25-33.
Weinberg, R. L., Veprintsev, D. B., Bycroft, M., and Fersht, A. R. (2005). Comparative binding of p53 to its promoter and DNA recognition elements. Journal of molecular biology *348*, 589-596.
Weisz, L., Zalcenstein, A., Stambolsky, P., Cohen, Y., Goldfinger, N., Oren, M., and Rotter, V. (2004). Transactivation of the EGR1 gene contributes to mutant p53 gain of function. Cancer research *64*, 8318-8327.
Wilhelm, M. T., Rufini, A., Wetzel, M. K., Tsuchihara, K., Inoue, S., Tomasini, R., Itie-Youten, A., Wakeham, A., Arsenian-Henriksson, M., Melino, G*., et al.* (2010). Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. Genes & development *24*, 549-560.
Will, K., Warnecke, G., Wiesmuller, L., and Deppert, W. (1998). Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing. Proceedings of the National Academy of Sciences of the United States of America *95*, 13681-13686.
Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997). Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Molecular and cellular biology *17*, 5598-5611.

Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R.*, et al.* (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nature genetics *17*, 141-143.
Wu, H. L., Hsu, C. Y., Liu, W. H., and Yung, B. Y. (1999). Berberine-induced apoptosis of human leukemia HL-60 cells is associated with down-regulation of nucleophosmin/B23 and telomerase activity. International journal of cancer Journal international du cancer *81*, 923-929.
Wu, M. H., Chang, J. H., and Yung, B. Y. (2002). Resistance to UV-induced cell-killing in nucleophosmin/B23 over-expressed NIH 3T3 fibroblasts: enhancement of DNA repair and up-regulation of PCNA in association with nucleophosmin/B23 over-expression. Carcinogenesis *23*, 93-100.
Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.
Xie, S., Xie, B., Lee, M. Y., and Dai, W. (2005). Regulation of cell cycle checkpoints by polo-like kinases. Oncogene *24*, 277-286.
Xie, S. Q., Wu, H. Y., Wang, Q., Cogswell, J. P., Husain, I., Conn, C., Stambrook, P., Jhanwar-Uniyal, M., and Dai, W. (2001). Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. Journal of Biological Chemistry *276*, 43305-43312.
Xu, J., Reumers, J., Couceiro, J. R., De Smet, F., Gallardo, R., Rudyak, S., Cornelis, A., Rozenski, J., Zwolinska, A., Marine, J. C.*, et al.* (2011). Gain of function of mutant p53 by coaggregation with multiple tumor suppressors. Nature chemical biology *7*, 285-295.
Xu, J., Wang, J., Hu, Y., Qian, J., Xu, B., Chen, H., Zou, W., and Fang, J. Y. (2014). Unequal prognostic potentials of p53 gain-of-function mutations in human cancers associate with drug-metabolizing activity. Cell death & disease *5*, e1108.
Xu, X., Sakon, M., Nagano, H., Hiraoka, N., Yamamoto, H., Hayashi, N., Dono, K., Nakamori, S., Umeshita, K., Ito, Y.*, et al.* (2004). Akt2 expression correlates with prognosis of human hepatocellular carcinoma. Oncology reports *11*, 25-32.
Xue, W., Zender, L., Miething, C., Dickins, R. A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S. W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656-660.
Yakes, F. M., Chinratanalab, W., Ritter, C. A., King, W., Seelig, S., and Arteaga, C. L. (2002). Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer research *62*, 4132-4141.
Yan, L. (2009). MK-2206: A potent oral allosteric AKT inhibitor. American Association for Cancer Research; April 19; Denver, CO.
Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Molecular cell *2*, 305-316.
Yap, T. A., Yan, L., Patnaik, A., Fearen, I., Olmos, D., Papadopoulos, K., Baird, R. D., Delgado, L., Taylor, A., Lupinacci, L.*, et al.* (2011). First-in-man clinical trial of the oral pan-AKT inhibitor MK-2206 in patients with advanced solid tumors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *29*, 4688-4695.
Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W. C., Stukenberg, P. T., Shenolikar, S., Uchida, T.*, et al.* (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nature cell biology *6*, 308-318.
Yin, C., Knudson, C. M., Korsmeyer, S. J., and Van Dyke, T. (1997). Bax suppresses tumorigenesis and stimulates apoptosis in vivo. Nature *385*, 637-640.
Yoneda-Kato, N., Look, A. T., Kirstein, M. N., Valentine, M. B., Raimondi, S. C., Cohen, K. J., Carroll, A. J., and Morris, S. W. (1996). The t(3;5)(q25.1;q34) of myelodysplastic syndrome and acute myeloid leukemia produces a novel fusion gene, NPM-MLF1. Oncogene *12*, 265-275.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature *352*, 345-347.
Yu, J., and Zhang, L. (2005). The transcriptional targets of p53 in apoptosis control. Biochemical and biophysical research communications *331*, 851-858.
Yun, C., Wang, Y., Mukhopadhyay, D., Backlund, P., Kolli, N., Yergey, A., Wilkinson, K. D., and Dasso, M. (2008). Nucleolar protein B23/nucleophosmin regulates the vertebrate SUMO pathway through SENP3 and SENP5 proteases. The Journal of cell biology *183*, 589-595.
Yun, J. P., Chew, E. C., Liew, C. T., Chan, J. Y. H., Jin, M. L., Ding, M. X., Fai, Y. H., Li, H. K. R., Liang, X. M., and Wu, Q. L. (2003). Nucleophosmin/B23 is a proliferate shuttle protein associated with nuclear matrix. Journal of cellular biochemistry *90*, 1140-1148.
Zalcenstein, A., Stambolsky, P., Weisz, L., Muller, M., Wallach, D., Goncharov, T. M., Krammer, P. H., Rotter, V., and Oren, M. (2003). Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. Oncogene *22*, 5667-5676.
Zalcenstein, A., Weisz, L., Stambolsky, P., Bar, J., Rotter, V., and Oren, M. (2006). Repression of the MSP/MST-1 gene contributes to the antiapoptotic gain of function of mutant p53. Oncogene *25*, 359-369.
Zeller, K. I., Haggerty, T. J., Barrett, J. F., Guo, Q., Wonsey, D. R., and Dang, C. V. (2001). Characterization of nucleophosmin (B23) as a Myc target by scanning chromatin immunoprecipitation. The Journal of biological chemistry *276*, 48285-48291.
Zelzer, E., Levy, Y., Kahana, C., Shilo, B. Z., Rubinstein, M., and Cohen, B. (1998). Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. The EMBO journal *17*, 5085-5094.
Zhan, Q. M., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Forance, A. J. (1999). Association with Cdc2 and inhibition of Cdc2/cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*, 2892-2900.
Zhang, C. Z. Y., Chen, G. G., Merchant, J. L., and Lai, P. B. S. (2012). Interaction between ZBP-89 and p53 mutants and its contribution to effects of HDACi on hepatocellular carcinoma. Cell Cycle *11*, 322-334.
Zhang, H., Cicchetti, G., Onda, H., Koon, H. B., Asrican, K., Bajraszewski, N., Vazquez, F., Carpenter, C. L., and Kwiatkowski, D. J. (2003). Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. The Journal of clinical investigation *112*, 1223-1233.
Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. Science *290*, 989-992.
Zhang, W., Patil, S., Chauhan, B., Guo, S., Powell, D. R., Le, J., Klotsas, A., Matika, R., Xiao, X., Franks, R.*, et al.* (2006). FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. The Journal of biological chemistry *281*, 10105-10117.
Zhao, Q., and Lee, F. S. (1999). Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. The Journal of biological chemistry *274*, 8355-8358.
Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., and Hung, M. C. (2001a). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nature cell biology *3*, 245-252.
Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001b). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nature cell biology *3*, 973-982.
Zhu, W. G., Abbas, T., and Dutta, A. (2005). DNA replication and genomic instability. Adv Exp Med Biol *570*, 249-279.
Zimmermann, S., and Moelling, K. (1999). Phosphorylation and regulation of Raf by Akt (protein kinase B). Science *286*, 1741-1744.

Zuckerman, V., Lenos, K., Popowicz, G. M., Silberman, I., Grossman, T., Marine, J. C., Holak, T. A., Jochemsen, A. G., and Haupt, Y. (2009). c-Abl phosphorylates Hdmx and regulates its interaction with p53. The Journal of biological chemistry *284*, 4031-4039.