The Role of the p14ARF Tumour Suppressor in Promoting Apoptosis

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Statement of Originality

The contents of this thesis have not been presented for the award of a degree or diploma at this or any other university. The data presented are the original work of the author except where specifically indicated in the text.

Data presented in Chapter 6 are derived from work involving a number of authors and which was recently submitted to Journal of Clinical Oncology. My contribution to this work is set out in Chapter 6. Statements from fellow authors attesting to my contribution, and outlining their own contribution, are presented in Appendix 1.

Stuart J. Gallagher

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Summary

The incidence of melanoma has risen dramatically during the past three decades, yet there has been little improvement in effective treatments for this intractable and aggressive disease. Melanoma tumours are notoriously resistant to apoptosis, a cell suicide program that is activated by most cancer therapies. This thesis explores the role of the melanoma susceptibility gene product p14ARF in promoting cell cycle arrest and apoptosis, in order to resolve the impact of this tumour suppressor in melanomagenesis and melanoma susceptibility.

The *p14ARF* tumour suppressor gene is mutated in almost half of all cancers, and germline mutations in *p14ARF* confer a greatly increased risk of developing melanoma. The primary function of p14ARF is to relay oncogenic signals to p53, a central regulator of cellular response to stress. There is conflicting evidence regarding the role of p14ARF in promoting apoptosis. Much of the current evidence is based on murine studies, which may not translate accurately to humans due to important differences in animal physiology and the primary sequence and functions of the mouse and human ARF proteins. Furthermore, results from previous studies are often confounded by supra-physiological expression of p14ARF, and are complicated by the fact that *p14ARF* shares its genomic sequence with the $p16^{INK4a}$ tumour suppressor gene.

This study demonstrates that p14ARF expression in human cancer and primary cell lines promotes rapid p53-dependent cell cycle arrest, rather than apoptosis. As p14ARF expression did not induce apoptosis, we investigated if p14ARF could modulate the sensitivity of a cell to apoptosis induced by cytotoxic agents. Using a p14ARF-

inducible U2OS osteosarcoma cell line model, we examined the impact of p14ARF expression on the apoptotic response of the cell to a panel of thirteen cytotoxic agents. p14ARF expression increased apoptosis caused by a sub-set of agents, including trichostatin A, sodium butyrate, DRB, Adriamycin and UVB radiation.

p14ARF-mediated chemosensitivity was p53- and caspase-dependent, and involved the loss of mitochondrial potential. While loss of mitochondrial potential was dependent on p53, it was not blocked by caspase inhibition, demonstrating that caspases play a role downstream of mitochondrial depolarisation. Inhibition of individual components of the apoptotic program showed that p14ARF-mediated chemosensitivity was not strictly dependent on the pro-apoptotic Bax or Fas proteins.

We also investigated whether p14ARF could sensitise melanoma to chemotherapeutics *in vivo*. We investigated the expression level of p14ARF, $p16^{INK4a}$ and MITFm and mutation status of *B-RAF*, *N-RAS* and *PTEN* in melanomas from 30 patients that had undergone isolated limb infusion - a palliative therapeutic strategy that results in much higher response rates than systemic treatment. Expression of p14ARF did not predict response to the drugs actinomycin D and melphalan . Instead, high expression of $p16^{INK4a}$ and presence of activating *N-RAS* mutation were independent predictors of response to high doses of these chemotherapeutic drugs.

This work suggests that p14ARF analogues may be beneficial adjuncts in cancer therapy, but are unlikely to be effective as single agents. Additionally, p14ARF mimetics will only be effective in tumours with intact p53 signalling. Melanomas

frequently carry functional p53, and may be susceptible to this mode of treatment providing the apoptotic pathway downstream of p53 is intact or can be restored.

Ethics Approval

All work contained in this thesis had appropriate Institutional Biosafety Committee ethics approvals for dealing with genetically modified organisms. All work was conducted in accordance with the regulations of the Gene Technology Regulator for the manipulation of genetically modified organisms.

Ethics approval for the work in Chapter 6 was obtained and is covered by the Sydney South West Area Health Service Protocol No X00-0274 - "Molecular profile of melanoma chemosensitivity and resistance".

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Abbreviations

A1	Bcl-2-related protein A1
ActD	Actinomycin D
ADR	Adriamycin
AIF	Apoptosis Inducing Factor
APAF-1	Apoptotic protease activating factor 1
APC	Allophycocyanin
APS	Ammonium persulfate
ARF	Alternate Reading Frame
ARF-BP1	ARF-binding protein 1/Mcl-1 ubiquitin ligase E3
ATR	Ataxia telangiectasia and Rad3 related
Bad	Bcl-X_L/Bcl-2-associated death promoter
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
Bcl-x_L	B-cell lymphoma x (large)
BH1-4	Bcl-2 homology domain 1 - 4
Bid	Bcl-2-interacting domain
Bim	Bcl-2 interacting mediator of cell death
BIP-NC	Bax-inhibiting peptide negative control
BIP-V5	Bax-inhibiting peptide V5
bmi-1	B lymphoma Mo-MLV insertion region 1
B-RAF	V-raf murine sarcoma viral oncogene homolog B1
CARF	Collaborates/cooperates with ARF
CBP	CREB binding protein
cdc25c	Cell division cycle 25c
CDK	Cyclin dependent kinase
cFLIP	Fas-associated death domain-like interleukin 1beta-converting enzyme inhibitory protein
Chk1	Checkpoint kinase 1
CMX-Ros	Chloromethyl-X-rosamine
c-myc	Cellular myelocytomatosis oncogene
CPT	Camptothecin
CTBP1	C-terminal binding protein 1
DD	Death domain
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DP1	E2F dimerization partner 1
DRB	5,6 dichloro benzimidazole ribosode
DTIC	Dacarbazine
$\Delta\Psi_m$	Mitochondrial electrochemical potential
EDTA	Ethylenediaminetetraacetic acid

EGFP	Enhance Green fluorescent protein
Fas	Fibroblast associated receptor
FasL	Fibroblast associated receptor Ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
Foxm1b	Forkhead box M1b
GADD45	Growth arrest and DNA-damage-inducible 45
GFP	Green Fluorescent Protein
GSPT1	G1 to S phase transition 1
hADA3	Human homologue of yeast alteration/deficiency in activation 3
HDAC	Histone deacetylase
HDM2	Human murine double minute
HECT	Homology to E6-AP C-terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia induced factor-1
IAPs	Inhibitor of apoptosis proteins
iCAD	Inhibitor of caspase-activated DNase
IFNß	Interferon beta
IgG	Immunoglobulin G
IPTG	Isopropyl ß-D-thiogalactopyranoside
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide
LM	Light microscopy
LZAP	Leucine zipper-containing ARF-binding protein
MAP kinase	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Murine double minute
MdmX	Murine double minute X
MEFs	Murine Embrionic Fibroblasts
MITF	Microphthalmia associated transcription factor
MOI	Multiplicity of infection
NaB	Sodium Butyrate
NF-kB	Nuclear factor kappa B
NoLS	Nucleolar localisation domain
Noxa	PMA-induced protein
NPM	Nucleophosmin
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
Omi/HtrA2	High temperature requirement protein A2
p16INK4a	Inhibitor of cyclin dependent kinases 4 and 6
PARF	Protein associated with ARF
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
PIDD	pro-apoptotic interacting domain
PIG3	p53-inducible gene 3
PTEN	phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
Ras	Rat sarcoma
Rb	retinoblastoma protein

RING	Really interesting new gene
RIPA	Radio-immunoprecipitation
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
SMAC/DIABLO	second mitochondria-derived activator of caspases/direct IAP binding protein with low pI
smARF	shorter mitochondrial ARF
SUMO	Short ubiquitin-related modifier
tBid	truncated Bid
TBP-1	Tat-binding protein-1
TBS	Tris buffered saline
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tip60	Tat interacting protein, 60kDa
TNF	Tumour necrosis factor
TNFR1/2	Tumour necrosis factor receptor 1/2
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRAIL-R1/2	Tumour necrosis factor-related apoptosis-inducing ligand receptor 1/2
TSA	Trichostatin A
TTBS	Tween-TBS
Tyr-Ras	Tyrosinase-Ras
Ubc9	Ubiquitin conjugating enzyme 9
UV	Ultra Violet
v-Abl	Abelson murine leukaemia viral oncogene homolog 1
VPA	Valproic Acid
XIAP	X-linked inhibitor of apoptosis
z-VAD-fmk	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

1. Literature review

1.1 Cancer and Melanoma

Cancer is the second largest cause of death in Australia. The rate of newly diagnosed cancer continues to increase with the increasing average age of the population (up 26% from 1993 to 2003) (AIHW and AACR, 2007); however on an age adjusted basis, cancer rates have decreased 0.7% over this period. Despite overall reduced age adjusted incidence and mortality rates for cancer, rates of melanoma continue to rise. In the UK, age-standardised melanoma rates have increased almost five-fold in males, and over three-fold in females since 1975 (Lens and Dawes, 2004; www.cancerresearchuk.org, 2007). Rates have also increased in the US - from 1973 to 1997, the age adjusted melanoma rate jumped from 6.7 to 19.3 per 100000 in men, and from 5.9 to 13.8 in women (Jemal *et al.*, 2001).

In Australia, the number of new melanoma cases rose by 40.8% between 1983 and 2003, and the age-standardised rate increased 13.9% in the same period to 46.9/100000 (AIHW and AACR, 2007). During this period the number of deaths from melanoma also increased by 34.3% to 1146, with age-standardised mortality rates increasing marginally from 5.3 to 5.6 per 100000. Melanoma death rates appear to have plateaued in the last decade, due to earlier detection rather than improved therapies (Howe *et al.*, 2001; Miller and Mihm, 2006).

The increased rate of melanoma development in the last decade is the result of increased exposure to ultraviolet (UV) radiation (Armstrong and Kricker, 2001). This has been

caused by changes in behaviour and attitude to sun exposure, and to a lesser extent, by ozone depletion (Beddingfield, 2003; Burton and Armstrong, 1994). Australians younger than 35 years of age have grown up with effective sun education programs such as the Cancer Council's "Slip, Slop, Slap" campaign and have adopted safer sun exposure behaviour. This has resulted in reduced melanoma rates in this cohort (Coory *et al.*, 2006; Marks, 1999). However, the reduced melanoma risk in this younger age group will not have a large impact on overall melanoma rates until they are older, and it is predicted that overall rates of melanoma will continue to increase for at least another 20 years, albeit more slowly (McDermid *et al.*, 2005).

1.1.1 Melanoma Prognosis

Increasing rates of melanoma are of particular concern because effective therapies are limited for metastatic melanoma. Although stage I melanoma (no signs of cancer spread and less than 2mm thick) has a 95% cure rate by surgical excision, patients presenting with metastatic melanoma have five year survival rates of 65% for patients with regional melanoma, and 15% for patients with distant melanoma (Jemal *et al.*, 2004). Less than 10% of patients with visceral involvement survive two years (Balch *et al.*, 2001a).

Therapeutic options for patients with metastatic melanoma have remained relatively unchanged for the past three decades and dacarbazine remains the best systemic treatment (Eggermont and Kirkwood, 2004). Overall response rates to dacarbazine are less than 15% (Avril *et al.*, 2004; Middleton *et al.*, 2000; Millward *et al.*, 2004) although certain patients have highly sensitive tumours and rare complete remissions

are sustained (Coates and Segelov, 1994). While much work is being done on immune and bio-chemotherapies and adjuvants, these have yet to yield significant therapeutic improvement (reviewed in (Eggermont and Kirkwood, 2004; Thompson *et al.*, 2005))

1.1.2 Melanoma and apoptosis

The source of melanoma's recalcitrance to treatment lies in its resistance to apoptosis (programmed cell death) (reviewed in (Soengas and Lowe, 2003)). Melanoma arises from melanocytes, cells of neural crest origin that are responsible for the generation of melanin - a pigment that is distributed to surrounding cells and protects the skin from sun damage. The role of the melanocyte in protecting the skin requires that they are intrinsically resistant to the damaging effects of UV radiation, and as a result melanocytes are inherently resistant to apoptosis. For example, whereas most cells exposed to UV radiation undergo cell death or proliferative arrest, melanocytes are stimulated to grow by α-melanocyte-stimulating hormone that is produced by melanocytes and surrounding keratinocytes (Chakraborty *et al.*, 1996; Schauer *et al.*, 1994). The intrinsic resistance of melanocytes to apoptosis is largely attributed to high expression levels of anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members. During melanomagenesis, melanoma cells acquire mutations that fortify these defences against death, while driving growth and metastatic potential (Bush and Li, 2003; Soengas and Lowe, 2003).

Over 80% of melanomas gain growth signals from oncogenic activation of the mitogen activated protein kinase (MAP Kinase) pathway members B-RAF (V-raf murine sarcoma viral oncogene homolog B1) and N-RAS (Neuroblastoma RAS viral (v-ras)

oncogene homolog), or the receptor tyrosine kinase c-Kit. Intriguingly, these oncogenes are altered with different frequencies in different types of melanoma, suggesting different pathways of melanomagenesis (Table 1.1). For example, c-Kit alterations occur in 30-50% of mucosal and acral melanomas, and in melanomas arising from chronically sun damaged skin, but are rare in melanomas arising from intermittently sun exposed skin (Curtin *et al.*, 2006). In contrast, B-RAF mutations occur in up to 70% of benign naevi and cutaneous melanoma, while they occur in less than 20% of acral or mucosal melanomas (Kumar *et al.*, 2003a; Kumar *et al.*, 2004; Sasaki *et al.*, 2004). Spitz naevi and blue melanoma - both rare melanocytic neoplasms - have a very low occurrence of B-RAF and N-RAS mutations and have not yet been thoroughly investigated for c-Kit alterations (Indsto *et al.*, 2007; Saldanha *et al.*, 2004).

Table 1.1 Prevalence of activating alterations of c-Kit, B-RAF and N-RAS in melanoma by subtype.

Gene	***Naevi***	***Non-CSD***	***CSD***	***Mucosal***	***Acral***	***Spitz***	***Blue***
B-RAF	30-70%	50-60%	50-60%	<10%	10-20%	<12%	0%
N-RAS	10-20%	10-25%	10-25%	10-20%	10-20%	<6%	0%
c-Kit	<10%	0%	30%	40%	35-50%	ND	ND

CSD, chronic sun damage, ND, not determined

References used in table: (Curtin *et al.*, 2006; Curtin *et al.*, 2005; Davies *et al.*, 2002; Gorden *et al.*, 2003; Indsto *et al.*, 2007; Janku *et al.*, 2005; Kumar *et al.*, 2003a; Kumar *et al.*, 2003b; Kumar *et al.*, 2004; Maldonado *et al.*, 2003; Ohashi *et al.*, 1996; Omholt *et al.*, 2002; Omholt *et al.*, 2003; Saldanha *et al.*, 2006; Sasaki *et al.*, 2004; Ugurel *et al.*, 2007; Yazdi *et al.*, 2003)

The progression from melanocyte to metastatic melanoma involves the transition through a number of stages, usually involving progression from naevi to dysplastic naevi, to primary melanoma spreading superficially and/or vertically, then finally to metastatic melanoma (Miller and Mihm, 2006). In an attempt to understand melanoma progression and provide effective prognostic information, studies have investigated genetic changes that occur during these different stages to determine the suite of genes

that are altered during progression. The Bcl-2 family of proteins, which are critical in transmitting apoptotic signals, are frequently altered during melanoma progression and play a central role in the resistance of melanoma tumours to apoptosis (Zhuang *et al.*, 2007). This family contains many pro- and anti-apoptotic proteins and their functions will be reviewed in more depth later in this chapter.

Unexpectedly, expression of the anti-apoptotic Bcl-2 protein is often decreased in melanomas and expression of pro-apoptotic Bax and Bak proteins is retained or increased (Table 1.2). Although this seems counter-intuitive, melanoma tumours frequently over-express other anti-apoptotic members of the Bcl-2 family - in particular Mcl-1 and Bcl-x_L. Thus melanomas do not require Bcl-2 and can overcome the pro-apoptotic signalling of Bax and Bak (Table 1.2). Additionally, the down-regulations of other pro-apoptotic Bcl-2 family members (such as PUMA) negate the requirement for Bak and Bax reduction.

Table 1.2 Percentage of naevi, primary and metastatic melanomas expressing high levels of various Bcl-2 family proteins.

Bcl-2 family members	*Melanocytes*	*Naevi*	*Primary*	*Metastatic*	*Association with Progression*
Anti-apoptotic					
Bcl-2	43	46-100	50-60	30-80	Mostly decreased[1]
Bcl-x_L	30-60	30-80	80-100	80-100	Increased
Mcl-1	0[3]	70-80	60-90	80-100	Increased
Survivin[2]	0	100	ND	70-100	Increased
XIAP[2]	0[3]	0	25-75	75	Increased
Pro-Apoptotic					
Bax	100	100	75	70-100	Increased
Bak	100	ND	85	85	Steady or increased
PUMA	100	100	60	30	Decreased

[1]The majority of reports indicate that decreased Bcl-2 expression is associated with melanoma progression

[2]Survivin and XIAP are inhibitor of apoptosis proteins (IAPs), not Bcl-2 members.

[3]Melanocytes express low levels of Mcl-1 and XIAP

ND, Not determined

References used in table: (Divito *et al.*, 2004; Emanuel *et al.*, 2008; Fecker *et al.*, 2006; Gradilone *et al.*, 2003; Grossman *et al.*, 1999; Karst *et al.*, 2005; Leiter *et al.*, 2000; Selzer *et al.*, 1998; Tang *et al.*, 1998; Zhuang *et al.*, 2007)

Many other proteins important for triggering apoptosis are also disabled in melanoma. The initiators of apoptosis located at the cell surface are often disabled - fibroblast-associated receptor (Fas) is mutated in 7% of metastatic melanomas (Shin *et al.*, 1999) and wild-type Fas is generally expressed at low levels in melanomas due to promoter methylation (Bullani *et al.*, 2002). Metastatic melanoma is frequently resistant to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), with 35-45% of tumours displaying decreased expression of the receptors TRAIL-R1 and TRAIL-R2 (Zhuang *et al.*, 2006). The apoptotic protease activating factor-1 (APAF-1), a downstream component of the apoptosis cascade, is critical for the activation of caspase 9, and may be decreased by loss of heterozygosity in up to 40% of melanomas (Dai *et al.*, 2004; Soengas *et al.*, 2001), although this figure may be much lower (Allen *et al.*, 2005; Peltenburg *et al.*, 2005). Additional protection from apoptosis occurs via the direct inhibition of activated caspases by the inhibitor of apoptosis proteins (IAPs).

IAPs include survivin and XIAP, and are increased in over 70% of melanomas (Emanuel *et al.*, 2008; Gradilone *et al.*, 2003; Grossman *et al.*, 1999).

One of the central components of a cell's response to stress is the tumour suppressor protein and "guardian of the genome", p53, which is mutated in over half of all cancers (Lane, 1992). Given that melanoma is an aggressive and intractable disease, it is surprising that fewer than 25% of melanoma tumours disable p53 (Albino *et al.*, 1994; Florenes *et al.*, 1994; Gwosdz *et al.*, 2006; Sparrow *et al.*, 1995). Transfection of p53 reporter plasmids into melanoma cells indicates that the endogenous p53 is functional and transcriptionally active (Kichina *et al.*, 2003). The low rate of p53-inactivation in melanomas suggests that the p53-cell cycle regulatory pathway is inactivated via alternative means in this cancer. A recent report found that melanoma cells express various isoforms of p53 and some of these variants may inhibit the full-length protein (Avery-Kiejda *et al.*, 2008). In mouse models of melanoma, disruption of the upstream p53 regulator ARF (Alternate Reading Frame) can functionally replace p53 loss during melanomagenesis, and melanomas arising in these animals carry wild-type p53 (Chin *et al.*, 1997).

The p14ARF and $p16^{INK4a}$ tumour suppressor proteins are of particular interest in melanoma. They are encoded by the *INK4a/ARF* locus on chromosome band 9p21 and are frequently inactivated in melanoma and other human cancers. Additionally, germline mutations affecting this locus confer a greatly increased risk of cutaneous melanoma (Sharpless and DePinho, 1999). The principal role of p14ARF is to promote the stabilisation of p53 by inhibiting the action of the p53-ubiquitin ligase human mdm2 (HDM2) (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998). p53 promotes

cell cycle arrest and apoptosis, raising the possibility that p14ARF may also be pro-apoptotic. However, the role of p14ARF in promoting apoptosis is equivocal as much of the evidence is conflicting and based on the murine homologue, p19ARF. In this thesis we investigated the role of human p14ARF in regulating cell cycle arrest and apoptosis.

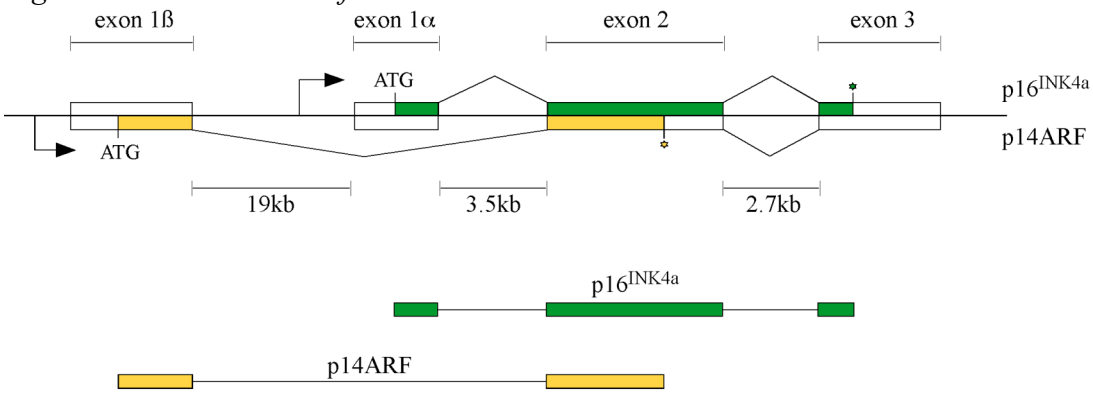
1.2 The ARF tumour suppressor

1.2.1 The *INK4a/ARF* locus

The Alternate Reading Frame (ARF) tumour suppressor protein (known as p14ARF in humans and p19ARF in mice) was originally identified as an alternative transcript of the *INK4a/ARF* tumour suppressor locus, a sequence that also encodes the $p16^{INK4a}$ inhibitor of cyclin dependent kinases 4 and 6 (Duro *et al.*, 1995; Quelle *et al.*, 1995). By virtue of unique first exons, the *ARF* and $p16^{INK4a}$ transcripts are translated in alternative reading frames and encode proteins bearing no homology. The $p16^{INK4a}$ gene utilises exons 1α, 2 and 3 and has a unique promoter. ARF utilises the upstream exon 1ß spliced to exons 2 and 3 and this transcript is translated in a different reading frame (Figure 1.1).

The *INK4a/ARF* locus is second only to p53 in the frequency of its disruption in human cancer and is mutated, deleted or hyper-methylated in half of all tumours (Haber, 1997; Sharpless and DePinho, 1999; Sherr, 1996). While the frequent mutation of the *INK4a/ARF* sequence demonstrates its importance in cancer progression, the two products of this locus are especially relevant in melanoma, as germline mutations are inherited in 39% of melanoma-prone kindreds (Goldstein *et al.*, 2006).

Figure 1.1 The structure of the INK4a/ARF locus



1.2.2 *INK4a/ARF* mutations in melanoma kindreds

Mutations in the *INK4a/ARF* locus confer increased risk of melanoma, pancreatic cancer and neural system tumours. Penetrance studies of families with mutations affecting this locus show that carriers have between 12-62% chance of developing melanoma before 50 years of age, rising to between 31-96% probability by 80 years of age (Bishop *et al.*, 2002). There is a suggestion that mutations affecting both p14ARF and $p16^{INK4a}$ confer a higher risk of melanoma than $p16^{INK4a}$-specific mutations (Berwick *et al.*, 2006). Families with *INK4a/ARF* mutations also have a higher risk of pancreatic cancer, with one study demonstrating that carriers have 39 times the risk of developing this cancer compared to non-carriers (Borg *et al.*, 2000). It is clear that p14ARF plays an important role in preventing pancreatic cancer, as the incidence of pancreatic cancer is higher in families carrying *INK4a/ARF* mutations that also affect the p14ARF amino acid sequence, compared to families carrying mutations that target $p16^{INK4a}$ exclusively (41% compared to 19% respectively) (Goldstein *et al.*, 2007).

Inherited mutations involving the *INK4a/ARF* locus are most commonly missense mutations (65%), but also involve deletions (16%), insertions or duplications (7%), nonsense mutations (5%) and splice site variants (5%) (Goldstein *et al.*, 2006; Goldstein *et al.*, 2007). In melanoma prone families, $p16^{INK4a}$ appears to function as the primary melanoma suppressor as mutations specifically targeting p14ARF are uncommon, affecting only 2% of high risk melanoma families with *INK4a/ARF* mutations (Table 1.3). Of the remaining families, 38% harbour mutations affecting the $p16^{INK4a}$-specific exon 1α, and 60% carry alterations affecting exons 2 or 3, or the entire locus (Table 1.3). It should be noted that 39% of the missense mutations affecting exons 2 and 3, specifically target $p16^{INK4a}$ and do not alter the p14ARF amino acid sequence. In contrast, exon 2 mutations that change the amino acid sequence of p14ARF but do not alter $p16^{INK4a}$ are rare (Goldstein *et al.*, 2006; Goldstein *et al.*, 2007). Mutations in both familial and sporadic melanoma cases are spread throughout the locus with 66% of mutations occurring in exon 2 (Figure 1.2).

Figure 1.2 The location of p16^{INK4a} mutations

The location of mutations affecting p16^{INK4a} reported in somatic tumours (n=782) or in the germline of melanoma-prone kindreds (n=104). Exon 2 mutations may also affect the primary sequence of p14ARF (source https://biodesktop.uvm.edu/perl/p16).

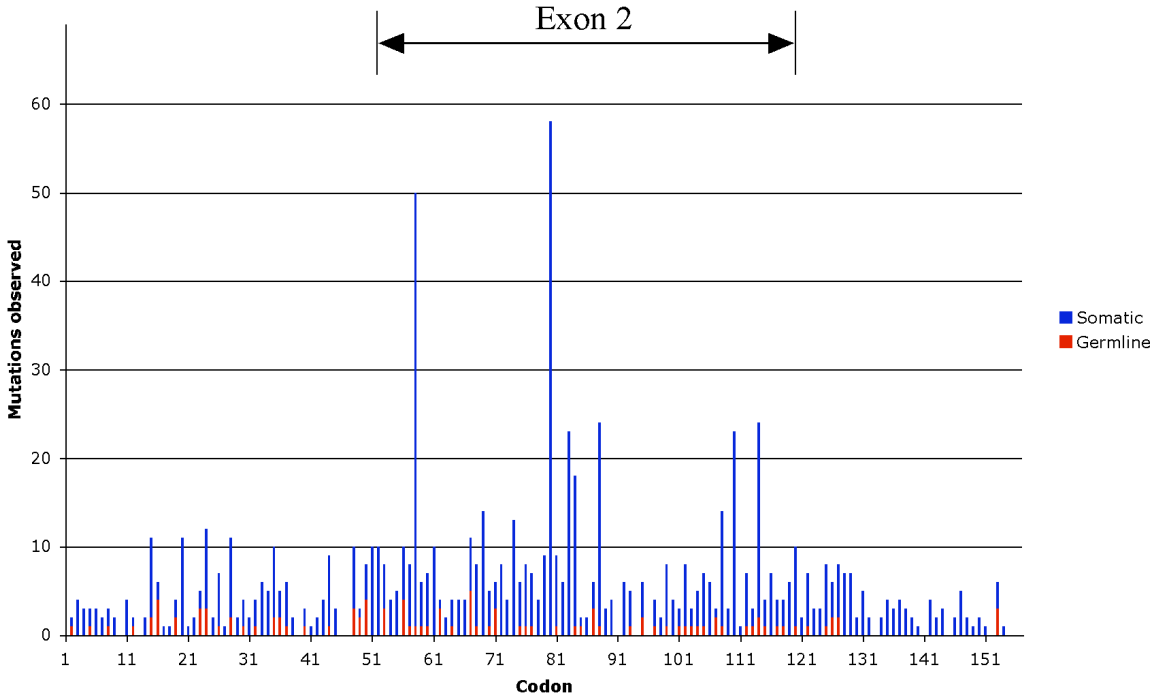


Table 1.3 Distribution of inherited *INK4a/ARF* mutations.

Exon of *Ink4a/ARF* locus	**Frequency mutated**
Exon 1ß	2%
Exon 1α	38%
Exon 2	51%
Exon 3	7%
Deletion of locus	2%

Data from 466 familial melanoma families (Goldstein *et al.*, 2006). All exon 1ß mutations are either of deletions (3/7) or splice variants (4/7).

While mutations in melanoma prone families target p16^{INK4a} predominantly, the importance of p14ARF mutations in melanoma susceptibility should not be underestimated. 43% of point mutations affecting the exon 2 coding region of p14ARF are radical as judged by the Grantham algorithm - which predicts functional effects of

amino acid changes - while 71% were non-conservative (compared to 35% and 76% respectively for $p16^{INK4a}$) (Goldstein *et al.*, 2007). Additionally, a number of studies have identified knockout mutations that target exon 1ß in melanoma prone families, and these families are also more likely to develop neural system tumours (Goldstein *et al.*, 2007; Hewitt *et al.*, 2002; Randerson-Moor *et al.*, 2001; Rizos *et al.*, 2001b).

1.2.3 *INK4a/ARF* mutations in sporadic cancer

The *INK4a/ARF* locus is disrupted in approximately half of all cancers by mutation, promoter methylation or deletion (Holland *et al.*, 1999; Pollock *et al.*, 1996; Sharpless and DePinho, 1999; Soufir *et al.*, 1998; Walker *et al.*, 1995). Although most *INK4a/ARF* mutations target $p16^{INK4a}$, 52% of somatic *INK4a/ARF* alterations also affect the p14ARF amino acid sequence (Pollock *et al.*, 1996) (Table 1.4). Alterations specifically affecting p14ARF are rare, but have been detected in human tumours and familial melanoma kindreds (Hewitt *et al.*, 2002; Randerson-Moor *et al.*, 2001; Rizos *et al.*, 2001b). p53 is mutated in only 7% of cancers that have also lost p14ARF, and significantly, the loss of p14ARF almost always involves the concomitant loss of $p16^{INK4a}$ (Sharpless and DePinho, 1999). This suggests that ARF functions primarily via the p53 pathway, as the inactivation of these two tumour suppressors is usually mutually exclusive. It also supports data generated in human fibroblasts that the loss of $p16^{INK4a}$ provides a growth advantage in a p53-null background (Voorhoeve and Agami, 2003).

Table 1.4 Proportion of mutations affecting p14ARF amino acid sequence in tumours with *INK4a/ARF* locus mutations.

	All cancers	**Melanoma**	**Skin - not Melanoma**	**Pancreatic**	**Head & Neck**	**Lung**	**Esophageus**	**Leukaemia**	**Brain**
p14ARF changed	52%	47%	48%	46%	62%	52%	58%	47%	48%
	Effect of all exon 2 mutations on p14ARF primary sequence								
Silent	22%	35%	30%	30%	19%	23%	8%	21%	22%
Frameshift	9%	7%	4%	14%	11%	9%	12%	11%	7%
Missense	55%	53%	57%	50%	49%	42%	55%	64%	63%
Nonsense	3%	2%	4%	0%	4%	7%	4%	0%	0%
Other	11%	4%	4%	7%	17%	19%	22%	4%	7%
Tumours tested	781	79	33	67	124	84	81	47	44

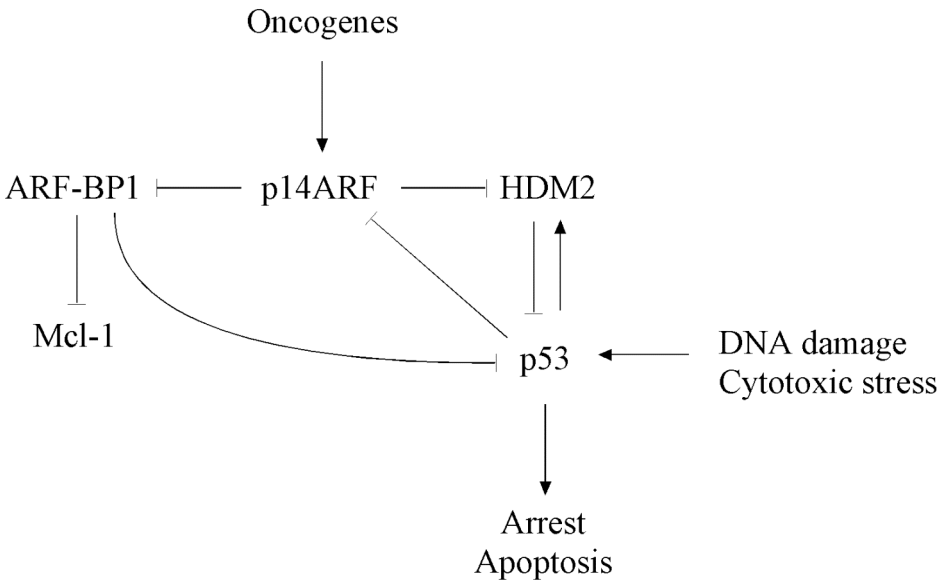
(Source: https://biodesktop.uvm.edu/perl/p16)

1.2.4 The ARF tumour suppressor is a critical activator of the p53 pathway

The ARF tumour suppressor protein suppresses aberrant cell growth in response to oncogene activation by inducing the p53-pathway (Figure 1.3). The p53 transcription factor is a central mediator of cell response to cytotoxic stress and oncogenic signalling. p53 is a labile protein that is rapidly stabilised by various cellular stress signals via post translational modification or inhibition of its negative regulators - most notably HDM2 (for reviews see (Harris and Levine, 2005; Lavin and Gueven, 2006)). Once activated, tetramers of p53 bind specific DNA promoter sequences containing the sequence motif: RRRCWWGYYY-spacer of 0-21 nucleotides –RRRCWWGYYY (where R is a purine, W is A or T and Y is a pyrimidine) (Levine, 1997). p53 initiates a transcriptional program that can result in cell cycle arrest, senescence or apoptosis. p53 targets over a hundred genes for transcriptional activation or repression by promoter binding (Ho and Benchimol, 2003). The DNA binding domain of the p53 protein lies between amino acid residues 102 to 292 and 90% of cancer-associated missense mutations in p53 target

this region, demonstrating the importance of transactivation in the role of p53 as a tumour suppressor (Levine, 1997).

Figure 1.3 The ARF tumour suppressor pathway
HDM2 and ARF-BP1 ubiquitinate p53, leading to its degradation. p14ARF is induced by oncogenic signalling and inhibits HDM2 and ARF-BP1 activity, leading to the stabilisation of p53. p53 acts in a auto-regulatory feedback loop by repressing p14ARF transcription and activating HDM2 transcription.



p14ARF is induced by various oncogenes, such as E2F1 and MYC (Drayton *et al.*, 2003; Parisi *et al.*, 2002b). Once induced, ARF stabilisation of p53 is mediated through two ubiquitin ligases: HDM2, a RING finger oncoprotein, and ARF-BP1/Mule (ARF-binding protein 1/Mcl-1 ubiquitin ligase E3), a HECT (homology to E6-AP C-terminus) containing protein (Figure 1.3) (Chen *et al.*, 2005a; Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). Both HDM2 and ARF-BP1 act as specific E3 ubiquitin ligases for p53, are highly expressed in various types of tumours, and have the potential to impede the tumour suppressor functions of p53. p53 can act in a self-regulatory pathway by

promoting HDM2 transcription and repressing ARF transcription (Figure 1.3) (Stott *et al.*, 1998).

HDM2 is an E3 ubiquitin ligase that contains a C-terminal RING domain which is responsible for its ubiquitin ligase activity (Boddy *et al.*, 1994; Fakharzadeh *et al.*, 1991). The transfer of ubiquitin onto target proteins is mediated by three separate enzymes: E1 (ubiquitin-activating enzyme); E2 (ubiquitin-conjugating enzyme); and E3 (ubiquitin-protein ligase). The E3 protein binds the target and determines substrate specificity. HDM2 ubiquitinates p53 on at least six C-terminal lysine residues, leading to its nuclear export and degradation (Haupt *et al.*, 1997; Honda *et al.*, 1997; Lohrum *et al.*, 2001; Nakamura *et al.*, 2000). Initially it was thought that HDM2 could poly-ubiquitinate p53 - which is required to target a protein for degradation by the 26S proteosome. However some *in vitro* studies have demonstrated that HDM2 mono-ubiquitinates p53, and further ubiquitin chains are added by other, as yet undetermined proteins (Lai *et al.*, 2001). One possibility is that HDM2 mono-ubiquitinates free p53, causing its nuclear export, while DNA bound p53 is initially mono-ubiquitinated by HDM2, then further poly-ubiquitinated by the p53 co-activator p300, leading to its proteosomal degradation in either the nucleus or cytoplasm (Grossman *et al.*, 2003; Xirodimas *et al.*, 2001; Yang *et al.*, 2004). As well as promoting the nuclear export and degradation of p53, HDM2 can directly inhibit p53 transcription. Amino acids 19-108 of HDM2 mediate binding to the N-terminal transactivation domain of p53 (Bottger *et al.*, 1997), thereby concealing this domain from co-activators and transcriptional machinery and repressing p53 transactivation (Momand *et al.*, 1992; Oliner *et al.*, 1993).

ARF associates with HDM2 to inhibit the ubiquitination, nuclear export and subsequent degradation of p53 (Tao and Levine, 1999; Zhang *et al.*, 1998). It has been proposed that ARF physically sequesters HDM2 in nucleoli, thus relieving nucleoplasmic p53 from HDM2-mediated degradation (Weber *et al.*, 2000b; Weber *et al.*, 1999; Zhang and Xiong, 1999). However, the nucleolar relocalisation of HDM2 does not appear to be required for p53 activation (Korgaonkar *et al.*, 2002) and the redistribution of ARF into the nucleoplasm enhances its interaction with HDM2 and its p53-dependent growth-suppressive activity (Korgaonkar *et al.*, 2005). This current model of ARF function supports the concept that nucleolar disruption contributes to p53 signalling (Rubbi and Milner, 2003) since many stress signals perturb the nucleolus, causing the release of nucleolar proteins such as ARF, L11, L23, L5 and NPM, that activate the p53 pathway.

It is less certain whether ARF can disrupt direct HDM2 inhibition of p53 transcription. ARF binds the central part of HDM2 (amino acids 210-304), which does not overlap with the N-terminal p53 binding domain of HDM2 (Bothner *et al.*, 2001). This suggests that ARF may not prevent p53 binding to HDM2 by steric hindrance, and p53/HDM2/ARF ternary complexes are observed *in vivo*, with HDM2 acting as a bridge (Kamijo *et al.*, 1998). These ternary complexes may somehow enhance p53 transcription, as ARF can increase transcription from p53 reporter promoters without increasing p53 levels (Kamijo *et al.*, 1998; Korgaonkar *et al.*, 2002).

In addition to HDM2, ARF-BP1 is a key regulator of the p53 cell cycle regulatory pathway; ARF-BP1 directly binds and ubiquitinates p53 in an HDM2-independent manner (Chen *et al.*, 2005a). Silencing of ARF-BP1 expression in p53-positive cells extended the half-life of p53, resulted in the transcriptional activation of the p53 targets

$p21^{Waf1}$ and Bax, and activated a p53-dependent apoptotic response (Chen *et al.*, 2005a). Unexpectedly, ARF-BP1 ubiquitinates and promotes the degradation of the anti-apoptotic Bcl-2 family member, Mcl-1, and down-regulation of ARF-BP1 expression can also render cells more resistant to killing by genotoxic agents (Zhong *et al.*, 2005). Thus, ARF-BP1 has been assigned both anti-apoptotic (via p53 degradation) and pro-apoptotic (via Mcl-1 degradation) functions. The divergent roles of ARF-BP1 may be regulated by ARF. Following aberrant oncogene activation, ARF expression is induced and inhibits ARF-BP1 activity toward p53 in the nucleus, thereby leading to p53-dependent apoptosis. In the cytoplasm, where ARF is not abundant, oncogene activation may lead to ARF-BP1 mediated Mcl-1 degradation, further promoting apoptosis.

1.2.5 Structure of ARF

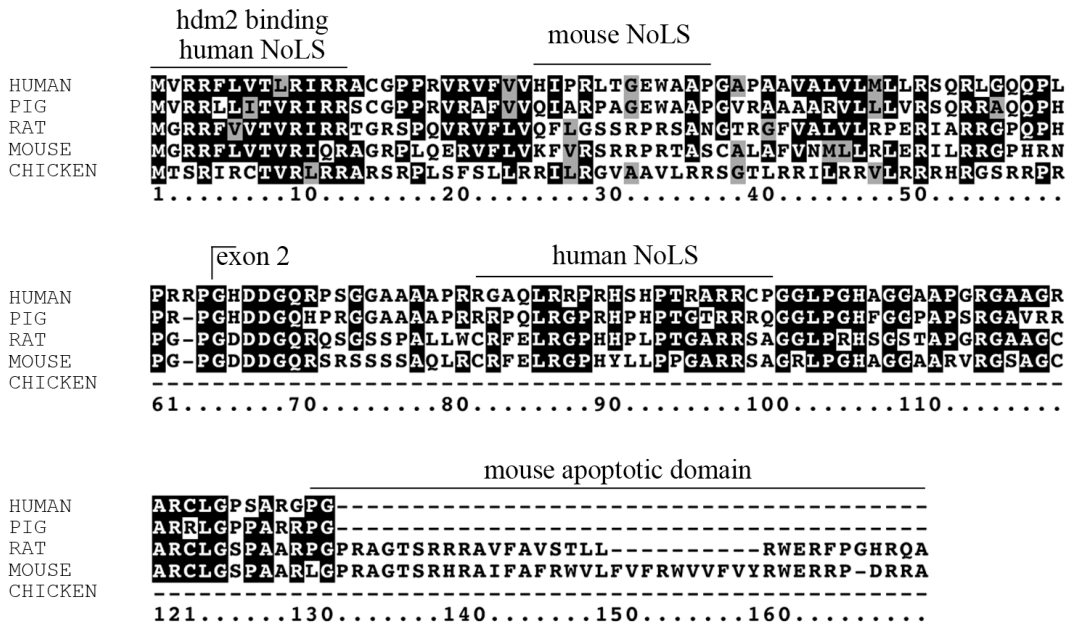
Human and mouse ARF proteins are arginine-rich, hydrophobic proteins of 132 and 169 amino acids, respectively (Figure 1.4). They display no homology to other known human proteins and are poorly conserved, sharing only 50% amino acid identity (Stott *et al.*, 1998). Exon 1ß shows lower homology between human and mouse proteins (45% identity) than exon 2 (57% identity) and, rather inexplicably for tumour suppressors, both *ARF* and *INK4a* sequences seem to be evolving rapidly and divergently at about three times the rate of other genes, including other members of the p53 and Rb tumour suppression network (Szklarczyk *et al.*, 2007).

Despite overall low homology, human and mouse ARF proteins show significant sequence similarity within their amino-terminal 14 amino acids (11/14 identity; see

Figure 1.4), and this region contains many of the known ARF functions, including nucleolar localisation, HDM2 binding and ability to induce cell cycle arrest. Furthermore, in chickens, only exon 1ß is translated to encode a smaller but functional 7kDa ARF protein (Kim *et al.*, 2003). Thus, it has been suggested that the *INK4a/ARF* exon-2 and -3 sequences simply act to stabilise the exon 1ß transcript (Sherr, 2006). However, the region encoded by the human *INK4a/ARF* exon 2 contains a second nucleolar localisation domain important for full HDM2 inhibition (Zhang and Xiong, 1999). Additionally, many mammals translate the region of ARF encoded by exon 2 (Szklarczyk *et al.*, 2007), even though a simulation of a million possible exon 2 sequence variants which did not alter the $p16^{INK4a}$ amino acid sequence showed only 4% of simulated sequences encoded full-length ARF (Szklarczyk *et al.*, 2007). Taken together, these data suggest that the exon 2-encoded segment of ARF is functionally important.

Figure 1.4 Comparison of the human, pig, rat, mouse and chicken ARF amino acid sequences

Identical residues highlighted in black and conserved substitutions in grey. Functional motifs, including nucleolar localisation sequences (NoLS) of human and mouse sequences are also marked.



Nucleolar localisation of ARF is mediated by one nucleolar localisation sequence (NoLS) in murine p19ARF (residues 26-37) and by two NoLS in human p14ARF (2-14 and 82-101) (Rizos *et al.*, 2000; Weber *et al.*, 2000b; Zhang and Xiong, 1999). These highly basic NoLS regions direct ARF to the granular compartment of the nucleolus (Bertwistle *et al.*, 2004; Itahana *et al.*, 2003; Lindstrom *et al.*, 2000).

Two arginine-rich sequences in the amino-terminus (aa 3-10 and 21-29) mediate binding to HDM2 (Bothner *et al.*, 2001; Clark *et al.*, 2002). These basic sequences interact electrostatically with the acidic residues (32%Asp/Glu) in the central region of HDM2 (amino acids 210-304) (Bothner *et al.*, 2001). In solution, the N-terminus of p14ARF becomes organised upon binding to the central section of HDM2 and forms two ß-strands. The HDM2 protein forms ß-strands at residues 245-253 and 275-282 upon binding to ARF (DiGiammarino *et al.*, 2001), revealing a cryptic nucleolar localisation sequence in the RING finger domain of HDM2 (Lohrum *et al.*, 2000).

1.2.6 Expression in normal tissue

The ARF promoter is a TATA-less CpG island that is characteristic of housekeeper genes (Robertson and Jones, 1998). It contains many Sp1 and E2F transcription factor binding sites. Low levels of p19ARF are expressed in many mouse tissues and levels increase in murine embryonic fibroblasts (MEFs) as they near senescence (Quelle *et al.*, 1995; Zindy *et al.*, 1998). Murine p19ARF is transcriptionally upregulated by Ras and p19ARF-null MEFs are refractory to growth arrest induced by Ras (Serrano *et al.*, 1997). In contrast, human p14ARF expression levels remain low as cells near

senescence. Further, ARF transcription is not induced by Ras (Brookes *et al.*, 2002; Wei *et al.*, 2001) and p14ARF-deficient fibroblasts still undergo arrest in response to Ras (Lindstrom *et al.*, 2000; Voorhoeve and Agami, 2003; Wei *et al.*, 2001).

Consistent with its role as a tumour suppressor, p19ARF is transcriptionally induced by a number of oncogenes, including E1A (de Stanchina *et al.*, 1998), E2F1(Bates *et al.*, 1998; Elliott *et al.*, 2001), E2F2/E2F3 (Berkovich *et al.*, 2003), c-myc (Zindy *et al.*, 1998), Ras (Palmero *et al.*, 1998),v-Abl (Radfar *et al.*, 1998), ß-catenin (Damalas *et al.*, 2001) and the Dentin matrix protein 1 tumour suppressor (Inoue *et al.*, 1999; Sreeramaneni *et al.*, 2005). In addition, several inhibitors of senescence (e.g. bmi-1, B-cell lymphoma 6 protein, chromobox 7, T-box 2, T-box 3) are known to repress the transcription of murine ARF (Brummelkamp *et al.*, 2001; Gil *et al.*, 2004; Jacobs *et al.*, 1999; Jacobs *et al.*, 2000). Much less is known about the regulation of p14ARF expression; p14ARF transcription is induced by E2F1 (Parisi *et al.*, 2002a), but not by oncogenic Ras alone. However, oncogenic Ras can cooperate with E2F1 to increase p14ARF transcription, and there is also some evidence that ß-catenin can induce ARF expression (Berkovich *et al.*, 2003; Damalas *et al.*, 2001). While p14ARF transcription is inhibited by T-box 2, T-box 3 and chromobox 7, it is not down-regulated by bmi-1 (Bernard *et al.*, 2005; Brummelkamp *et al.*, 2001; Gil *et al.*, 2004; Lingbeek *et al.*, 2002). Additionally, the oncogenic transcription factors Twist and AML-ETO repress transcription of ARF (Linggi *et al.*, 2002; Maestro *et al.*, 1999).

1.2.7 ARF Degradation

ARF is a relatively stable protein, with half-life estimations ranging from approximately one to six hours (our unpublished observations support an estimate at the higher end of

this range) (Kuo *et al.*, 2004; Rodway *et al.*, 2004). p19ARF and p14ARF (both lysineless proteins) undergo amino-terminal ubiquitination and their degradation depends on the ubiquitin-proteosome pathway and may be enhanced by HDM2 expression (Kuo *et al.*, 2004). Stress induced nucleoplasmic redistribution of ARF destabilises the protein, and targeting of a p14ARF fragment encompassing amino acids 2-29 to the nucleolus increased its half-life (Rodway *et al.*, 2004). Consistent with these data, the association of ARF with nucleophosmin (a nucleolar endoribonuclease) restricts ARF to the nucleolus and stabilises ARF by blocking its ubiquitination (Colombo *et al.*, 2005; Colombo *et al.*, 2006; Korgaonkar *et al.*, 2005). In contrast, HDM2-ARF complex formation occurs preferentially in the nucleoplasm and this may allow for enhanced ARF ubiquitination and degradation (Korgaonkar *et al.*, 2002).

1.2.8 Nucleolar functions of the ARF tumour suppressor

ARF is predominantly a nucleolar protein and rather than residing in inactive "storage" within the nucleolus (Korgaonkar *et al.*, 2002; Rodway *et al.*, 2004), ARF may regulate ribosome biogenesis by retarding the processing of early 47S/45S and 32S rRNA precursors (Sugimoto *et al.*, 2003). These effects do not depend on HDM2 or p53 but may involve the interaction of ARF with nucleophosmin (NPM/B23). NPM is an abundant nucleolar endoribonuclease that is required for the maturation of 28S rRNA and interacts with many cellular proteins, including p53, HDM2, ARF and the BRCA1 associated RING domain 1/breast cancer-1 ubiquitin ligase. The interaction of ARF and nucleophosmin retains both proteins in the nucleolus, bound to the pre-60S ribosome (Rizos *et al.*, 2006), where they reduce ribosome biogenesis and nucleolar size, and as a result, protein synthesis and cell volume (Apicelli *et al.*, 2008). The actual role of the nucleolar ARF-NPM complex remains unclear; data demonstrating that p19ARF

promoted the degradation of NPM (Itahana *et al.*, 2003) are not supported by our findings with p14ARF (Rizos *et al.*, 2006) nor indeed by other studies (Brady *et al.*, 2004; Korgaonkar *et al.*, 2002). In response to cytotoxic drugs (such as actinomycin D) and DNA damaging agents (including UV light), NPM and ARF undergo nucleoplasmic redistribution, where HDM2 and NPM compete for ARF binding (Lee *et al.*, 2005). The nucleoplasmic translocation of ARF and NPM promotes the formation of the ARF-HDM2 and the NPM-HDM2 binary complexes and induces potent activation of the p53 pathway (Lee *et al.*, 2005). Thus, cell growth may be affected by ARF localised in either the nucleolus, via its association with NPM and ribosomes, or in the nucleoplasm, by its interaction with HDM2 and ARF-BP1.

1.2.9 Other ARF binding partners

ARF can also suppress the proliferation of mouse cells lacking both MDM2 and p53, implying interactions with other regulators. Consistent with these findings, the tumour susceptibility of knock-out mice lacking ARF and p53, with or without MDM2, is significantly greater than that associated with animals lacking either gene alone (Weber *et al.*, 2000a). In searching for ARF binding proteins that are likely to underlie the p53-independent effects of ARF, many other ARF-interacting proteins have been identified (see Table 1.5). However, the biological significance of these ARF-interactions on the tumour suppressor functions of ARF has not been well established. Most of these targets have been identified from yeast-2-hybrid screens and co-immunoprecipitation experiments involving over-expression of ARF. Determining the significance of these interactions is difficult as ARF is a highly basic protein with an isoelectric point of over 12, making it notoriously “sticky”. In fact, only ARF’s interaction with HDM2 and perhaps with ARF-BP1 and nucleophosmin, has been shown to be relevant for its

tumour suppressing function (Chen *et al.*, 2005a; Colombo *et al.*, 2005; Pomerantz *et al.*, 1998).

Table 1.5 Cellular protein partners of the ARF tumour suppressor protein.

ARF binding partner	Biological Effect of ARF binding	References
ANCO1	Restrict nuclear receptor transcriptional activity	(Tompkins *et al.*, 2006)
APA-1	No apparent effects	(Benanti *et al.*, 2002)
ARF-BP1/Mule1	Inhibition of ARF-BP1 ubiquitin ligase activity	(Chen *et al.*, 2005a)
ATR	ATR/Chk1 activation and p53 phosphorylation	(Rocha *et al.*, 2005a)
BCL6	Inhibition of BCL6 transcriptional activity	(Shvarts *et al.*, 2002)
CARF	Enhanced ARF-mediated cell cycle arrest	(Hasan *et al.*, 2002)
CTBP1	Degradation of CtBP1 and apoptosis	(Paliwal *et al.*, 2006)
c-MYC	Inhibition of c-MYC transcriptional activity	(Qi *et al.*, 2004)
DP-1	Inhibition of ARF-induced E2F proteolysis	(Datta *et al.*, 2005)
E2F-1, -2, -3	Degradation of E2F	(Eymin *et al.*, 2001)
Foxm1b	Inhibition of Foxm1b transcriptional activity	(Kalinichenko *et al.*, 2004)
GSPT1	Effect on activity of GST binding protein unknown	(Tompkins *et al.*, 2006)
HIF-1α	Inhibition of HIF-1α transactivation	(Fatyol and Szalay, 2001)
LZAP	Suppresses of ARF inhibition of HDM2	(Wang *et al.*, 2006)
Mdm2	Inhibition of mdm2 ubiquitin ligase activity	(Pomerantz *et al.*, 1998)
MdmX	Enhanced p53 transactivation	(Jackson *et al.*, 2001)
Neurabin	Enhanced ARF-mediated cell cycle arrest	(Vivo *et al.*, 2001)
Nucleophosmin	Degradation of NPM, inhibition of NPM shuttling	(Bertwistle *et al.*, 2004)
$p120^{E4F}$	Enhanced ARF-mediated cell cycle arrest	(Rizos *et al.*, 2003)
p63	Inhibition of p63 transcriptional activity	(Calabro *et al.*, 2004)
PARF	Enhanced ARF signalling	(Tompkins *et al.*, 2006)
Peroxin 19p	Inhibition of p19ARF, does not bind human ARF	(Sugihara *et al.*, 2001)
Tat-binding protein-1	Induces ARF stabilization	(Pollice *et al.*, 2004)
Tip60	CHK2 phosphoylation and G2 arrest	(Eymin *et al.*, 2006b)
Topoisomerase I	Enhanced topoisomerase I activity	(Karayan *et al.*, 2001)
Ubc9	Involvement in p14ARF-mediated sumoylation	(Rizos *et al.*, 2005)
Werners helicase	Nucleolar exclusion of Werners helicase	(Woods *et al.*, 2004)
YY1	Decreased YY1/HDM2 binding	(Sui *et al.*, 2004)

1.2.10 ARF promotes the sumoylation of its binding partners

p14ARF has recently been shown to promote the sumoylation of some of its binding partners, including HDM2 and nucleophosmin (Rizos *et al.*, 2005; Tago *et al.*, 2005; Xirodimas *et al.*, 2002). Sumoylation is analogous to ubiquitination, and is the process by which the SUMO protein is conjugated to a target protein. The effects of this modification are target specific and include control of protein stability, formation of subnuclear structures and regulation of transcription factor activities (Melchior, 2000). The diverse functional consequences of sumoylation provide a possible explanation for the versatility of p14ARF downstream effects. Although ARF can promote sumoylation *in vivo,* the biological impact of target sumoylation has not been demonstrated for ARF binding partners, nor has any ARF-targeted sumoylation site been identified. Although the biological significance of ARF-mediated sumoylation remains to be established, the fact that a subset of melanoma-associated p14ARF mutations failed to sumoylate HDM2 *in vivo* (Rizos *et al.*, 2005), suggests that sumoylation may provide a mechanism for the diverse actions of the ARF tumour suppressor proteins.

1.2.11 Tumour suppressor functions – *in vivo* mice work

The overlapping design of the *INK4a/ARF* locus has made it difficult to tease apart the relative contribution of ARF and $p16^{INK4a}$. Mouse models have assisted in answering this question (Table 1.6). Initial mouse models knocked out exon 2 and thus disabled both p19ARF and $p16^{INK4a}$. These *INK4a/ARF*-null mice were highly tumour-prone, but did not develop spontaneous melanomas and derivative cells exhibited an immortalised phenotype (Serrano *et al.*, 1996). Disruption of exon 1ß in the mouse germline was subsequently used to generate mice lacking only p19ARF. Despite the fact that these animals continued to express wild-type $p16^{INK4a}$, they exhibited a similar cancer-prone

phenotype to the *INK4a/ARF*-null mice (Kamijo *et al.*, 1997). In contrast, the $p16^{INK4a}$-null mice were not especially tumour prone (~20% developed tumours by two years of age, mainly B-cell lymphomas); however these animals were susceptible to spontaneous melanoma development, albeit at a very low frequency (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). Intriguingly, when the *INK4a/ARF*–null mice were crossed with ARF hemizygotes, the animals developed melanomas and the penetrance rose from 8% to 50% after treatment with the carcinogen DMBA (Krimpenfort *et al.*, 2001). The effect of *ARF* haploinsufficiency in the *INK4a*-null background suggests that the INK4a and ARF pathways cooperate in melanoma development. Similarly, *INK4a/ARF*-null mice expressing melanocyte-specific oncogenic H-RAS (Tyr-RAS) developed melanoma spontaneously with high penetrance (Chin *et al.*, 1997). The Ras oncogene also co-operated with both ARF-null and $p16^{INK4a}$-null mouse models to promote spontaneous melanoma (Sharpless *et al.*, 2003). While Tyr-RAS mice are not tumour prone, 50% of Tyr-RAS/$ARF^{-/-}$ mice develop melanoma (40 week latency) and 35% of Tyr-RAS/$INK4a^{-/-}$ mice develop melanoma (75 week latency) (Sharpless *et al.*, 2003). In summary, both products of the *INK4a/ARF* locus are involved in mouse melanoma development.

The close functional connection between ARF and the p53 pathway is supported by the observation that $p53^{-/-}$, p53/$ARF^{-/-}$ and p53/ARF/$MDM2^{-/-}$ (triple knockout; TKO) mice develop cancer with a similar latency (Kamijo *et al.*, 1999; Moore *et al.*, 2003; Weber *et al.*, 2000a). Consistent with the role of ARF in activating the p53 pathway, tumours from ARF-null mice often retain p53 (Kamijo *et al.*, 1997). Tumours from ARF-null mice have a wider spectrum than p53 null mice, demonstrating that the longer latency of ARF-null mice alters the tumour spectrum (Table 1.6).

Given the large number of binding partners that have been reported for ARF, it has been proposed that p14ARF may possess p53-independent tumour suppressor activity. Certainly, inactivation of p19ARF in $p53^{-/-}$ and $p53/MDM2^{-/-}$ mice expands the range and frequency of tumour (Weber *et al.*, 2000a). However these results are not straightforward, as the interbred ARF-null and MDM2/p53 double knock out mice were all of mixed 129 X C57BL/6 background, and strain specific differences may have affected the result. The majority of the current data suggest that most functions of ARF action require intact p53.

Table 1.6 Tumour spectrum and latency in different strains of ARF deficient mice.

Genotype	Mouse Strain	Tumour latency (Weeks)	% Melanomas	% Lymphomas	% Sarcomas	% Carcinomas	% Neural Tumours	% Other	References
$p19ARF^{-/-}$	FVB	62	0	33	39	17	9	0	(Krimpenfort *et al.*, 2001;
$p16^{INK4a\ -/-}$		76	12	18	41	29	0	0	Sharpless *et al.*, 2001;
$INK4a/ARF^{-/-}$		38	0	90	0	10	0	0	Sharpless *et al.*, 2004)
$ARF^{+/-}/INK4a^{-/-}$		52	9	18	45	27	0	0	
$p19ARF^{-/-}$	C57BL/6	38	0	29	43	17	11	0	(Kamijo *et al.*, 1999;
$p53^{-/-}$	x 129Sv	16	0	70	30	0	0	0	Moore *et al.*, 2003; Weber
$p19ARF/p53^{-/-}$		16	0	72	6	11	6	6	*et al.*, 2000a)
$p19ARF/p53/MDM2^{-/-}$		16	0	68	26	0	5		
Tyr-Ras[1]	C57BL/6		0	0	0	0	0	0	(Chin *et al.*, 1997;
Tyr-Ras[1] $p19ARF^{-/-}$	x CBA x	40	52	19	10		5	14	Sharpless *et al.*, 2003)
Tyr-Ras[1] $p16^{INK4a-/-}$	129Sv	75	35	6	0	6	0	53	
Tyr-Ras[1] $INK4a/ARF^{-/-}$		24	88		12				

[1]Tyr-Ras mice express oncogenic Ras driven by the melanocyte specific tyrosinase promoter.

1.2.12 smARF

Interestingly, *ARF* mRNA also encodes a shorter, mitochondrial ARF (smARF) protein (Reef *et al.*, 2006). smARF is produced in both the human and mouse as a result of

internal initiation of translation at Met45 in p19ARF and Met48 in p14ARF. It is a short-lived protein, with a half-life of under 1 h and accounts for only 1-5% of total ARF protein in the cell. Lacking the N-terminal nucleolar localisation signal of full length ARF, smARF localises to an internal mitochondrial compartment where it interacts with, and is stabilised by, the mitochondrial protein, p32 (Reef *et al.*, 2007). Accumulation of smARF reduces mitochondrial membrane potential and induces autophagy and eventual cell death. The mitochondrial depolarisation induced by smARF does not lead to the leakage of mitochondrial products into the cytoplasm, nor is it inhibited by over-expression of anti-apoptotic Bcl-2 or Bcl-x_L or dependent on p53, Bax, Bak or caspases. This demonstrates that smARF induced cell death is not an apoptotic process. Instead, smARF appears to alter the potential across the inner mitochondrial membrane, leading to loss of mitochondrial function. Cell death by autophagy ensues after 72 hours, presumably as a result of the energy crisis caused by mitochondrial dysfunction (Reef *et al.*, 2007). The existence of smARF provides a possible explanation for p53-independent growth retardation observed by expression of full length *ARF* mRNA. However, because smARF production is so low, most data regarding smARF function relies on ectopically over-expressed smARF and thus the role of endogenous smARF in tumour suppression is yet to be defined.

1.3 Apoptosis

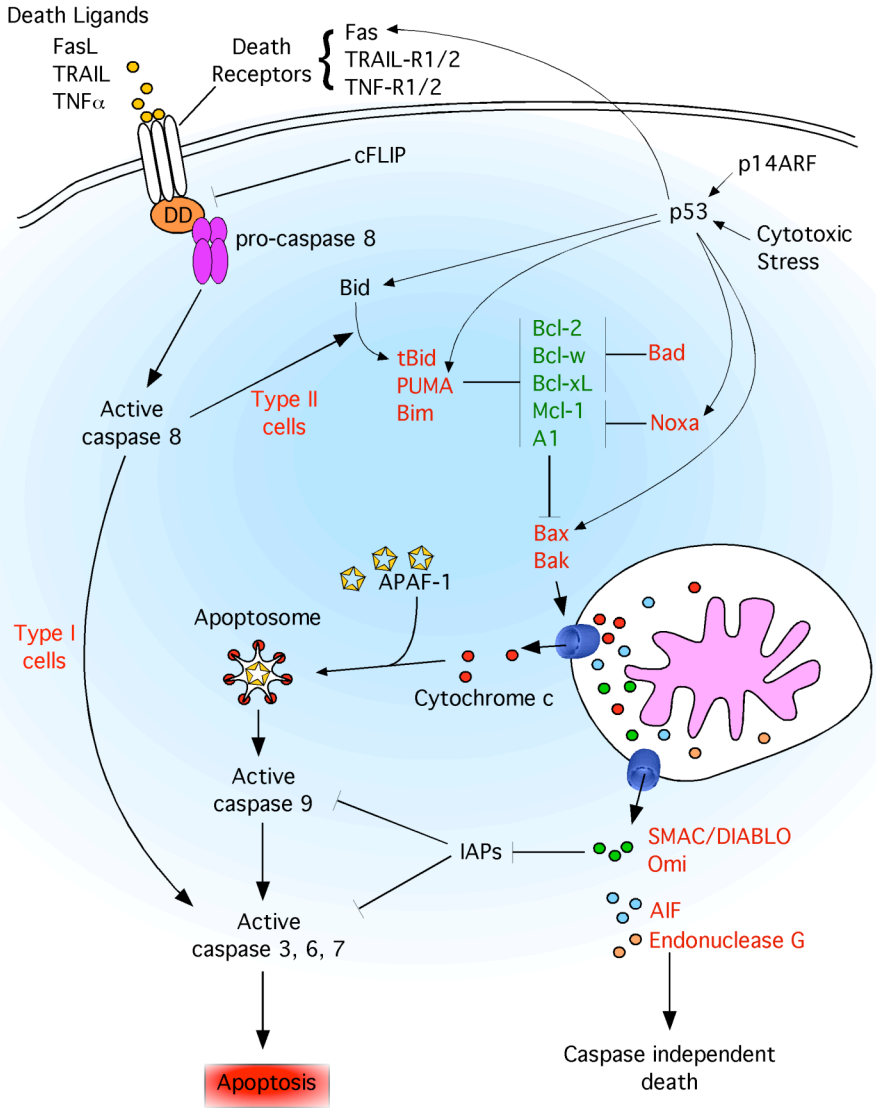
Although cell death has been the subject of research for over a century, the field of apoptosis was launched in earnest by a seminal article by Kerr describing common morphological features of cells undergoing programmed cell death (Kerr *et al.*, 1972).

Kerr's article coined the term "apoptosis" derived from the Greek "from falling", a term used over 2000 years ago to describe leaves and petals falling from plants.

Apoptosis is an essential process used to remove unwanted cells during development and to cull damaged, infected or potentially cancerous cells to prevent progression of disease or pathology. Apoptosis is a deliberate and active process, mediated by a network of proteins (Figure 1.5). As expected for a program that leads to cell death, there are many checks and balances. The central players in apoptosis are caspases (cysteine aspartyl proteases), proteases containing cysteine in their proteolytic domain which cleave their target at an aspartic acid residue (Stennicke and Salvesen, 1999). Caspases are expressed as an inactive pro-caspase that must be cleaved to become active. The caspase cascade begins with cleavage of the initiator caspases (including caspase 2, 8, 9, 10), which then cleave and activate downstream effector caspases (caspase 3, 6 and 7). Effector caspases 3, 6 and 7 cleave substrates such as the inhibitor of caspase-activated DNase (iCAD), lamin and poly(ADP-ribose) polymerase (PARP), leading to the phenotypic changes characteristic of apoptosis. These changes include chromatin condensation, exposure of phosphatidyl-serine to the exterior cytoplasmic membrane and DNA fragmentation (Clarke, 1990; Enari *et al.*, 1998; Fadok *et al.*, 1992; Ruchaud *et al.*, 2002; Sakahira *et al.*, 1998; Tewari *et al.*, 1995). The cascade of events that cause apoptosis allow for signal amplification, and once activated provide a surge of pro-apoptotic signalling that ensures cell death. The cascade also provides an opportunity for many checkpoints to control this potentially dangerous cellular program.

Figure 1.5 Overview of apoptosis pathways

Anti-apoptotic Bcl-2 family members are in green text. Pro-apoptotic Bcl-2 family proteins and mitochondrial proteins are in red text (see text for details). DD; death domain.



1.3.1 The extrinsic apoptotic pathway

Initiating apoptotic signals may come from either membrane bound death receptors (the extrinsic pathway) or via the mitochondria (the intrinsic pathway). At the apex of the extrinsic pathway is the TNF (tumour necrosis factor) receptor superfamily which

includes death receptors such as fibroblast-associated receptor (Fas/Apo1/CD95), tumour necrosis factor receptor (TNFR1/2) and TNF-related apoptosis inducing ligand receptor (TRAIL-R1/TRAIL-R2/DR5) (Wajant, 2003; Wajant *et al.*, 2005). Binding of the respective ligands (FasL, TNF- α and TRAIL) to their receptors results in receptor trimerisation and leads to the formation of the death inducing signalling complex (DISC) (Kischkel *et al.*, 1995). During DISC formation, the cytoplasmic death domain (DD) containing region of the receptor recruits Fas-associated DD containing protein, which in turn recruits caspase 8. The high local concentration of caspase 8 at the DISC increases its auto-proteolytic capacity by 100 fold, resulting in self-cleavage and activation of caspase 8 (Medema *et al.*, 1997; Muzio *et al.*, 1998). Caspase 10 can also be recruited to, and activated by, the DISC complex, but its role in initiating apoptosis has not been fully elucidated (Kischkel *et al.*, 2001).

The activity of the DISC, and therefore caspase 8 activation, can be inhibited by FLICE inhibitory protein (cFLIP) (Irmler *et al.*, 1997). cFLIP is highly expressed in 83% of melanomas, providing resistance to TRAIL mediated apoptosis (Bullani *et al.*, 2001; Chawla-Sarkar *et al.*, 2004). Additionally cFLIP expression is induced by nuclear factor kappa B (NF-κB) in a cell specific manner (for example, it is induced by NF-κB in transformed murine fibroblast SV40 and human breast cancer MCF-7 cells, but not in human embryonic kidney HEK293 or Jurkat T-cells), and is one way that NF-κB mediates cell protection from apoptosis (Kreuz *et al.*, 2001). p14ARF accumulation can inhibit NF-κB, thereby increasing the sensitivity of some cell types to extrinsic pathway activation (Rocha *et al.*, 2005a).

Cells can be classified as type I or type II depending on the potency of their activated caspase 8 response. In Type I cells, active caspase 8 cleaves effector caspases, leading to apoptosis directly (Martin and Fearnhead, 2002; Scaffidi *et al.*, 1998). In type II cells, activation of effector caspases by caspase 8 is insufficient to directly cause apoptosis. In this case, caspase 8 may cleave the Bcl-2 family member Bid, which in its truncated form (tBid) causes mitochondrial depolarisation, thereby linking the extrinsic and intrinsic pathways (Figure 1.5) (Ozoren *et al.*, 2000).

1.3.2 The intrinsic apoptotic pathway

The mitochondria are central to the intrinsic apoptotic pathway. Programmed cell death is induced by changes in mitochondrial permeability and the release of mitochondrial proteins such as cytochrome c, apoptosis inducing factor (AIF), endonuclease G and second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO) via pores in the outer mitochondrial membrane (Figure 1.5). Once cytochrome c is released into the cytoplasm, it binds to apoptotic protease activating factor 1 (APAF-1) and, with the co-activator ATP, recruits caspase 9 to its caspase recruiting domain, leading to caspase 9 activation (Riedl *et al.*, 2005; Yu *et al.*, 2005). Caspase 9 can then cleave and activate down-stream effector caspases 3 and 7 (Li *et al.*, 1997; Twiddy *et al.*, 2006), with caspase 6 then being activated by caspase 3 (Slee *et al.*, 1999). Caspase 9 activation can also positively regulate its own activation by cleaving targets that lie upstream of mitochondrial depolarisation. These including Bid, caspase 2 and caspase 8, thereby promoting further mitochondrial depolarisation and reinforcing the apoptotic signal (Henderson *et al.*, 2003; Samraj *et al.*, 2007). As a result, once initiated, all mitochondria in a cell depolarise rapidly and completely (Goldstein *et al.*, 2000).

Pore formation in the outer mitochondrial membrane is facilitated by the concerted action of members of the Bcl-2 family. Members of this family contain up to four Bcl-2 homology domains (BH1-BH4) that mediate interaction with other members of the family. The Bcl-2 superfamily contains both pro- and anti-apoptotic members and can be divided into three groups. The first group are anti-apoptotic members that contain all four homology regions, including B-cell lymphoma 2 (Bcl-2), Bcl-x_L, myeloid cell leukemia sequence 1 (Mcl-1), Bcl-2-related protein A1 (A1) and Bcl-w (Boise *et al.*, 1993; Gibson *et al.*, 1996; Kozopas *et al.*, 1993; Lin *et al.*, 1993; Vaux *et al.*, 1988). The second group comprises of the pro-apoptotic members Bcl-2-associated X protein (Bax) and Bcl-2 antagonist killer 1 (Bak) which contain BH domains 1-3 (Farrow *et al.*, 1995; Oltvai *et al.*, 1993). The last group are pro-apoptotic members that only contain the BH3 domain and are termed BH3-only proteins. This sub-set of the Bcl-2 family includes Bcl-2-interacting domain (Bid), Bcl-x_L/Bcl-2-associated death promoter (Bad), Bcl-2 interacting mediator of cell death (Bim), PMA-induced protein (Noxa) and p53-upregulated modulator of apoptosis (PUMA) (Nakano and Vousden, 2001; O'Connor *et al.*, 1998; Oda *et al.*, 2000; Wang *et al.*, 1996; Yang and al, 1996). While there is debate regarding the dynamics of Bcl-2 family member activation, the prevailing model posits that activated Bax and Bak can oligomerise and form pores in the mitochondrial outer membrane, but are inactivated by the anti-apoptotic Bcl-2 members. BH3-only pro-apoptotic proteins alleviate this inhibition by binding with anti-apoptotic Bcl-2 members, facilitating the assembly of Bax and Bak into pores in the outer mitochondrial membrane (Figure 1.5) (Willis *et al.*, 2007).

Preventing Bax and Bak activation are the anti-apoptotic members of the Bcl-2 family: Bcl-2; Bcl-x_L; Mcl-1; A1 and Bcl-w. These proteins bind to Bax and Bak, sequestering them into inactive dimers that are unable to oligomerise (Cheng *et al.*, 2001; Ross *et al.*, 2001; Ruffolo and Shore, 2003; Youle and Strasser, 2008). The BH1, 2 and 3 domains of the anti-apoptotic Bcl-2 proteins form a hydrophobic pocket that binds to the BH3 domain of Bax and Bak (Lalier *et al.*, 2007). High expression of anti-apoptotic Bcl-2 members confers a survival advantage to cells, and melanomas often express high levels of Bcl-2, Bcl-x_L and Mcl-1 (Table 1.2). Bcl-2 expression is essential for developing melanocytes, and Bcl-$2^{-/-}$ mice are deficient in melanocytes, and lose pigmentation after birth (McGill *et al.*, 2002; Yamamura *et al.*, 1996). The *Bcl-x_L* and *Mcl-1* genes are essential for normal embryogenesis and mice deficient either of these genes die before implantation or shortly after birth (Motoyama *et al.*, 1995; Rinkenberger *et al.*, 2000).

The pro-apoptotic BH3-only proteins bind and inhibit the anti-apoptotic Bcl-2 family members, thereby relaying apoptotic signals to the mitochondria (Uren *et al.*, 2007). As a result, the induction of BH-3 only proteins (which include Bid, Bad, Bim, Noxa, and PUMA), facilitate the oligomerisation of Bax and Bak and apoptosis.

In what has been called the "Bax/Bak rheostat model", the balance between opposing pro- and anti-apoptotic Bcl-2 proteins determines cell fate (Korsmeyer *et al.*, 1993). The various Bcl-2 members are regulated at transcriptional and post-translational levels. Cell stress can increase the level of Bax, Bid, PUMA and NOXA by p53-mediated transcriptional activation (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda *et al.*, 2000; Sax *et al.*, 2002; Yu *et al.*, 2003). While Bid is a transcriptional target of p53, it is an inactive zymogen that is mainly regulated by activating cleavage by

caspase 8 or cathepsins (Garnett *et al.*, 2007; Li *et al.*, 1998; Stoka *et al.*, 2001). Additionally, p53 binds and represses transcription from the *Bcl-2* and *Mcl-1* promoter (Pietrzak and Puzianowska-Kuznicka, 2008; Wu *et al.*, 2001). Bcl-2 is also a target of microphthalmia associated transcription factor (MITF), a master regulator in melanocytes that controls the growth and differentiation of melanocytes (Lekmine *et al.*, 2007), and MITF loss in melanocytes causes apoptosis that can be rescued by Bcl-2 over-expression (McGill *et al.*, 2002). Mcl-1 can also be poly-ubiquitinated by ARF-BP1, leading to its degradation by the 26S proteosome (Zhong *et al.*, 2005). While p14ARF can inhibit the ability of ARF-BP1 to ubiquitinate p53, it is not certain if this function extends to ARF-BP1 degradation of Mcl-1.

1.3.2.1 Specificity of Bcl-2 binding interactions

Apoptosis is also regulated by the selective binding activity of the Bcl-2 family of proteins (Fletcher and Huang, 2008). Bim, PUMA and tBid, bind all anti-apoptotic Bcl-2 members while Bad only binds Bcl-2, Bcl-x_L and Bcl-w, and NOXA only binds Mcl-1 and A1 (Figure 1.5) (van Delft and Huang, 2006; Willis *et al.*, 2005; Youle and Strasser, 2008). Therefore, in cells expressing Bcl-2 and Mcl-1, over expression of Bad or Noxa alone will not lead to apoptosis, while they do kill when expressed together (Chen *et al.*, 2005b). Additionally, the anti-apoptotic Bcl-2 members differ in their ability to inhibit Bax and Bak. Mcl-1 and Bcl-x_L bind both Bak and Bax, while Bcl-2, Bcl-w and A1 bind Bak alone (Willis *et al.*, 2005). Expression of the Bcl-2 members differs between cell types and tumours. Therefore, the BH-3 mimetic ABT-737, which acts like Bad to inhibit Bcl-2, Bcl-x_L and Bcl-w, is unlikely to be effective as a single agent in melanomas, which often have high Mcl-1 expression levels (Adams *et al.*, 2005; Zhuang *et al.*, 2007).

1.3.2.2 Other mitochondrial pro-death molecules

As well as cytochrome c release, mitochondrial outer membrane permeabilisation also causes the release of other mitochondrial inter-membrane space proteins that contribute to cell death. These include SMAC/DIABLO, high temperature requirement protein A2 (Omi/HtrA2), endonuclease G and apoptosis inducing factor (AIF). SMAC/DIABLO and Omi contribute to caspase dependent cell death by inactivating the function of the inhibitor of apoptosis proteins (IAPs). IAPs are negative regulators of activated caspases, that may prevent run-away caspase activation from auto-proteolysis. IAPs include X-linked inhibitor of apoptosis (XIAP), c-IAP1, c-IAP2, neuronal IAP and survivin (Deveraux and Reed, 1999; Liston *et al.*, 1996; Roy *et al.*, 1997; Uren *et al.*, 1996). They inhibit caspases 3, 7 and 9 directly and inhibit activation of caspase 8 by Fas (Deveraux *et al.*, 1999; Deveraux *et al.*, 1997; Takahashi *et al.*, 1998). Melanomas frequently express high levels of survivin and XIAP which contribute to their apoptotic resistance and is associated with poor prognosis (Chawla-Sarkar *et al.*, 2004; Gradilone *et al.*, 2003; Grossman *et al.*, 1999; Liu *et al.*, 2006).

Release of the AIF protein from mitochondria can cause caspase-independent cell death. Newly synthesised AIF is imported from the cytoplasm to the mitochondrial inter-membrane space, where its mitochondrial localisation signal is cleaved to generate the mature protein. Over-expression or micro-injection of mature AIF leads to caspase independent peripheral chromatin shrinkage, phosphatidyl-serine exposure and cleavage of genomic DNA into high moleculare weight fragments (<50kbp) (Susin *et al.*, 1999). Release of AIF from the mitochondria is required for its apoptotic activity, and it is not

inhibited by z-VAD-fmk, a pan-caspase inhibitor. AIF does not have any intrinsic nuclease activity itself, and its downstream effectors have not been elucidated (Daugas *et al.*, 2000; Susin *et al.*, 2000). Another potential mediator of cell death that may be released by mitochondrial outer membrane permeabilisation is endonuclease G, a DNase that causes caspase-independent, large-scale DNA fragmentation (Li *et al.*, 2001). The role of endonuclease G in promoting cell death in melanoma is uncertain; a variety of apoptotic stimuli promoted mitochondrial release of cytochrome c, SMAC/DIABLO, and AIF but not endonuclease G (Liu *et al.*, 2006). Endonuclease G may be important to promoting death in cells with low caspase activity (van Gurp *et al.*, 2003).

1.3.3 Caspase independent cell death

Although caspase-driven apoptosis has been the main focus of cell death study in the past few decades, the contribution to disease of other programmed cell death pathways is now being recognised. Alternate death mechanisms may be mediated by autophagy, cathepsins and calpains.

1.3.3.1 Autophagy

An alternate pathway leading to cell death is autophagy (used here to refer to macroautophagy), a process that can be triggered by the ARF variant smARF (Reef *et al.*, 2006). Autophagy is an self-cannibalisation process characterised by the formation of double membrane enclosed cytoplasmic vesicles that engulf cytoplasmic contents and organelles such as mitochondria and endoplasmic reticulum (for review see (Mizushima, 2007)). These vesicles (autophagosomes) deliver their contents to

lysosomes, where they are degraded and recycled. Autophagy was long viewed as a cell survival mechanism as it can provide cells with essential metabolic substrates in starvation conditions. However, it is now recognised that autophagy can also contribute to cell death (Clarke, 1990). Autophagy can cause cell death via the destruction of vital cellular components, leading to cellular dysfunction and atrophy (Gozuacik and Kimchi, 2007). While autophagic death causes chromatin shrinkage, in contrast to caspase mediated death it does not result in DNA fragmentations or the formation of apoptotic bodies (Gozuacik and Kimchi, 2007).

1.3.3.2 Calpain and Cathepsin proteases

Calpains are cytosolic proteases that are activated by high Ca^{2+} levels and their activation can lead to cell death (Goll *et al.*, 2003). Calpains do not have strict cleavage specificity, and cleave a number of proteins involved in apoptosis. Cleavage of Bcl-2 and Bcl-x_L by calpains can tip the balance of the Bcl-2 family towards apoptosis and cause mitochondrial depolarisation (Vandenabeele *et al.*, 2005). Additionally, Bax is cleaved by calpains, forming an 18kDa product that potently induces apoptosis (Gao and Dou, 2000). Calpains also cleave caspases, but at different sites to upstream caspases, often resulting in caspase inactivation (Chua *et al.*, 2000). Caspase 3 can also cleave and inactivate the negative calpain regulator calpastatin, to create a network between the caspase and calpain death pathways (Porn-Ares *et al.*, 1998).

Another class of proteases, cathepsins, can also mediate cell death. Cathepsins mainly reside in the lysosome, where they degrade and recycle lysosomal contents and process antigens for presentation to MHC class II receptors (Chwieralski *et al.*, 2006). Like

calpains, cathepsins have less site specificity than the caspases, and if released into the cytoplasm by extensive lysosomal rupture they can cause massive protein degradation and necrosis (Bursch, 2001). However small amounts of released cathepsins can lead to apoptosis, probably via cleavage and activation of Bid, and degradation of anti-apoptotic Bcl-2, Mcl-1 and Bcl-x_L (Cirman *et al.*, 2004; Stoka *et al.*, 2001; Turk and Stoka, 2007).

1.4 ARF and Apoptosis

The role of p14ARF in the apoptotic pathway remains controversial. There are important differences between the murine and human ARF homologues, and conflicting observations in different cellular contexts. MEFs over-expressing p19ARF undergo cell cycle arrest (Quelle *et al.*, 1995), but cells overexpressing p19ARF, together with E1A, Myc or RAS oncogenes undergo apoptosis (de Stanchina *et al.*, 1998; Russell *et al.*, 2002; Zindy *et al.*, 1998). Activation of murine ARF alone can also trigger apoptosis; the ectopic expression of p19ARF with a cre/loxP-regulated adenoviral vector induced apoptosis in p53-null MEFs, p53-intact human U2OS cells, and p53-deficient human SAOS-2 cells (Matsuoka *et al.*, 2003; Suzuki *et al.*, 2003; Tsuji *et al.*, 2002).

The C-terminal region of the murine ARF protein (amino acids 130-169) is important for p19ARF-mediated apoptosis, but this region is not conserved in human p14ARF (Matsuoka *et al.*, 2003) (see Figure 1.4). Thus, p14ARF may not be as potent an apoptotic regulator as its murine counterpart; ectopic Myc expression did not induce extensive apoptosis in human diploid fibroblasts despite obvious upregulation of

p14ARF and p53 (Drayton *et al.*, 2003). Additionally, long-term stable transient p14ARF expression did not lead to apoptosis in p53 intact lung cancer or glioma cells (Gao *et al.*, 2001; Simon *et al.*, 2006) or p53-null SAOS-2 (osteosarcoma) and H358 (bronchioloalveolar carcinoma) cells (Eymin *et al.*, 2001). Nor did p14ARF induction cause apoptosis in HT-1080 fibrosarcoma cells (p53 defective, with p14ARF expressed under the Tet-on promoter (Magro *et al.*, 2004).

Many groups have used adenoviral expression systems to investigate the functions of ARF. These studies show that p14ARF induces the death of p53-intact cells (Deng *et al.*, 2002; Kim *et al.*, 2004; Matsuoka *et al.*, 2003; Yang *et al.*, 2000; Yarbrough *et al.*, 2002) but not p53-null cells (Deng *et al.*, 2002; Kim *et al.*, 2004; Matsuoka *et al.*, 2003; Yang *et al.*, 2000; Yarbrough *et al.*, 2002). In contrast, others have shown that p14ARF induces apoptosis independently of p53 (Hemmati *et al.*, 2002). Knockout/knock-in models of HCT116 and DU145 colorectal cancer cells showed p14ARF caused apoptosis independent of p53 and Bax after 72 hours, and death occurred via mitochondrial depolarisation and was caspase dependent (Hemmati *et al.*, 2002; Hemmati *et al.*, 2006; Hemmati *et al.*, 2005). Titrations of virus levels show that apoptosis increases with p14ARF expression levels (Deng *et al.*, 2002; Hemmati *et al.*, 2002) and abnormally high expression levels may have contributed to the p53-independent apoptosis observed by Hemmati *et al* (Hemmati *et al.*, 2002).

In some cases, extended p14ARF signalling is needed to cause apoptosis. In these cases over six days of p14ARF expression was required to cause apoptosis in a p53-dependent (Sekaric *et al.*, 2007) or independent (Eymin *et al.*, 2003) manner. Long-term p14ARF expression can also lead to a senescent phenotype, with cells enlarging,

becoming vacuolated, and in some cases becoming ß-galactosidase positive (Sekaric *et al.*, 2007; Shamanin and Androphy, 2004).

ARF might reasonably be expected to cause apoptosis by up-regulating p53. p53 is a major apoptotic regulator and many of its transcriptional targets are pro-apoptotic, including Bax, Fas, p53-regulated apoptosis-inducing protein 1, PIDD, PUMA and NOXA (reviewed in (Das *et al.*, 2008; Fridman and Lowe, 2003)). p53 also represses the pro-survival proteins survivin, Bcl-2 and Mcl-1 (Hoffman *et al.*, 2002; Pietrzak and Puzianowska-Kuznicka, 2008; Wu *et al.*, 2001). Both Bax and Fas have been reported to be key mediators of ARF induced apoptosis (Kim *et al.*, 2004; Suzuki *et al.*, 2003). Bax (but not Bak) is required for ectopic p19ARF killing of SV-40 transformed MEFs (Suzuki *et al.*, 2003) and in human cells, apoptosis and chemosensitisation induced by adenovirally-expressed p14ARF is associated with increased Bax expression (Li *et al.*, 2006; Nakazawa *et al.*, 2003; Yuan *et al.*, 2007). Bax is not always required for adenovirally-expressed p14ARF-induced apoptosis; in HCT116 cells Bak can functionally replace Bax, and loss of both abrogates apoptosis caused by adenovirus-mediated p14ARF over-expression (Hemmati *et al.*, 2006). Expression levels of the different Bcl-2 family members could explain differences between Bax and Bak requirement in specific cell types. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-x_L, Mcl-1 among others) target Bax and Bak differentially; Bcl-x_L/Mcl-1 inhibit Bak more potently than Bax, while Bcl-2, Bcl-w and A1 specifically inhibit Bax (Willis *et al.*, 2005). Therefore, cells with high levels of Mcl-1 and/or Bcl-x_L that effectively disables Bak would be dependent on Bax activity for intrinsic pathway activation.

In many cases p53 stabilisation does not lead to apoptosis, but rather promotes cell cycle arrest (Draus *et al.*, 2001; Soddu *et al.*, 1996). p53-mediated transcription of pro-arrest targets can also inhibit apoptosis. Stabilisation of p53 by ARF results in increased $p21^{Waf1}$ – a direct target of p53 that inhibits cell cycle progression by binding CDK/cyclin complexes. $p21^{Waf1}$ can inhibit apoptosis by inhibition of cyclin-dependent kinase-mediated caspase-9 activation (Sohn *et al.*, 2006), inhibition of death receptor activation of caspase 8 and 10 (Xu and El-Deiry, 2000), and binding to caspase 3 (Suzuki *et al.*, 2000; Suzuki *et al.*, 1998). Additionally, loss of $p21^{Waf1}$ increases apoptosis in response to cytotoxic agents (Wendt *et al.*, 2006) and ARF over-expression (Hemmati *et al.*, 2005).

1.4.1 Post-translational modification of p53 by ARF

The transcriptional spectrum of p53 is altered by its many post-translational modifications. While ARF has not been reported to directly modify p53, ARF accumulation increases sumoylation of p53 by HDM2, with as yet undetermined effects (Chen and Chen, 2003). Additionally, p53 accumulated in response to ARF may be acetylated by the p53 co-activator and histone acyltransferase p300 (Mellert *et al.*, 2007; Sekaric *et al.*, 2007). Acetylation of p53 at lysine 382 by p300 requires hADA3, and is inhibited by HDM2, thus ARF-mediated HDM2 inhibition favours p53 acetylation (Sekaric *et al.*, 2007; Wang *et al.*, 2001a).

1.4.2 ARF and chemosensitivity

There have also been a few studies suggesting that p14ARF accumulation may influence chemosensitivity. In most cases, p14ARF only increased the chemosensitivity

of cells with functional p53. Adenoviral-mediated p14ARF over-expression sensitised MCF-7 breast cancer cells to cisplatin, but not to taxol (Deng *et al.*, 2002) and stable p14ARF expression increased the radiosensitivity of p53-intact lung cancer (Gao *et al.*, 2001) and glioma cells (Simon *et al.*, 2006). Although p14ARF expressing glioma cells were more radiosensitive, they were not rendered more sensitive to vincristine (an inhibitor of tubulin polymerisation) or the nitrosourea BCNU (Simon *et al.*, 2006). Although p53 dependence is suggested by these three reports, it has not been conclusively demonstrated with the use of isogenic p53-null/knock-down cell lines.

There is also evidence that p14ARF can modulate chemosensitivity in a p53 independent manner. p14ARF expressed under the Tet-on system in p53-defective HT-1080 fibrosarcoma cells decreased death induced by the antifolates methotrexate, trimetrexate and raltitrexed (Magro *et al.*, 2004). This was found to be due to decreased dihydrofolate reductase levels and increased thymidine salvage; ARF binds DP1 (DRTF1 polypeptide 1), a co-activator of E2F1, thereby reducing transcription of the E2F1 target, dihydrofolate reductase (Datta *et al.*, 2002; Datta *et al.*, 2005). ARF mediated inhibition and degradation of E2F1 may regulate the apoptotic program as E2F1 can cause apoptosis independent of p53 and E2F1 also represses the anti-apoptotic Mcl-1 (Croxton *et al.*, 2002; Elliott *et al.*, 2001)

1.4.2.1 ARF represses NF-κB via ATR

ARF has also been shown to repress the transcriptional activity of the nuclear factor kappa B (NF-κB) subunit RelA (p65) (Rocha *et al.*, 2003; Rocha *et al.*, 2005a). p14ARF expression induced threonine-505 phosphorylation of RelA via a mechanism

dependent on telangiectasia mutated and Rad -3 related protein (ATR) and checkpoint kinase 1 (Chk1), leading to repression of anti-apoptotic targets such as Bcl-x_L and, potentially, cFLIP, increasing sensitivity to TNF (Kreuz *et al.*, 2001; Rocha *et al.*, 2005a). It is proposed that ARF activates ATR, leading to the phosphorylation of Chk1. Chk1 phosphorylation induced by ARF expression is low compared to UV radiation induced Chk1 phosphorylation, but is independent of p53 and HDM2. As NF-κB has many pro-survival targets, p14ARF repression of RelA could provide a pro-apoptotic stimuli in many settings.

1.5 Scope of this thesis

The ARF tumour suppressor monitors abnormal proliferative signals and initiates defensive mechanisms that either halt cell division or trigger cell death. Accordingly, the loss of ARF strongly predisposes to spontaneous cancer development in humans and in mice. The precise mechanisms of ARF action are critical to understanding the genesis and control of melanoma as well as many other human cancers. In this work we have focussed on dissecting the impact of the human p14ARF protein on the survival and chemosensitivity of human cells.