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FUNCTIONAL CHARACTERIZATION OF THE ALTERNATIVE READING FRAME PROTEIN P14ARF

by

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Stockholm 2004

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-917-X

Till Mamma och Pia

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1 ABSTRACT

A deeper understanding of the molecular events underlying tumor development is a prerequisite for the design of novel and efficient therapies. Inactivation of the p53 and retinoblastoma (Rb) tumor suppressor pathways appears as a common theme in most malignant human tumors. Most intriguingly, both p53 and Rb proteins are in part regulated through the *CDKN2A* (*INK4a/ARF*) locus on human chromosome 9p21, a region frequently lost in tumors. This region encodes two structurally and functionally distinct tumor suppressor proteins known as ARF (human p14ARF, mouse p19ARF), and p16INK4a. Exons 1 alpha, 2 and 3 encode p16INK4a, while exon 1 beta, spliced to exon 2 in an alternative reading frame encodes ARF. p16INK4a induces cell cycle arrest by inhibiting CDK4/6 whereas expression of ARF induces cell cycle arrest in part through inhibition of MDM2, a negative regulator of p53. This thesis is focused on the expression, localization and function of human p14ARF.

We discovered that p14ARF was overexpressed and localized to nucleoli, in human tumor cell lines deficient for p53 function as described in **paper I**. p14ARF had a similar intracellular localization as the major nucleolar phosphoprotein B23 (also known as nucleophosmin or NPM) during interphase, mitosis and in response to RNA polymerase I transcriptional inhibition that causes nucleolar dysfunction. B23 was identified as a *bona fide* p14ARF associated protein (**paper II**). The results indicated that B23 could be involved in efficient nucleolar localization and possibly stability of p14ARF protein. In **paper III**, the status of the p14ARF-MDM2-p53 pathway was investigated in a panel of Burkitt´s lymphoma (BL) cell lines. Loss of p14ARF (and p16INK4a) occurred in wildtype (wt) p53 containing BL lines only, whereas other BLs with wt p53 contained abundant levels of MDM2. Thus, inactivation of the p53 pathway was frequent in BL cell lines, presumably as a mechanism to escape or attenuate Myc induced p53-dependent apoptosis. Next, we were interested in the role of p14ARF as a regulator of p53 activity in human fibroblasts after the activation of Myc or E2F-1 oncogenes (**paper IV**). Both Myc and E2F1 stabilized p53, along with phosphorylation on serine-15, induction of p21 and MDM2. Only E2F-1 markedly induced p14ARF. Both Myc and E2F-1 stabilized p53 in primary fibroblasts also after depletion of ARF. Caffeine blocked p53 accumulation after Myc or E2F-1 activation. Thus, the p53 response to activated oncogenes in normal human fibroblasts was not critically dependent on p14ARF. Interestingly, activation of Myc led to a strong accumulation of p16INK4a protein in primary human fibroblasts (**paper V**).

In summary, ARF is a nucleolar protein having some properties in common with previously characterized nucleolar proteins. But ARF is also a peculiar protein. Being both an inhibitor of MDM2 and of the ribosomal RNA processing machinery, ARF represents an interesting and almost unique link between p53 on one side, and the nucleolus on the other. The relative roles of ARF and p16INK4a in human tumor development remain enigmatic, but recent advances in the field indicate a predominant role of p16INK4a in protecting human cells from oncogenic transformation.

Keywords: p14ARF, p19ARF, alternative reading frame, CDKN2A, p53, B23, nucleophosmin, Burkitt´s lymphoma, nucleolus, senescence, Myc, E2F, phosphorylation, RNA interference, ribosome biogenesis

ISBN: 91-7349-917-X

2 LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals throughout the text:

I. **Lindstrom MS**, Klangby U, Inoue R, Pisa P, Wiman KG, Asker CE. Immunolocalization of human p14 (ARF) to the granular component of the interphase nucleolus. Exp. Cell Res. 2000, 256:400-410.

II. Russek N*, **Lindstrom MS***, Wiman KG, Oren M. The ARF tumor suppressor protein interacts with the nucleolar B23 protein. Submitted

III. **Lindstrom MS**, Klangby U, Wiman KG. p14ARF homozygous deletion or MDM2 overexpression in Burkitt lymphoma lines carrying wild type p53. Oncogene. 2001, 20:2171-2177.

IV. **Lindstrom MS**, Wiman KG. Myc and E2F1 induce p53 through p14ARF-independent mechanisms in human fibroblasts. Oncogene. 2003, 22:4993-5005.

V. **Lindstrom MS**, Wiman KG. Activation of Myc induces p16INK4a expression in human primary fibroblasts. Manuscript.

*Equal contribution

3 LIST OF OTHER RELATED PUBLICATIONS

1. Henriksson M, Selivanova G, **Lindstrom MS**, Wiman KG. Inactivation of Myc-induced p53-dependent apoptosis in human tumors. Apoptosis. 2001, 6: 133-137. Review.

2. **Lindstrom MS**, Wiman KG, Asker CE, "p14ARF/p19ARF", in Encyclopedia of Molecular Medicine, John Wiley & Sons, 2001

3. Jackson MW, **Lindstrom MS**, Berberich SJ. MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. J. Biol. Chem. 2001, 276:25336-25341.

4. **Lindstrom MS**, Wiman KG. Role of genetic and epigenetic changes in Burkitt lymphoma. Semin. Cancer Biol. 2002, 12:381-387. Review.

5. Hashemi J*, **Lindstrom MS***, Asker C, Platz A, Hansson J, Wiman KG. A melanoma-predisposing germline CDKN2A mutation with functional significance for both p16 and p14ARF. Cancer Lett. 2002, 180:211-221.

6. Wang JL, Zheng BY, Li XD, Angstrom T, **Lindstrom MS**, Wallin KL. Predictive significance of the alterations of p16INK4a, p14ARF, p53 and PCNA expression in the progression of cervical cancer. Clin. Cancer Res. 2004, 10:2407-2414.

7. Wang JL, Zheng BY, Li XD, Nokelainen K, Angstrom T, **Lindstrom MS**, Wallin KL. p16INK4A and p14ARF expression patterns in HPV related cervical neoplasia, submitted

8. **Lindstrom MS**. Modulation of p53 and MDM2 functions by ribosomal protein L5, manuscript

4 ABBREVIATIONS

aa	amino acid(s)
ARF	alternative reading frame
BL	Burkitt´s lymphoma
bp	base pair
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CpG	cytosine-guanine dinucleotides
DNA	deoxyribonucleic acid
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescent protein
GC	granular component
HPV	human papilloma virus
INK	inhibitor of cyclin dependent kinase
kb	kilobase
kD	kilodalton
LOH	loss of heterozygosity
M	mitosis
mAb	monoclonal antibody
MDM2	mouse double minute 2
MEFs	mouse embryo fibroblasts
mRNA	messenger ribonucleic acid
mt	mutant
MTS	multiple tumor suppressor
NES	nuclear export signal
NHL	non-Hodgkin lymphoma
NLS	nuclear localization signal
NoLS	nucleolar localization signal
PCR	polymerase chain reaction
PD	population doubling
Rb	retinoblastoma protein
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription (or real-time)-polymerase chain reaction
SA-β-gal	senescence associated beta galactosidase
siRNA	short (or small) interfering RNA
wt	wild type

5 INTRODUCTION

5.1 IS TUMORIGENESIS A MULTISTEP GENETIC AND EPIGENETIC DISEASE?

It is widely accepted that the evolution and establishment of a tumor (cancer) is a multistep process. Each step reflects progressive transformation from normal tissue to a highly malignant (aggressive) rapidly growing cell mass that is lethal to its host [1,2]. Hanahan and Weinberg delineated six traits necessary for tumor development. These were defined as self-sufficiency in growth signals, insensitivity to anti-growth signals, disruption of apoptosis (cell death) mechanisms, unlimited replicative potential, angiogenesis (formation of new blod vessels) and ability to metastasis and invade other tissues [2]. Green and Evan suggested that deregulated proliferation and reduced apoptosis creates a platform, necessary and sufficient for tumor development [1]. The wannabe tumor cell will acquire these capabilities through various genetic and epigenetic changes. Genetic alterations include point mutations, deletions, and also larger chromosomal alterations (e g translocations and inversions). The epigenetic inheritance can be divided into DNA methylation (hypo or hyper), genomic imprinting and histone modifications (e g methylation, acetylation and phosphorylation) [3]. Epigenetic changes will ultimately lead to differences in transcription of genes including oncogenes and tumor suppressor genes. Vogelstein and co-workers recently presented an interesting example of how histone modifications and DNA methylation act together in regulating p16INK4a (CDKN2A) gene transcription [4]. Weinberg´s group defined a minimal set of genetic changes required for transformation of normal human cells to “tumor cells” by introducing Simian Virus 40 (SV40) early region, oncogenic H-ras and telomerase activity (TERT) into primary human fibroblasts cultured *in vitro* [5]. One has to remember that all of the components TERT, SV40 early region (encoding both Large and Small T SV40 antigens) and H-Ras have multiple downstream effects, many of which are poorly defined, and more important, seem to differ between mouse and human. For instance, it is much easier to transform mouse cells than humans and in part, this has been attributed to the erosion of telomeres in primary human fibroblasts. However, Seger et al. found that human fibroblasts expressing E1A, MDM2 and Ras could form tumors in mice without the need for introducing telomerase activity [6]. Mouse tumor models indicate that activation of one oncogene (*Myc*) only could drive tumor development and that turning off the oncogene

could make the tumor regress [7-9]. Thus, even if tumorigenesis is a multistep process, the inactivation of *Myc* is sufficient to reverse tumor development.

5.2 THE CELL CYCLE

To duplicate, a cell needs to reproduce its DNA and separate the chromosomes so that a complete genome is inherited by each daughter cell. In order to accomplish this, the cell enters the cell cycle, artificially divided into four phases: DNA replication (S phase), chromosome separation or mitosis (M phase), which are separated by gap phases (G1, G2). By definition quiescent cells (or non-proliferating cells) are supposed to be in a G0 state. The cell cycle has several "checkpoints" such as the G1/S and the G2/M at which progression can be arrested if the monitoring safety systems detect errors in DNA replication, and for instance the Rb and p53 tumor suppressors are among the key players being active at these checkpoints [10]. Progression through the cell cycle is controlled by cyclins and associated cyclin dependent kinases (CDKs). Notable examples of cyclin-CDK complexes are the cyclin D/cdk4 and cyclin E/cdk2 complexes involved in G1 to S phase transition [11]. Although the assumed essential role of cyclin E in the normal cell cycle has been challenged [12]. CDKs, are negatively regulated by CDK inhibitors (CDKIs). The CDKIs can be classified as belonging to either the INK4 family consisting of p16INK4a, p15INK4b, p18INK4c and p19INK4d that only bind and inhibit CDK4 and CDK6 (D-type CDKs) or into the Cip/Kip family composed of p21Cip1/waf1, p27Kip1 and p57Kip2 (that apparently inhibit all types of CDKs), reviewed in [13,14]. Members of the INK4 family of CDKs are small proteins with quite high homology and species conservation, but with different functions as tumor suppressors or regulators of developmental processes [13,14].

5.3 RETINOBLASTOMA PROTEIN

The retinoblastoma gene (*RB*) is involved in the eye cancer disease known as retinoblastoma, and is the perfect prototype gene for the classical Knudson two-hit model of tumor development [15,16]. The *RB* gene product is a nuclear phosphoprotein sharing homology with two other Rb-like proteins p107 and p130, reviewed in [17]. Rb is a regulator of the G1 cell cycle checkpoint and it binds to members of the E2F transcription family that is needed for transcription of genes necessary for S-phase entry, reviewed in [18]. Several members of the E2F family have been cloned and characterized. The key member in the family is E2F-1, isolated by exploiting its direct association with Rb [19,20]. When Rb is hyperphosphorylated by the G1/S cyclin

dependent kinases it will release E2Fs which otherwise is bound to hypophosphorylated Rb and will thus allow for transcription of genes needed for cell cycle progression and replication of DNA, reviewed in [18,21].

5.4 P53 AND ITS ROLE IN CANCER

p53 was originally discovered in 1979 in many laboratories as a Simian virus (SV40) Large T associated protein, see e g [22-24]. p53 was first thought to be an oncogene, but 10 years later a team headed by Bert Vogelstein, then studying colon cancer, showed p53 to be a tumor suppressor, see e g ref [25]. p53 is mutated in about half of all the tumors detected and evaluated at the clinic, reviewed in [26]. p53 is considered a fundamental gatekeeper of the cell cycle and guardian of the cellular genome, reviewed in [27,28]. The p53 protein is activated in response to cellular stress. These stressors include oncogene activation (i e abnormal and sustained mitogenic stimuli and/or unrestricted cell cycling), hypoxia, heat shock, electroporation, transfection of DNA, various types of genotoxic chemicals that cause DNA damage, γ-irradiation, UV, ribonucleotide depletion, shear forces and nutrient deprivation, reviewed in [29]. Accumulation of nuclear p53 can also be achieved using the nuclear export inhibitor Leptomycin B (LMB) [30] and a wide range of proteasomal inhibitors, such as MG132. Rubbi and Milner put forward the hypothesis, and provided some compelling evidence, that p53 stabilization and accumulation is a response to disturbances in nucleolar function that occurs under most of the above mentioned stress conditions, except after exposure to LMB or proteasomal inhibitors. It is thus tempting to speculate that p53 stabilization and degradation is somehow linked to the nucleolus [31].

p53 is a nuclear phosphoprotein of 393 amino acids and with a short half life of about 15 minutes, see e g [32]. The regulation of p53 is controlled on several levels such as transcription, protein-protein interactions, post-translational regulation or subcellular localization [33]. Post-translational modifications include direct or indirect phosphorylation on p53 serine 15 and 20 residues by the ATM/ATR and Chk1/Chk2 DNA damage activated kinases [34]. Other major sites on p53 for phosphorylation are threonine 18 and serine residues 37, 46 and 392, reviewed in [35]. p53 acetylation occurs at lysine residues in the C-terminal part of p53 and is associated with an increased DNA binding activity [36]. The role of each of these modifications for p53 function remains unclear and it should be noted that these residues are not “hot spots”

for mutations, possibly indicating a certain degree of redundancy. Alternatively, modifications of these residues are less important for p53´s tumor suppressive activities and of course, differences would also be expected between species and cell types. The p53 protein is also both mono- and polyubiqutinated by MDM2 protein [37] with help from p300/CBP [38,39].

The power of p53 lies within its ability to act as a transcription factor capable of either activating or repressing gene transcription [40]. Specific co-factors and post-translational modifications of p53 will determine whether the p53 response will be directed towards apoptosis, growth arrest or involved in other processes such as angiogenesis, differentiation or senescence [41]. Many hundreds of genes are regulated by p53 [42] and potentially several thousands of genes have putative p53 binding sites [43]. Genes regulated by p53 can be broadly categorized into groups of genes involved in apoptosis (Scotin, Apaf-1, Bax, Fas, Puma, Noxa, Killer/Dr5, Igf-bp3, Perp), cell cycle arrest (B99, cyclin G, p21/waf1/cip1, 14-3-3-o, Gadd45), p53 autoregulation (Mdm2, p73, Pirh2) and angiogenesis/metastasis/invasion (Maspin, Tsp1) (see [41] and [44] and references therein). There are also other proteins that are more difficult to categorize, but that potentially could be involved in other cellular processes e g Wig-1 [45,46] and Mcg10 [47]. On the other hand, these proteins have also been attributed anti-growth or pro-apoptotic effects.

Two p53 relatives have been cloned and named p63 and p73. They share significant sequence homology and have some functions in common with p53, but p63 and p73 are more related to each other than to p53 although all three of them have high similarity in the central DNA binding domain, reviewed in [48]. Both p63 and p73 have the ability to transactivate approximately the same set of target genes and also induce cell cycle arrest and apoptosis [48]. The most important roles for p63 and p73 seem to lie in developmental processes. Mice lacking p63 have severe defects in limb and skin development [49,50] and loss of p73 causes neurological, pheromonal and inflammatory defects [51]. Both p63 and p73 have complex expression patterns and exist as multiple isoforms, reviewed in [52,53]. In addition, p63 and p73 expression differentially regulate p53, MDM2 and MDMX functions further complicated by the presence or absence of various isoforms of p63 and p73 [52,53]. With no doubt, p63 and in particular p73, should be regarded as important participants in the p53 network when considering key publications showing that p73 fulfills a role in response to

chemotherapy and that this function of p73 is antagonized by mutant p53 [54]. Even more surprising and important is the observation that this interaction is also affected by a common polymorphism in p53 at codon 72 [55]. Based on findings in mouse models both p63 and 73 are required for efficient p53 apoptosis and transactivation of certain target genes [56].

5.5 MDM2

A deeper insight into the structure and function of MDM2 is needed in order to understand the function of ARF (as will be discussed later). MDM2 (murine double minute 2) was cloned from a transformed murine cell line 3T3-DM (derivative of a BALB/c cell line) in which the MDM2 gene was amplified, and MDM2 was also found to cause transformation of NIH-3T3 or Rat-1 fibroblasts [57,58]. Human MDM2, also known as HDM2, was cloned in 1992 from the Caco-2 cell line [59]. Henceforth, MDM2 will be used for both MDM2 and HDM2. MDM2 is a multifunctional protein with E3 ubiquitin ligase activity towards p53 and many other substrates. MDM2 contains several distinct domains that confer binding to various cellular proteins (p53, E2F1, p73 and Rb), a zinc binding region and binding to RNA, reviewed in [60]. MDM2 also contains signals for nuclear and nucleolar localization (NLS, NoLS), nuclear export (NES) and the RING finger domain containing E3 activity [60]. MDM2 contains several sites for phosphorylation, auto-ubiqutination, sumoylation and acetylation, reviewed in [61].

Overexpression of MDM2 is frequent in human tumors by gene amplification, or enhanced translation and numerous splice variants have been found in human tumors with potential oncogenic properties, reviewed in [62]. In an outstanding seminal paper, MDM2 was found amplified in human sarcomas and this occurred most frequently in tumors carrying wt p53 [59]. Thus, MDM2 is considered oncogenic because it inactivates p53. Some tumors have both p53 and MDM2 alterations indicating p53 independent functions of MDM2. MDM2 can affect p53 in several ways, but the physiological relevance of each of these mechanisms in the control of p53 is debated. First, MDM2 binding to the N-terminal transactivation domain interferes with p53 induced gene expression [63]. Second, MDM2 promotes export of p53 to the nucleoplasm [64]. Third, MDM2 is a RING finger domain and targets p53 for polyubiquitination and 26S proteasomal degradation [65]. MDM2-mediated degradation of p53 occurs through the proteasome pathway mainly in the cytoplasm

[66,67]. The fact that MDM2 exerts an E3 ubiquitin ligase activity towards p53 has further implicated MDM2´s role in regulating p53 [68]. MDM2 and p53 are controlling each other in a fine tuned auto-regulatory system reviewed in [69] and that is a strong candidate for interventional cancer therapy, reviewed in [70]. How nuclear versus cytoplasmic degradation and ubiquitination of p53 and MDM2 occurs are intensely studied. Apparently, high levels of MDM2 cause p53 monoubiquitination whereas low levels enhance p53 export and polyubiquitination [37]. Presumably, many of the post-translational modifications seen in p53 and MDM2 ultimately aim at reducing the interaction between them. Small molecule inhibitors preventing binding of MDM2 to p53 [71], MDM2 E3 ligase inhibitors or molecules with ARF-like properties are under development [70]. Combined with mutant p53 reactivation strategies these approaches give some hope for future cancer therapy, reviewed in [72].

Despite its role as a growth promoting protein due to its control of p53, expression of MDM2 cDNA in some primary cells (as well as in some tumor cell lines) most often results in growth arrest. Subsequently growth inhibitory domains in MDM2 have been mapped and defined. Approximately 40 different splice variants have been found in human tumors (not all forms results in a protein it seems), reviewed in [73]. Whether these MDM2 variants are oncogenic or growth inhibitory appears to be context dependent. Dang et al. reported that expression of MDM2 in primary MEFs or IL-7 dependent pre-B cells accelerated their proliferation whereas expression of some isoforms lacking p53 binding resulted in growth inhibition [74]. On the other hand, Fridman et al. also investigated the ability of MDM2 splice variants in Eμ-myc transgenic mice and concluded that they were as oncogenic as wt MDM2 [75]. MDM2 can therefore promote both growth arrest and tumorigenesis dependent on cellular background. These studies also show that our understanding of MDM2 function is incomplete, and the relevance and implication of MDM2 “overexpression" is thus unclear from my point of view (see discussion about paper III).

MDM2 has p53 independent functions and interacting partners and exert E3 activity towards other proteins than p53. MDM2 has been shown to interact with proteins such as ARF, CBP/p300, Numb, Rb, E2F1, p73 and ribosomal proteins L5, L11 and L23. For a complete up to date list of MDM2 interacting proteins, see ref [60]. The MDM2 regulatory protein is subject to ubiquitination, sumoylation, phosphorylation and acetylation resulting in changes in localization, stability, protein interactions and

enzymatic activities, reviewed in [61]. Phosphorylation of MDM2 at certain amino acid residues changes the affinity of some commonly used monoclonal antibodies [76,77]. Notably, MDM2 undergoes ATM dependent phosphorylation on Ser 395 in response to DNA damage [77], cell cycle dependent phosphorylation by cyclin A/cdk2 on Thr 216 [76]. In addition, proteins such as c-abl, DNA-PK, Akt/PKB and CK2 can also modify MDM2 and hence its ability to bind p53, ARF, p300 and also affect MDM2´s ability to regulate p53 transactivation, degradation and nuclear export [61]. For a more comprehensive review about MDM2 posttranscriptional modifications, I recommend the article by Meek and Knippschild [61].

MDM2 has a structural homologue known as MDMX or MDM4 (human HDMX) [78] and the proteins can associate through their RING fingers [79]. MDMX is quite similar to MDM2 but there are some interesting differences. Both MDM2 and MDMX have p53 binding domains, putative zinc finger domains and RING fingers but MDMX seems to lack nuclear localization signal (NLS) and nuclear export signal (NES), reviewed in [80]. Some divergence between MDM2 and MDMX is also noted in the central acidic domain [81]. Furthermore, MDMX does not possess MDM2´s ability to ubiquitinate or degrade p53 [82] but can inhibit p53´s transcriptional activity at least *in vitro*. MDMX null mice die during the embryonic stage as is the case for MDM2 null mice [83], but this lethality can be rescued by crossing over with p53 null mice, indicating that both MDM2 and MDMX are critical regulators of p53 in vivo [84]. Expression of MDMX is not induced by DNA damage, rather downregulated, and it seems as if MDMX is regulated by MDM2 mediated ubiquitination [85]. Similar to MDM2, MDMX also exists as multiple splice variants and has been found overexpressed in human tumors with wt p53 [86]. Undoubtedly, mouse models demonstrate that both MDM2 and MDMX are essential regulators of p53 *in vivo* and cancer therapy targeting p53/MDM2 will therefore also probably interfere with the p53/MDMX complex.

5.6 INK4A/ARF

The *INK4a/ARF* (or *CDKN2A*) locus at 9p21 is commonly altered in human tumors by deletion mutations, missense mutations and hypermethylation of CpG islands in the two promoter regions upstream of exon 1α and exon 1β [11]. For instance, chromosome region 9p21 is often involved in chromosomal inversions, translocations, heterozygous deletions, and homozygous deletions in a variety of malignant cell lines

including glioma, nonsmall cell lung cancer, leukemia, and melanoma [87]. It is suggested that this locus is second or equivalent to p53 with regard to the number of mutations found in human tumors. Huot et al. claimed the figure of 75% [88]. The *INK4a/ARF* locus has a complicated structural organization (Fig. 1). The first and most important gene found to be located here was *p16INK4a* (also known as *p16, CDKN2, MTS1* or *CDKN2A*). p16INK4a has been established and verified as a *bona fide* tumor suppressor in humans and rodents [89,90].

The role of the *INK4a/ARF* locus in tumorigenesis is complicated by the fact that exon 2 of this locus is shared by another gene called *ARF* (alternative reading frame). The mRNA transcript was discovered simultaneously in several groups [91-94], but Dawn Quelle in Charles Sherr´s group made the first functional characterization of the protein and published this work in the journal "Cell" the same year [94]. ARF has a unique first exon 1β which is located upstream of p16INK4a exon 1α. Exon 1β is under the control of its own promoter, and splices onto the shared exon 2 but in the alternative reading frame (Fig. 1).

Alternative reading frames are extremely rare in eukaryotes. Some other examples are 4E-BP3 and MASK encoded by overlapping reading frames within the same cellular transcript [95] and the XLαs/ALEX loci [96] and a comment in [97].

The human ARF protein (henceforth denoted p14ARF) is shorter than mouse p19ARF. Both p19ARF and p14ARF are highly basic polypeptides with no similarity with any other known protein, and they share less than 50% identity [98]. The human and mouse *ARF* promoters are TATA-less CpG islands having similarity to the members in the housekeeping gene family [99] and [100]. In pancreas, a third alternative transcript encoding a putative 12 kDa protein named p12 has been discovered [101].

Nomenclature definitions

The nomenclature is difficult and is unfortunately not always consistent, also in the articles presented in this thesis. It differs depending on whether one is referring to the mouse or human variant, the gene (in italics) or the protein, and the person you write your article with. Finally, it also differs depending on whether one is regarding *ARF* and *p16INK4a* as one gene or two. In this thesis, I will use the nomenclature p14ARF (for human ARF), p19ARF when referring to mouse ARF and sometimes simply

ARF (or p19ARF/p14ARF) when speaking about things applicable to both. I prefer to avoid the term "ARF" since it is easily confused with abbreviations for acute renal failure (ARF), Alan R Fersht, or ADP-ribosylating factor (ARF) among others. One could also distinguish ARF (human) and Arf (mouse) Perfectly acceptable expressions are also $p19^{ARF}$, $p19^{Arf}$ or $p14^{ARF}$, but I avoid those mainly for technical reasons and simplicity. With regard to the entire locus, most of often I will use "*INK4a/ARF*" in particular when discussing mouse models but also *CDKN2A* will be used. With regard to p16INK4a protein, alternative expressions are $p16^{INK4a}$, $p16^{Ink4a}$ and sometimes INK4a (human) or Ink4a (mouse). I will mostly use p16INK4a for simplicity. Sometimes brackets are seen e g p16 (INK4a) but that often relates to technical issues because PubMed and OMIM don´t use superscript. When specifically referring to the gene it will thus look like: *p16INK4a, p16Ink4a,* $p16^{INK4a}$, $p16^{Ink4a}$, *INK4a and Ink4a.* Probably I got it all wrong anyway.

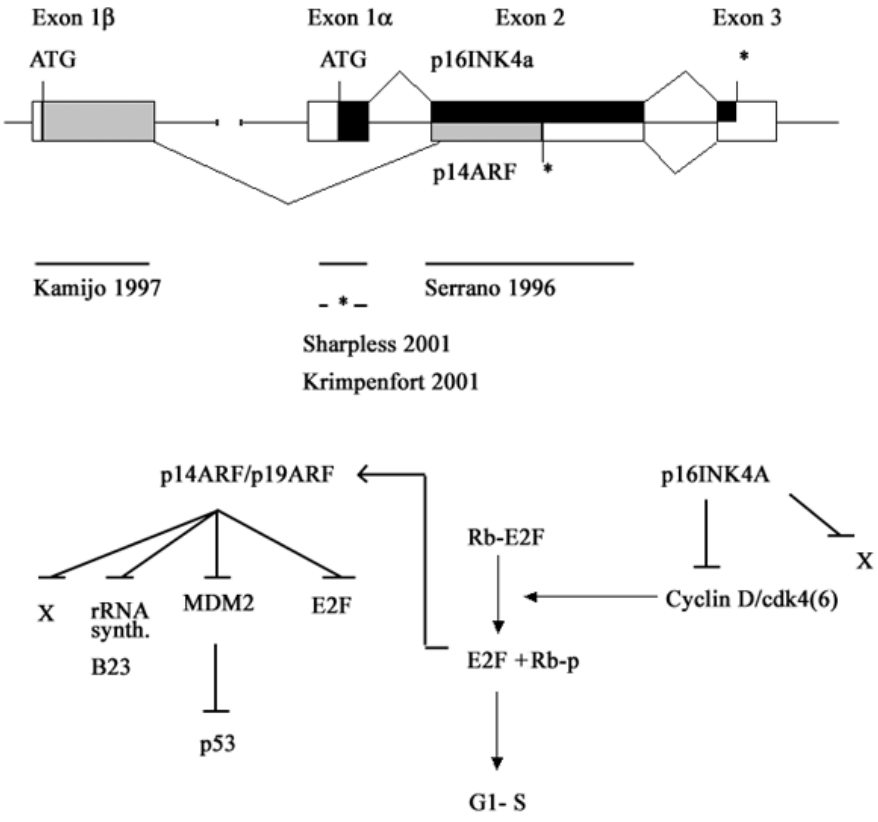

Fig 1. The INK4a/ARF locus. The upper panel illustrates a simplified view of the INK4a/ARF locus and its organization. Knock-outs/knock-in mice are indicated. In the lower part of the figure, pathways in which p14ARF/p19ARF and p16INK4a proteins are supposed to be active are indicated (* indicates stop codon and X denotes unknown targets).

5.6.1 P16INK4A structure and function

The p16INK4a protein inhibits phosphorylation of the retinoblastoma protein (Rb) by binding to cyclin D-cdk4/6 complexes [102]. As mentioned previously, function of p16INK4A is frequently lost in human (and murine) tumors by genetic and epigenetic mechanisms [103]. Mutations in this small, ankyrin repeat protein disrupt the native secondary and/or tertiary structure, leading to protein unfolding, aggregation and loss of function [104]. Increased expression of p16INK4a is observed in cells undergoing premature or replicative senescence *in vitro* [105,106]. Inappropriate cell culture conditions, oxidative stress and chromatin remodelling have been shown to induce premature senescence and expression of p16INK4a [107]. Accumulation of p16INK4a is also a direct consequence of overexpression or constitutive activation of oncogenes such as Ras [105] and E2F1 [108]. Introduction of DNA double strand breaks by cisplatin and topoisomerase inhibitors subsequently results in premature senescence and delayed accumulation of p16INK4a protein in human fibroblasts [109]. Studies also show that p16INK4a participates in a G1 cell cycle arrest following DNA damage in lung cancer cells whereas others have demonstrated induction p16INK4A in response to UV irradiation of *in vitro* cultured melanocytes [110].

5.6.2 P14ARF/P19ARF inhibits MDM2

p14ARF/p19ARF potently suppresses oncogenic transformation in primary cells and this function is abrogated when p53 is neutralized by viral oncoproteins and dominant negative mutants but not by the p53 antagonist MDM2 [111,112]. Stott (1998) specifically examined human p14ARF and stated that it has the ability to elicit a p53 response, manifest in the increased expression of MDM2, and resulting in a distinctive cell cycle arrest in both G1 and G2/M phases [98]. p14ARF/p19ARF physically and functionally interacts with MDM2 respectively in binary complexes and also in ternary complexes together with p53 [111,113,114]. It has also been reported that p19ARF and p53 forms binary complexes *in vitro* [114]. Both p19ARF and p14ARF inhibit MDM2´s function. p14ARF/p19ARF can inhibit MDM2´s E3 ubiquitine ligase activity [115], or by sequestration of MDM2 in nucleoli [116] and/or finally inhibition of MDM2 mediated p53 nuclear export [117,118]. Evidently, both p19ARF and p14ARF interferes with MDM2´s E3 ubiquitine ligase activity or by “trapping” or “sequestering” MDM2 in the nucleus ultimately preventing nuclear export and degradation of MDM2 and p53. More specifically, p14ARF interferes with MDM2-mediated polyubiquitination of p53 [119] but re-localization of MDM2 and

stabilization of p53 is not absolutely essential in the case of p19ARF to activate p53 [120]. It is likely that some discrepancies relate to differences in protein structure-function between p19ARF and p14ARF, level of expression, type of expression vector and cellular background. These models explain the raised levels of nuclear p53 but do not point out a universal model, due to difficulties in establishing physiologically relevant levels of p53, MDM2 and p14ARF/p19ARF [121].

5.6.3 What have we learnt from INK4A/ARF transgenic mice?

Four major publications describing different mouse models for loss of various *p16Ink4a/p19Arf* functions have been published [122-125] (see also Fig. 1). The main conclusion that can be drawn from these studies is that both *p19Arf* and *p16Ink4a* are *bona fide* tumor suppressors and that *p19Arf* is the most important one in mice at least in some settings. In some cell types, the two proteins collaborate and seem to be equally important. Kamijo et al., (1997) used a conventional targeting vector to ablate exon 1β in mouse embryonic stem cells [125] and expression of *p16Ink4a* was not abolished in these mice. These mice lacking *p19Arf*, but expressing functional *p16Ink4a*, developed tumors early in life with a phenotype resembling *p53-/-* mice, although the longer latency time in *p19Arf -/-* for tumor development made it possible for a broader spectrum of tumors to arise [125,126]. *p19Arf* -/- MEFs did not senescence and were transformed by oncogenic H-Ras alone [125]. In support, conversion of *p19Arf* +/+ or *p19arf* +/- mouse embryo fibroblasts (MEFs), to continuously proliferating cell lines involved loss of either *p19Arf* or *p53*. Further studies, with *p16Ink4a* specific knockouts indicated that also *p16Ink4a* is a *bona fide* tumor suppressor in mice [123,124]. Inactivation of *p16Ink4a* by promoter methylation seems to be an early event in carcinogen-induced tumorigenesis such as skin cancer, lung adenomas and induced mouse plasmacytomas [123]. For a more in depth analysis, I refer to those papers mentioned above.

5.6.4 P14ARF is (not only) a nucleolar protein

p14ARF is expressed at high levels and is predominantly nucleolar in p53 deficient tumor cell lines [98] or when activated by the E2F-1 transcription factor [118]. In addition, mouse p19ARF also accumulates in nucleoli of late passage MEFs and in response to oncogenic stimuli [116]. Interestingly, p14ARF is not only nucleolar. A significant fraction of the protein is also detected in the nucleoplasm [127] and sometimes it is predominantly nucleoplasmic without nucleolar localization [128].

Human cervical carcinomas express nucleolar p14ARF but sometimes strong nucleoplasmic staining is also detected [129,130]. Nucleoplasmic staining (mainly) has also been noted in aggressive B-cell lymphomas [131] and in tumors in the nervous system [132,133]. Thus, p14ARF protein is not only restricted to the nucleolus but show a much more dynamic localization. It is unclear how much of this relates to fixation techniques and sample handling/ preparation or simple diffusion of p14ARF. Sometimes, p14ARF leaves the nucleolus and accumulate in dense nuclear inclusions of various sizes but in some settings, these are highly regular that they could actually represent true functional nuclear bodies (although distinct from the PML nuclear bodies) [118,134,135]. Later on, these bodies attract the PML bodies though, and also accumulate Hsp 70 and other proteins such as p53 and MDM2 [135]. This pattern of p14ARF localization (see also fig. 2) i e nucleolus, nuclear bodies/foci, and diffuse nucleoplasmic/cytoplasmic is reminiscent of the patterns that have been described for the nucleolar Werner protein [136], and other nucleolar proteins. Intranucleolar inclusions have also been observed (M Lindstrom, unpublished results).

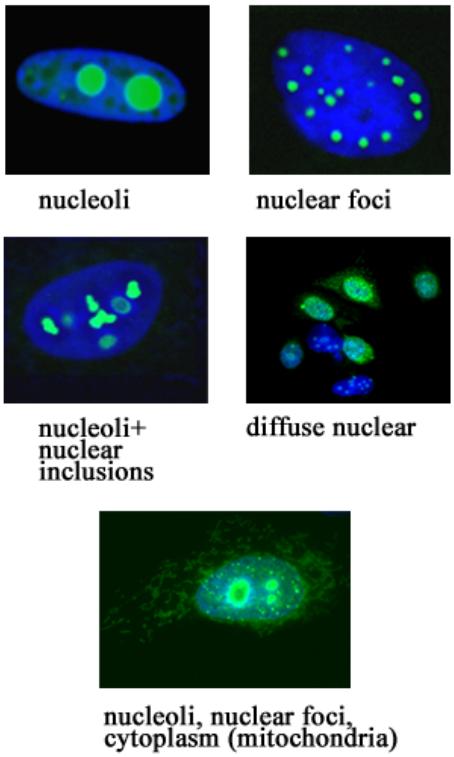

Fig 2. Localization of p14ARF. In this figure, I put together a few images of p14ARF gathered over the years. p14ARF can localize both to nucleoli (upper left, middle left and lower images), nuclear foci (upper right and lower images), non-nucleolar large inclusions (middle left image) in addition to having a more diffuse nucleoplasmic and cytoplasmic staining (middle right, lower images).

5.6.5 Hunting for functional domains

ARF has been cloned and sequenced from several species such as mice (*Mus Musculus*) [94], humans (*Homo Sapiens*) [92], rat (*Rattus Norvegicus*) [137], opossum, (*Monodelphis Domestica*) [138] and chicken (*Gallus Gallus*) [139]. Sequences are also available in public DNA sequence databases for a number of other species including monkeys (*Gorilla Gorilla*), pig (*Suf Scrofa)* and the Syrian (golden) hamster (*Mesocricetus auratus*) but ARF is not found in fish (*Fugu Rupries*) [140]. A partial sequence alignment of ARF from a subset of species with known ARF is presented in fig. 2.

Fig 3. Partial sequence alignment of ARF from different species. Please note that exon 1β sequences above are prematurely terminated in the alignment for technical reasons and that the very N-terminal part of monkey ARF was not available. Sequence alignment was done using the Clustal W (1.82) multiple sequence alignment method.

Secondary structure predictions for ARF proteins suggest that there is a common fold in the beginning of exon 1β, in the form of a β-sheet structure (M Lindstrom, unpublished data and ref [141]). So far, p14ARF has not been shown to undergo post-translational modifications *in vivo* such as phosphorylation or arginine methylation. However, p14ARF has several cysteine residues and p14ARF can homo-oligomerize and mutations of these cysteine residues impair oligomerization with implications for functional activity [142]. It is notable, that p14ARF lacks lysine residues that can undergo ubiquitination for protein degradation. However, ARF proteins from all species contain numerous putative phosphorylation sites. Protein phosphorylation is a common way to regulate nucleolar protein function. p14ARF has three sites for protein kinase C phosphorylation (PKC), where the first is in exon 1β and conserved between species (amino acid 8-10). p14ARF also contains sites for casein kinase II (CKII). It is reasonable to suggest that p14ARF/p19ARF is subject to phosphorylation/

dephosphorylation *in vivo*. The role of putative arginine methylation in p14ARF/p19ARF remains to be investigated.

Initial experiments indicated that the functional domain in p19ARF as well as p14ARF was encoded by exon 1β [94,98,143]. This has now been well established in many other studies [127,134]. An initial controversy, and to some extent still ongoing, was dealing with the role of a nucleolar localization signal/sequence (NoLS) in p14ARF exon 2. In fact, point mutations or small deletions in this region impair p14ARF´s nucleolar localization and ability to inhibit p53´s nuclear export [118]. A similar functional domain is not found in p19ARF exon 2 but is located in exon1β (residues 27-34) [144]. This initial study by Weber et al. also showed that p19ARF interacts with MDM2 residue 210-304 through residues 1-14 and 26-37 [145]. The motif 26-37 is required for nucleolar localization [145]. Korkgaonkar et al., 2003 mapped residues 6-10 and 21-25 in p19ARF and found them to be required for cell cycle arrest [120]. Residues 82-101 in human p14ARF contributed to nucleolar localization but made little contribution to the biological effect of p14ARF [145]. Midgley et al. used a 20 amino acid peptide ("Peptide 3") derived from the N-terminal beginning of p14ARF fused with GFP [146]. Peptide 3 had a prominent biological effect as indicated by stabilization and activation of p53 as well as block of in vitro ubiquitination of p53 mediated by MDM2. Similarly, Lohrum et al. used a 22 amino acid p14ARF peptide and draw the same conclusions [147]. But Lohrum et al. also noted that residues 65-132 in p14ARF weakly contributed to MDM2 binding besides having nucleolar localization, albeight less than the wt protein.

Clark et al. showed that p14ARF makes multiple contacts with MDM2 and that in general it is difficult to pinpoint one single motif. Perhaps most important was that they noted that motifs beyond amino acid 20 contribute to MDM2 binding and p53 stabilization (that is residues 46-132) [148]. Residues 51-132 of p14ARF had very little or no biological effect in terms of a p53 response. These findings could explain why deletions rather than point mutations are seen in ARF as suggested in ref. [148]. A peptide containing the first 37 amino acids of p19ARF was used for structural studies [149]. This peptide had the ability to interact with MDM2 and to cause cell cycle arrest. Under aqeous conditions, this peptide was unstructured but in TFE (2,2,2-trifluoroethanol), this peptide adopted into two alpha helices (residue 4-14 and 20-29). Each motif contained the p19ARF motif RxFLVxxVR and when taken into

account the sequence of p14ARF, RxFxVxxxR. The identification of this putative "ARF motif" could serve as a structural starting point in the development of small molecules with similar conformation and function. Results obtained with TFE should be interpreted with caution though, as indicated by the authors [149]. The NoLS in p19ARF residues 31-34 was consistently unstructured in both water and TFE. In a follow up paper from the same group, the structure of p19ARF N37 in complex with MDM2 was studied [141]. In contrast to the observed α-helices observed in TFE, p19ARF N37 and MDM2 formed β-sheet structures that extended into supramolecular assemblies (networks) *in vitro*. The authors also mapped the p19ARF interacting domains in MDM2 and found it to be mediated by amino acid residues 235-264 and 270-289 [141]. An interesting twist in this field was the characterization of chicken ARF. Well, it turned out to be no alternative reading frame in chickens and surprisingly chickens does not have p16INK4a either [139]). Chicken ARF is only 60 amino acids (22 arginines) but can still stabilize p53 and bind MDM2, but not as efficient as p14ARF [139]. The common theme in all ARF proteins is a high arginine content rather than perfect sequence conservation!

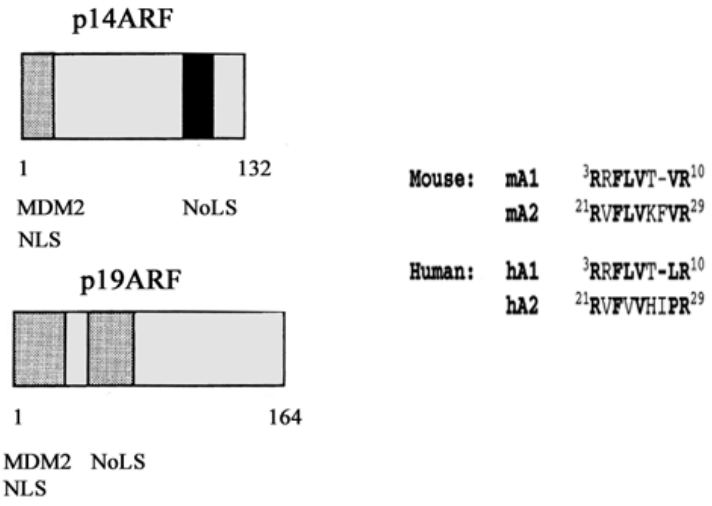

Fig 4. Simplified p14ARF/p19ARF domain map. To the left, the structure of (h) p14ARF and (m) p19ARF is shown in which motifs considered essential for MDM2 inhibition and nuclear/nucleolar localization (NLS/NoLS) is indicated. To the right the "ARF motif" is illustrated. Adapted from ref [141,145].

In conclusion, the N-terminal part of all ARF species appears to contain a structural important motif. ARF is highly arginine rich and this seems to be conserved but the exact localization of these "R-motifs" could be less important as long as the charge remains constant.

5.6.6 P14ARF/P19ARF and p16INK4A proteins in melanoma

Much of what we have learnt about ARF structure and function stem from studies of melanoma. Melanoma, or malignant skin cancer, is cancer of the melanocyte and is usually spontaneous. Melanomas frequently harbors Ras mutations and wt p53 but are still notoriously resistant to chemotherapy [150], in part this could be explained by frequent loss of downstream proteins involved in apoptosis execution such as Apaf-1 and caspases [151,152]. Some melanomas are of an inherited familiar type [153] and most of the germline mutations in these families target *p16INK4a* but not *p14ARF* [153]. For instance, there are many mutations in exon 1α that have been linked to predisposition for hereditary melanoma [153]. It was concluded that point mutations in *p16INK4a* did not affect ARF [143], but that study was performed using mouse *p19ARF* as a template for mutagenesis and there is some sequence divergence in this region when comparing p19ARF and p14ARF as discussed earlier, *but in general that conclusion holds true*. A quite common situation is to have loss of one allele whereas the other suffers from small deletions or point mutations [153]. Studies in mouse models of melanoma indicate that both p19ARF and p16Ink4a act as tumor suppressors in mouse melanomagenesis [89,124,154]. In contrast, studies of human melanocyte senescence *in vitro* indicate a crucial role for p16INK4a [155], but the p53 pathway (and ARF) could play a role in p16INK4a deficient melanocytes during telomere crisis and perhaps during unrestricted cell growth after crisis [155].

However, a series of publication have described germline mutations that specifically affect p14ARF but (presumably) not p16INK4a functions. For instance, a 16 bp insertion in exon1β was shown to severely impair p14ARF´s function and localization [156]. It should also be mentioned that a subset of metastatic melanoma cell lines of human origin was found to have exclusive loss of *p14ARF* but not *p16INK4a* [157]. Germline deletion of *p14ARF*, but not the *p16INK4a* coding sequences, was shown to occur in a melanoma-neural system tumor syndrome family [158]. However, loss of upstream regulatory elements that might have affected expression of p16INK4a in tissues could have occurred in that case [158]. A splice mutation in exon 1β that resulted in p14ARF haploinsufficiency has also been described [159]. Other germline splicing mutations affecting both *p14ARF* and *p16INK4a* have been described in detail elsewhere [160-162]. Majority of point mutations in exon 2 do not affect p14ARF, however a subset of these mutations impair the p53 activating function of p14ARF and its localization to the nucleolus as was shown for the R98Q mutation [118,163]. In

general, these mutations only partially impair p14ARF [118,163]. Nevertheless, *in vitro* assays indicate that the nucleolar localization motif in exon 2 of human p14ARF is necessary for efficient MDM2 sumoylation [164] besides its ability to confer nucleolar localization [118]. Since ARF also interferes with ribosomal rRNA processing [165] and functionally interacts with the important nucleolar protein nucleophosmin/B23 [166], ARF is likely to play a prominent role in the nucleolus. ARF mutants that are localized to the nucleoplasm and the cytoplasm could be less active than the wt protein, and in addition one must take into account the fact that p14ARF appears more stable in the nucleolus than non-nucleolar p14ARF mutants [127]. Hashemi et al. reported a 24 bp deletion in exon 2 in a patient with hereditary melanoma [167]. The cellular distribution and function of the resulting p14ARFΔ77-84 and p16Δ62-69 mutant proteins were studied in our laboratory. Interestingly, p14ARFΔ77-84 had decreased nucleolar localization and stabilized p53 less effectively than wt p14ARF [167].

Evidence favoring p14ARF as a melanoma tumor suppressor in humans is mostly indirect and not especially convincing, and the use of human fibroblasts with "genetically defined" alterations in exon 2 implies and verifies the prominent role of p16INK4a as a tumor suppressor in melanoma development [88,168,169]. In conclusion, loss of p16INK4a is a key to development of hereditary melanoma at least in humans. Loss of p14ARF functions could play a role in enhancing growth, proliferation and might increase the likelihood for escape from p53-induced apoptosis but on basis of the accumulated evidence and from the strictest point of view, it is difficult to say that p14ARF is a *bona fide* melanoma tumor suppressor in humans.

5.6.7 Discovery of novel ARF interacting proteins

Many novel p19ARF/p14ARF interacting proteins have been identified using yeast-two hybrid screen, GST pull down assays or immunoprecipitation that I have summarized in table 1. This list now includes MDM2 [111], MDMX [170], HIF1α subunit [171], E2F-1 [172-174], DP1 [175], topoisomerase I [176], p120E4F [177], CARF [178], Pex19p [179], spinophilin/neurabin II [180], Epstein-Barr virus encoded EBNA5 protein [181], tat binding protein [182] and B23/ nucleophosmin [183]. In general we don't know much more about the physiological relevance of many these interactions mentioned above *in vivo* and it is likely that some of these interactions results from strong electrostatic forces imposed by the highly arginine rich ARF proteins, and in some cases could be mediated by RNA structures. Bertwistle recently described the

interaction of p19ARF with numerous ribosomal and nucleolar proteins strongly indicating the association of the bulk of p19ARF (and p14ARF) protein as to be associated with pre ribosomal particles [183] or other types of RNP complexes within the nucleolus and possibly elsewhere in the cell.

Table 1. p14ARF/p19ARF interacting proteins

MDM2	yeast two hybrid/ IP	[111,113]
Cyclin G	GST pull down	[184]
E2F1	GST pull down/IP	[172-174]
Spinophilin	yeast two hybrid screen (brain cDNA library)	[180]
Pex19p	yeast two hybrid screen (testis cDNA library)	[179]
Topoisomerase I	GST pull down/IP	[176]
CARF	yeast two hybrid screen (testis cDNA library)	[178]
P120E4F	yeast two hybrid screen (fetal brain cDNA library)	[177]
HIF1a	GST pull down/IP	[171]
B23/NPM	IP	[166,183]
TBP1	yeast two hybrid	[182]
MDMX	GST pull down/IP	[170]
Ribosomal proteins	IP	[183]
EBNA-5	yeast two hybrid	[181]
DP1	IP	[175]

5.6.8 p53-independent functions of ARF

That p14ARF/p19ARF stabilize and activate p53 resulting in cell cycle arrest [113], and that MEFs derived from p19ARF null mice usually lack p53 mutation [125] taken together with the finding that p14ARF/p19ARF expression is increased in p53 deficient cells strongly suggest that p14ARF/p19ARF is an upstream regulator of p53. But, several lines of evidence indicate that p14ARF/p19ARF also has profound p53 independent activities. p19ARF can suppress cell growth without p53 as evidenced by the broader tumor spectrum seen in triple *p19ARF-MDM2-p53* knockout mice in comparison with *MDM2-p53* null mice [144]. Mouse B-cells derived from the Eμ-*Myc* lymphoma model lacking both p19ARF and p53 functions are more resistant to Myc induced apoptosis in addition to having a higher proliferation than cells lacking p19ARF or p53 [185]. Also, p19ARF could promote cellular senescence and suppress immortalization independent of p53 [186] and

finally p19ARF arrests MEFs lacking both MDM2 and p53 [144].

Early observations indicated that p14ARF (at that time called p16beta) could significantly inhibit cell growth *in vitro* [187] and also suppress growth of HeLa cells that are deficient in p53 function due to expression of viral proteins [188]. It has later been shown that adenovirus-p14ARF induces apoptosis in both p53 wt (U2OS) and p53 deficient Saos-2 cells and also independent of Bax [189]. Similar findings were reported elsewhere [190]. In support, an adenovirus encoding only the exon 1β domain of p14ARF suppressed cell growth by killing and arresting cells in G1 and G2 phases of the cell cycle independently of p53 and Rb status and also independently of p21, Bax and Mdm2 induction [191]. Modestou et al. noted that p19ARF triggered cell growth inhibition independent of p21 status [192], further underscored by the ability of oncogenic Ras to induce p19ARF, causing growth arrest independently of p21 [193]. It was also suggested that p19ARF is a more efficient trigger of apoptosis than human p14ARF in part due to sequence differences in the C-terminus, but this finding requires further substantiation [194]. Using p14ARF adenovirus it was also shown that p14ARF inhibits growth through a delay in S phase progression and this effect was independent of p53 [195]. The same authors also observed co-localization of p14ARF and replication protein A (RPA) in discrete nucleoplasmic bodies [195]. Eymin et al. showed that p14ARF induced G2 cell cycle arrest and apoptosis independently of p53 and could inhibit growth of *p53* null tumors in nude mice thus confirming results obtained in various cell lines [196]. One must recall that most studies rely on high expression of p14ARF that seldom is observed under physiological conditions and that expressing a protein with such characteristics as p14ARF/p19ARF (hydrophobic, arginine rich) could have profound side effects. In contrast to the studies by Modestou, Eymin and others Weber et al. draw the conclusion based on the HCT116 p53/p21+/+ and p53 -/- cells that cell cycle arrest by human p14ARF strictly depended on p53, p21 and to some extent 14-3-3sigma [197]. As a novel p53-independent function of ARF, it was shown that independently of MDM and p53, ARF represses the transcriptional activation domain of the NF-kappa B family member RelA by inducing its association with the histone deacetylase, HDAC1. Furthermore, the authors demonstrated that the response of NF-kappa B to the oncogene BCR-ABL was determined by the ARF status of the cell [198].

Kuo et al. used microarray studies to identify genes that were regulated by p19ARF independently of p53 and the list includes members of the B-cell translocation gene family (Btg 1, 2, 3 and Tob1) and these could inhibit growth of MEFs with or without p53 [199]. p19ARF and Btg2 could collaborate in limiting ribosome synthesis since Btg2 binds PRMT1 (an arginine methyltransferase) and stimulates its enzymatic activities [199]. Fibrillarin, a protein necessary for rRNA processing, is a target for PRMT1 and arginine methylation of fibrillarin reduces binding of fibrillarin to rRNA. Sherr´s group went on to demonstrate that p19ARF inhibits rRNA processing [165]. It seems as if p19ARF (and likely also p14ARF) expression inhibits production of ribosomal RNA retarding the processing of 47/45S and 32S species, and these effects do not require p53 or MDM2 and rRNA transcription was unaffected [165]. Sugimoto et al. also found that p19ARF binds the 5.8S rRNA species. p53 has also been found covalently linked to 5.8S [200].

An interesting and unexpected p53 independent role for p19ARF was found when studying p19ARF null mice. p19ARF is required for the regression of the hyaloid vascular system in mouse eye [201]. Mice lacking p19ARF have eye abnormalities similar to a condition in humans known as persistent hyperplastic primary vitreous. Thus, abnormalities in human p14ARF could be one cause for this condition and indicates a role for p14ARF/p19ARF in promoting vascular regression [201].

5.7 SOME WORDS ABOUT RIBOSOMAL PROTEINS AND MDM2

Ribosomal proteins have essential roles together with rRNA in forming the small and large ribosomal subunits or other types of accessory ribonucleoprotein complexes (RNPs) [202]. Assembly of pre-ribosomal particles occurs in the nucleolus as a consequence of the initiation of rDNA transcription and subsequent synthesis and processing of rRNA [202]. Ribosomal proteins can obviously be detected in the nucleolus but also in other cellular compartments such as the cytosol [203]. Interestingly, some studies indicate extra-ribosomal functions for some of these proteins [204]. A subset of ribosomal proteins (S29, L13, L7) can induce cell cycle arrest and cell death (apoptosis) when overexpressed in cells [204-206]. For instance, ribosomal protein L7 arrests cells in G1 and induce apoptosis in Jurkat T cells [206]. It was suggested that ribosomal proteins could control multiple aspects of uncontrolled cell growth and proliferation [207]. Not surprisingly, enhanced cellular growth is associated with increased expression of ribosomal proteins. Activation of the Myc

transcription factors results in up-regulation of many proteins involved in rRNA processing, ribosome biogenesis and protein translation [207].

Ribosomal protein L5 binds to the 5S RNA species and could also form ternary complexes with the 5.8S RNA [208]. Notably, both p53 and p19ARF can also bind 5.8S RNA. Ribosomal protein L5 contains a defined domain for binding 5S RNA and NLS, NoLS and NES [203,209]. Previously, ribosomal protein L5 had been found in a RNP complex with MDM2 or MDM2-p53 together with rRNA [210]. The nature and physiological role of this complex remains unknown. Interestingly, ribosomal protein L11 binds MDM2 and in a manner similar to p14ARF/p19ARF, activates a p53 response as evidenced by G1 cell cycle arrest and prevention of MDM2 mediated p53 degradation [211]. Thus, p14ARF/p19ARF and L11 all bind to the central domain of MDM2 and appear to antagonize MDM2´s activity and p53´s nuclear export. Interaction between L11 and MDM2 is enhanced by low doses of Actinomycin D that triggers nucleolar dysfunction due to inhibition or RNA pol I [211]. This might increase the interaction between nucleoplasmic MDM2 and L11 released from nucleoli. One must bear in mind that many nucleolar/ribosomal proteins have marked charge distributions (highly acidic or basic regions) that when overexpressed could cause disturbances in vital cellular processes including nucleolar function. Expression of p14ARF/p19ARF is suppressed in cells with wt p53 by as yet undefined mechanisms, while its expression is elevated in mutant p53 containing tumors [98]. With this in mind an interesting analogy emerges because the expressions of certain ribosomal proteins are elevated in presence of mutant p53 [212]. It has also been reported that wt p53 indirectly can repress ribosomal gene transcription by Pol I [213]. Finally, another issue that needs to be addressed here concerns the role of L5 in nuclear export of proteins and RNA. For instance, L5 participates in control of Rev nucleocytoplasmic shuttling and p53 could use the same route [214-216]. The role of L5 in MDM2-p53 localization and activity (if any) remains to be resolved as well as the critical questions why, when and how ribosomal proteins interact so specifically with MDM2.

5.8 SENESCENCE

Most normal cells such as dermal fibroblasts undergo replicative senescence when cultured and passaged *in vitro*. They loose their proliferative potential after a finite number of doublings [217,218]. Senescent arrest resembles terminal differentiation and appears to involve repression of proliferation-promoting genes [219]. Senescence

is accompanied by several other changes including increase of CDKIs, changes in methylation pattern and telomere shortening. By definition the criteria of senescence is based on morphological criteria (flattened and extended cell growth, vacuolization), arrest of proliferation (but not cell death) and finally a positive staining by senescence-associated β-gal [220]. Multiple pathways regulating the proliferative lifespan of human cells and human cells show a more complex and multifactorial regulation of the senescence process than murine cells [106,221]. Expression of telomerase needed for telomere maintenance also extend the lifespan of human fibroblasts *in vitro*. The shortening of the telomeres may lead to activation of p53 and other growth inhibitory pathways such as the pathway involving p16INK4a and Rb, reviewed in [222]. In most human cells both the p53-p21 pathway and the p16INK4a-Rb pathway are activated, and will act in synergy to cause cell cycle arrest by preventing phosphorylation of Rb [223]. Both p19ARF and p16INK4a proteins are strongly implicated in senescence and immortalization in both humans and mice but in a cell a species dependent manner [106,122,125,224,225].

5.9 ONCOGENIC SIGNALING THROUGH P14ARF AND P16INK4A

Several oncogenes can stabilize and activate p53, eventually leading to apoptosis or cell cycle arrest or premature senescence, reviewed in [226]. For instance, Gerard Evan and co-workers demonstrated that overexpression of Myc induces apoptosis in rat fibroblasts [227]. Later on Hermeking and Eick (1994) and also Wagner et al. (1994) provided evidence that p53 is likely to be a key mediator in the Myc apoptotic response [228,229]. Debbas and White demonstrated one year earlier that p53 mediates apoptosis in response to adenoviral protein E1A [230,231]. Findings by Symmonds et al. provided clues that p53-dependent apoptosis suppresses tumor growth *in vivo* using an SV40 mouse model [232]. In addition, p53 is involved in E2F1 induced apoptosis and E2F1 and p53 co-operate to induce apoptosis whereas p53 itself mainly induces growth arrest in G1 [233,234]. Thus, from these and other studies it was established that cellular (c-myc, E2F1 and ras) and viral oncogenes (eg SV40 Large T antigen, *v*-abl, adenoviral product E1A) when overexpressed resulted in p53 accumulation and thus p53 could play an important role in eliminating these cells.

Oncoproteins such as E2F1 [235], Ras [236], Myc [237], E1A [238], v-abl [239], β-catenin [240] and Raf [241] stabilize and activate p53 at least in part by up-regulation

of p19ARF in primary mouse embryo fibroblasts (MEFs). Also, the CD43 antigen activates the p19ARF-p53 pathway in MEFs [242] (see also Table 2).

Overexpression of p19ARF in the absence of oncogenic stimuli usually results in p53 dependent cell cycle arrest in G1 and G2, but after oncogene activation (e g c-Myc, E2F1) apoptosis is seen. This indicates that additional signals are needed in order to generate apoptosis. In addition, oncoproteins such as Myc, E2F1 and E1A signal both p53 dependent and p53 independent apoptosis. The discovery of ARF as a putative link to p53 provides a possible explanation for the p53-dependent apoptosis observed in the developing mouse lens in RB null mice [111]. ARF is also a putative link to p53 that could explain the induction of apoptosis in fibroblasts by c-myc [228,237] as well as the apoptotic effects by E1A and E2F-1 observed in other experimental systems.

5.9.1 E2F1

E2F-1 was isolated by exploiting its direct association with Rb [19], this is also the most interesting member of the entire E2F family since it has, in addition to its cell cycle promoting effect, a potent apoptotic function [243]. Studies of *E2F-1* -/- mice indicate the possibility of E2F-1 as tumor suppressor despite its role in triggering cell cycle progression because these mice develop tumors [244]. E2F-1 can induce both apoptosis and S-phase transition and whether E2F-1 is acting as an oncogene or tumor suppressor gene will depend on the extent to which E2F-1 induces apoptosis or G1/S transition, reviewed in [245]. Intriguingly, E2F-1 is stabilized in response to DNA damage by the ATM and Chk2 kinases in a manner analogous to p53 [246-248]. In addition to its interaction with pRb, E2F-1 also interacts with MDM2 [249]. Overexpression of E2F1 in wt MEFs induces cell cycle arrest, apoptosis and senescence see e g [234]. E2F1 induced apoptosis is increased by high levels of exogenous p53 and can be inhibited by overexpression of mutant p53 [233]. Like c-Myc, enforced expression of E2F1 results in cell cycle arrest and p53-dependent and p53-independent apoptosis, in part by inducing pro-apoptotic genes and thereby sensitising cells for apoptosis. E2F1 also activates the p53 homologue p73 by binding to the promotor [250-252]. Another E2F1 target is Apaf-1 (apoptosis protease-activating factor) that is also activated by p53 [253]. Interestingly, E2F1 induces caspases 3, 7, 8 and 9 by direct transcriptional activation [254].

E2F "DNA binding activity" consists of a heterodimer between E2F and DP proteins [255]. Dimerization of E2F and DP increases the transcriptional activation mediated by E2F [255]. DP1 in itself does not however affect cell cycle or apoptosis, indicative that it is the enhancement of E2F DNA binding that plays a critical role. A functional interaction between DP-1 and p53 has been observed and *in vitro*, p53 and E2F competes for DP1 binding [256].

Both the *p14ARF* and the *p19ARF* promoters contain putative binding sites for E2F-1 [99,235,257] and [100]. It is therefore tempting to conclude that oncogene activation of *p14ARF/p19ARF* is mediated by E2F-1 (although E2F-1 induces rather high levels of p53 and MDM2 in *p19ARF*-/- MEFs as well). Studies in other model systems have demonstrated that mouse p19ARF is not always required for activation of p53 upon E2F1 activation. A further level of complexity originates from the observation that p14ARF/p19ARF interacts with E2F1-3 on the protein level and inhibits the functions of E2F [172-174]. p19ARF also interacts with DP1 and E2F/DP1 complexes but differentially regulates the localization and activity of free and heterodimeric E2F1 and DP1 [175]. Thus, in addition to the ARF-MDM2-p53 regulatory system ARF also participates in a feedback loop system with E2F1 and E2F1/DP. In a further complication, Rb/E2F complexes have been proposed as downstream targets of ARF-p53 anti-proliferative signalling [258].

5.9.2 Myc

Many human tumors carry activated versions of cellular proto-oncogenes. One example of an oncogene frequently involved in human tumors is Myc (also c-Myc or MYC). This gene can drive tumor growth when overexpressed due to chromosomal translocation, gene amplification, or other mechanisms [259]. The Myc oncoprotein belongs to the helix-loop helix leucine zipper transcription factor family. Myc is involved in multiple cellular functions such as cell cycle control, DNA replication, apoptosis, metabolism and cell growth, differentiation and adhesion [260]. By orchestrating a wide range of target genes containing so called E-box motifs Myc, together with its interacting partner Max, will affect all these biological processes in a species, cell and tissue specific manner [261]. Notable genes that are influenced either directly or indirectly by Myc include those involved in cell cycle control (Cdk4, cyclins, CDC25A) [262]. Myc also suppresses expression and activity of negative cell cycle regulators such as p15INK4b [263], p21 [264] and p27 [265]. Myc facilitates

immortalization through up-regulation of the catalytic subunit of telomerase (hTERT) [266] while antagonizing cellular senescence by inducing Werner protein [267]. With regard to apoptosis, Myc expression sensitizes cells to apoptosis by inducing (either directly or indirectly) expression of apoptosis promoting genes such as p19ARF [237] and p53 [228].

Of note is the spectacular ability of Myc to enhance cellular growth [268] through simultaneous induction of proteins needed for ribosome biosynthesis [269] [270] and constituents of the ribosome itself [260,271]. Outstanding examples include nucleolar proteins B23/ nucleophosmin, C23/nucleolin and fibrillarin that all are essential keyplayers in nucleolar function [260,271]. Myc also has a general positive influence on protein translation through induction of tRNA and 5S rRNA species [272]. Current research is focused on Myc as a general modifier of chromatin, reviewed in [273]. The N-terminal domain of c-Myc interacts with TRRAP, a protein that recruits the GCN5 histone acetyltransferase [274] and TRRAP binding is required for c-Myc's oncogenic activity. This has led to the hypothesis that Myc–Max heterodimers activate transcription of target genes through recruitment of histone acetyltransferases, histone acetylation, and chromatin remodeling. TRRAP also binds E2F1 another growth- promoting and potentially oncogenic protein [275].

5.9.3 ARF, E1A and the ONYX-015 story

DNA tumor viruses drive cells into S-phase to facilitate DNA replication. Adenoviruses are viruses that have the ability to master both p53 and Rb pathways by direct interference from certain viral proteins [230,231]. The products of the early region 1A (E1A) can induce both p53-dependent and p53-independent apoptosis [276]. Adenoviruses also encode the E1B-55K protein that binds and inactivates p53 thereby neutralizing the effect E1A has i e stabilization of p53 [277]. E1A proteins antagonize the function of the p300 co-activator family and also the Rb protein. E1A inhibition of Rb activates E2F-1 and subsequently results in de-regulated cell cycle control and apoptosis [278]. Expression of E1A induces accumulation of p53 and E1A mediated apoptosis is mainly p53 dependent [230]. It was suggested that accumulation of p53 is caused by induction of unscheduled DNA synthesis [279]. However, p53-independent apoptosis induced by E1A protein has been documented and in this regard E1B (Bcl-2 like) might block other apoptosis pathways, not only p53 regulated [280]. E1A recruits the TRRAP/GCN5 histone acetyltransferase complex [281] similar to what has been

shown for Myc and E2F1 [275,282]. de Stanchina and co-workers found that accumulation of p53 in response to E1A expression in MEFs was strictly dependent on p19ARF but independent of DNA damage pathways [238]. Recently, adenoviral infection was shown to induce DNA damage like responses as indicated by activation of ATM and an increase in phosphorylated H2AX [280].

The adenovirus mutant dl1520 also known as ONYX-015 does not have the E1B-55K protein and the virus is thought to replicate in cells that lack p53 and hence induce cell lysis [283]. However, there is no good correlation between ONYX-015 replication and p53 status. Based on the fact that E1A "selects" for p19ARF-p53 pathway it was suggested that loss of p14ARF/p19ARF could explain ONYX-015 activity in cells with wt p53 [284] and studies also indicated that this was the case [285]. Others did not observe this and instead provided some evidence that ONYX-015 replication was independent of p14ARF, and p53 status [286]. Despite uncertainty about the absolute role of the p53 pathway components for virus replication, ONYX-015 shows promising synergistic effect in treatment of certain tumor forms [283].

5.9.4 Other regulators of ARF

Oncogenic **Ras** can transform most immortal rodent cells to a tumorigenic state. Transformation of primary cells by Ras requires either a cooperating oncogene or the inactivation of tumor suppressors such as p53 or p16INK4a in human cells (p19ARF in mouse cells) [105]. Expression of oncogenic Ras in primary human or rodent cells results in a permanent G1 arrest [105]. Palmero (1998) suggested that Ras activation of p53 involves p19ARF and this is largely independent of E2F-1, 2 [236,287]. Intriguingly, Ras expression in human fibroblasts does not induce p14ARF instead it only up-regulates p16INK4a [88,168,288,289]. The mechanism of induction of p19ARF by Ras remains at present unclear but could involve the MAPK cascade [226]. **Raf** activation in IMR-90 human lung fibroblasts also leads to premature senescence accompanied by up-regulation of p21 and p16INK4a [290]. Another aspect of Ras seems to be it capacity to regulate p53 acetylation and subsequently premature senescence [291]. p53 is acetylated at lysine 382 upon Ras expression, an event that seems to be critical for p53 function. Ras induces re-localization of p53 and CBP to PML bodies and formation of p53-PML-CBP trimeric complexes [291]. One of the genes induced during Ras-induced arrest is promyelocytic leukemia (PML) protein, a potential tumor suppressor that encodes a component of nuclear structures known as

promyelocytic oncogenic domains (PODs) [289,292]. PML levels increased during Ras-induced arrest and replicative senescence.

Moreover, oncogenic ras can activate the p19ARF-p53 program to suppress epithelial cell transformation and it was concluded by the authors that disruption of this program could be important during skin carcinogenesis and the development of other carcinomas [293]. Activation of the MAP kinase pathway in primary mouse keratinocytes leads to a p53 and p21-dependent cycle arrest and to terminal differentiation [294]. However, the response to **Raf** in p19ARF null keratinocytes was indistinguishable from wt controls [294]. Therefore, and paradoxically p19ARF is not essential for Raf-induced p53 induction and cell cycle arrest in keratinocytes indicating that oncogenes engage p53 activity via multiple mechanisms.

Aberrant accumulation of β**-catenin** in tumors is often associated with mutational inactivation of p53. A tumor-derived β-catenin mutant induces accumulation and activation of p53 [295]. Overexpression of wt p53 down-regulates β-catenin expression in both human and mouse cells [296]. Stabilization of p53 after β-catenin overexpression in wt MEFs is mediated through p19ARF in a manner partially dependent on the transcription factor E2F1 [240]. In wt MEFs, mutant β-catenin inhibits cell proliferation and imposes a senescence-like phenotype but not in cells lacking p19ARF or p53 [240]. An interesting twist indicating a tumor suppressor role for p14ARF is the frequent epigenetic silencing of p14ARF in several human colorectal cell lines and primary colorectal carcinomas [297]. p14ARF hypermethylation was slightly overrepresented in tumors with wild-type p53 compared to tumors carrying p53 mutations this difference was not significant [297].

The oncogene **v-abl** from the Abelsson murine leukaemia virus (Ab-MLV) also activates p19ARF and hence induces p53 dependent apoptosis during v-abl mediated pre-B cell transformation [298,299]. v-abl causes cell cycle arrest in primary mouse embryonic fibroblasts (MEFs) and elevated levels of both p53 and p21 [239] and *p53-/-* or *p19ARF-/-* MEFs were resistant to v-abl induced cell cycle arrest [239].

TBX-3 has the ability to downregulate p19ARF expression in MEFs, and p14ARF as well, leading to bypass of cellular senescence [300]. TBX-3 is a T-box protein

(transcription factor) and the gene is mutated in a human syndrome known as Ulnar-Mammary Syndrome (UMS) and these mutants failed to repress ARF expression and the authors speculated that the hypoproliferation seen in the UMS could be attributed to deregulated expression of p14ARF [300]. Even more exciting was the finding that also, **TBX-2** represses expression of p19ARF (and p16INK4a), and co-expression of TBX-2 antagonizes induction of p19ARF in response to oncogenic E2F1 or Myc [301]. TBX-2 is amplified in a subset of human breast cancers, and could potentially reduce expression of p16INK4a and p14ARF [301]. Although ARF and p16INK4a seem to play different roles depending on cell type and species the ability of TBX-2 [301] as well as the polycomb protein **BMI-1** [302,303] to repress (presumably through chromatin remodeling) the entire *INK4a/ARF* locus makes this difference less important. AML1 (or RUNX1) transcription factor is frequently mutated in acute myeloic leukemia (AML) which is a severe disease characterized by low frequency of p53 mutations. The fusion protein in AML, resulting from the t(8;21) translocation, **AML1-ETO** represses the p14ARF promoter whereas wt AML1 actually induce ARF [304]. An essential activator of p19ARF is **DMP-1**, a cyclin D-Myb like binding protein that has functional binding sites in the *p19ARF* promoter [235]. DMP-1 overexpression results in growth arrest due to activation of the p19ARF-p53 pathway but not apoptosis *per se* [235,305]. **DAP kinase** is a Ca^{2+}/calmodulin regulated serine/thronine kinase harboring a "death domain" [306]. DAP kinase is quite frequently lost in human cancers through methylation of the promoter, for instance in Burkitt´s lymphoma [307]. DAP kinase was found to suppress oncogene induced transformation of MEFs by activating the p19ARF-p53 checkpoint [308]. Additional p14ARF/p19ARF regulators include **Twist** (and possibly Dermo1) [309], **Jun D** [310], **BRCA1** [311] and **CTCF** [312].

Table 2. Examples of proteins that directly or indirectly modulate p14ARF/p19ARF transcription/translation or results in changes in p14ARF/p19ARF protein levels or stability. Please check the references for detailed information about the model systems used.

E2F-1, -2, -3	[99,118,313,314]
Myc	[237]
E1A	[238]
Abl	[239]
Ras	[236,287]
Raf	[241]
β-catenin	[240]
Dmp-1	[235]
Tbx-3	[300,315]
Tbx-2	[301,315,316]
Bmi-1	[302,303]
AML-ETO t(8;21)	[304]
CTCF	[312]
DAPK	[308]
Twist	[309]
Jun D	[310]
BRCA1	[311]

5.9.5 Viral proteins and the ARF-p53 pathway

Some viral proteins are implicated in the regulation of ARF and the ARF-p53 pathway. Epstein-Barr virus encoded **EBNA-5**, a peculiar protein, binds p14ARF *in vitro* and in living cells and by doing so it can inhibit the function of p14ARF and prolong the survival of p14ARF expressing cells [181]. **Polyoma virus middle T** antigen activates an ARF-p53 dependent checkpoint in murine fibroblasts *per se* [317] but somehow the polyoma virus small and large T antigens block (rat) ARF signaling to p53 [317]. It is also interesting to note that the authors observed a p53 response in the absence of a detectable (rat) ARF-MDM2 interaction. Human papilloma virus (HPV) encoded proteins **E6** and **E7** have interesting effects on p53 and p14ARF/p19ARF. In theory, expression of E6 targets p53 for degradation and hence increases levels of p14ARF/p19ARF, while also simultaneous expression of E7 inactivates Rb and results in the release of free E2F that could activate the p14ARF/p19ARF promoter. E7 expression in fibroblasts activates p53, but independent of p19ARF [318]. In a putative feedback loop, enforced expression of p19ARF inhibits E7 oncoprotein by sequestration and by blocking proteolysis of Rb by E7 [319].

5.10 THE NUCLEOLUS

Mammalian nuclei most often contain between 1-4 nucleoli but this number is usually increased in tumor cells as a response to increased growth. The size and morphology also differs somewhat between cell types. The prototype nucleolus consists of three morphologically distinct components. Fibrillar centres (FC) contain rRNA genes in tandem arrays found at several chromosomal loci known as nucleolar organising regions (NORs). The dense fibrillar component (DFC) contains actively transcribing rRNA genes and nascent rRNA transcripts. The third component is the granular component (GC) that is the site of late processing events in the biogenesis of pre-ribosomes, reviewed in [320,321]. Nucleoli also seem to play a role in viral infections [322], nuclear export [117], sequestration of regulatory molecules [116,323], modification of small RNAs, shuttling of mRNA species [324], RNP assembly [325] and control of aging [326,327]. Nucleolar structure is coupled to on-going RNA polymerase I transcription reviewed in [328] and see also [329]. Hundreds of protein components of the nucleolus were identified using a proteomics approach [330,331]. The analysis points out that many biological functions and pathways operating within the nucleolus [332] including, ribosome biogenesis; mRNA metabolism and also cell cycle regulation [330,331]. Nucleoli are dynamic structures and disassemble and reassemble during the cell cycle (reviewed in [333] and [334]). Upon entry into mitosis, an ordered disassembly of first the GC and then the DFC of the nucleolus occurs (reviewed in [335]). Ending of rDNA transcription is controlled by the activity of CDK1-cyclin B [329]. However, during mitosis the rDNA transcription machinery but not the processing machinery, remains attached to chromosomal NORs [336]. Proteins involved in pre-rRNA processing, including, B23; fibrillarin; nucleolin; and Nop52 associate with the periphery of mitotic chromosomes and become associated with pre-nucleolar bodies (PNBs) in telophase and early in G1 [336,337]. PNBs (or their components) are then recruited to NORs resulting in the formation of the nucleolus [336,337]. The rebuilding of the nucleolus is also dependent on the activity of CDKs (reviewed in [338]). Fluorescence Recovery After Photobleaching (FRAP) has shown that it is common that nucleolar proteins involved in the rDNA transcription machinery is in constant motion, binding only transiently to the rDNA and then being released [339]. Many sub-nuclear compartments are often associated with nucleoli including, the perinucleolar compartment (PNC); the Cajal (coiled bodies) and Sam68 bodies; and the recently discovered paraspeckles (reviewed in [340] and references therein). Nucleolar “sequestration” of proteins appears to be a

novel theme in cell cycle regulation and perhaps also regulation of many other cellular processes (reviewed in [333]). For example, in S. cerevisiae the exit from mitosis is regulated by the nucleolar sequestration of the protein phosphatase Cdc14p [323]. Upon inhibition of the proteasome many proteins (e g p53, MDM, PML, EBNA-5) seem to accumulate in nucleoli, indicating a putative role for the nucleolus in protein degradation and/or nuclear export [341-343].

6 MAIN AIMS OF THE STUDY

*To analyze the localization and expression of p14ARF in human tumor cell lines and normal cells

*To investigate if p14ARF and B23/nucleophosmin interact, and if, what is the significance?

*Assess the status of the p14ARF-MDM2-p53 pathway in Burkitt´s lymphoma cell lines

*Defining the role of p14ARF in Myc and E2F1 mediated stabilization of p53 in primary human fibroblasts

7 RESULTS AND DISCUSSION

7.1 PAPER I

In this paper, we demonstrated that endogenous p14ARF is a nucleolar protein in human tumor cell lines. In agreement with other studies, high expression of p14ARF was found only in human tumor cell lines deficient for p53 function indicating a negative feedback loop. We examined p14ARF in (more than) 32 tumor cell lines by immunofluorescence. Nucleolar p14ARF was detected in (more than) 10 lines, all of which lacked functional p53. This is the strongest evidence, although indirect, that p14ARF acts in the p53 pathway. For this we used an antiserum (denoted 271) raised against a peptide corresponding to a region in exon 2 unique to p14ARF. While the work was in its final stages, a commercially available antibody 14P02 was made public and it confirmed our results with 271. It later turned out that 14P02 (Ab2) was identical to the DCS240 monoclonal that was provided by Dr. Jiri Bartek. Further analysis using Ab2 and Ab3 (14P03/DCS241) has revealed that also lines with wt p53 and intact p14ARF (gene) also have very low but detectable expression of nucleolar p14ARF. One example of these is the wt p53 containing cell line U2OS (M Lindstrom, unpublished data).

Detection of p14ARF protein using Western blotting is not straightforward. First, the levels of p14ARF is very low/not expressed in most normal human and murine cells and in addition many tumor cell lines have loss of ARF expression due to deletion, promoter methylation or presence of wt p53. Second, p14ARF is predominantly nucleolar and mild lysis conditions only releases a subfraction of p14ARF whereas the rest remains attached to nucleolar structures. Thus, the yield with for instance "NP40 lysis buffer" or mild IP buffers is usually quite low. Preparation of nuclear and nucleolar extracts is advantageous when it comes to produce protein preparations with quite high yield of p14ARF. Whole cell lysates directly prepared by scraping cells in SDS sample buffer also works well for detection of p14ARF in most cases. Third, since p14ARF is sensitive to proteases the protein isolation protocols must include a coctail of inhibitors. The combination of genomic PCR, RT-PCR with western and immunofluorescence techniques usually gives clear answer on the status of p14ARF in cell lines. Note that antibodies quite often cross-reacts with nucleolar antigens!

The NoLS (nucleolar localization signal) is not defined by a consensus motif but instead one has to rely on empirical findings. Proteins that are destined for the nucleolus are often bound to other components of the nucleolus: rRNA, ribosome particle subunits or specific shuttle proteins. Many NoLS motifs have emerged with no speicifc consensus. The most common themes appear to be a series of basic residues such as two or more NLS in sequence, distinctive secondary structure folding of basic residues, and glycine-arginine rich domains involved in pre-rRNA processing. Those are all candidates for a NoLS (see e g [145,344,345]). Using GFP-p14ARF deletion mutants we found that nucleolar localization of p14ARF was mediated primarily by the exon 2 encoded regions, but the exon 1β segment tagged to GFP was not excluded from nucleoli but showed a more pronounced nucleoplasmic expression rather than nucleolar. This also differed depending on cell type. Results with chicken ARF would argue that the exon 2 encoded part of p14ARF (human) is not so important (if any role at all). From my point of view, I find it hard to believe that p14ARF got a "perfect" NoLS without any purpose at all and that evolution could have played a little trick.

Several complications exist in the studies concerning ARF domains. First, motifs in ARF that show binding to MDM2 also confer nuclear and nucleolar localization. Second, two proteins in different compartments that do not interact *in vivo* could indeed interact provided they are put together in the same place *in vitro*. Third, sequence divergence in ARF species creates obvious difficulties in comparing results. Fourth, the type of assay and biological endpoint (p53 stabilization, p53 activity, MDM2 binding, localization, or cell cycle arrest in combination with use of different ARF fusion tags (HA, Flag, GFP, Tx) that seem to affect stability and localization and fuction of ARF [127,181] will clearly give rise to differences in results and interpretation.

The nucleolus consists of several “subnucleolar” structures that are easily identified in the electron microscope. These structures are the FC (fibrillar centers), the DFC (dense fibrillar center) and the GC (granular component). Transcription of rDNA occurs in the nucleolar FCs by RNA polymerase I and is the basis for proper function of the nucleolus [320,321]. The protein B23/nucleophosmin is considered a protein marker for the GC, whereas fibrillarin is a marker for FC and DFC and RNA pol I is thought to be specific for FC, reviewed in [346] and references therein. On the other, one could also say that there are no distinct boundaries within the nucleolus, rather the nucleolus should be considered as a gradient in which different proteins are active at various

stages [333]. The maturing rRNA is processed and added to the assembly of ribosomes by the aid of additional proteins and chaperones. On basis of p14ARF´s co-localization with B23/nucleophosmin but not fibrillarin (or RNA pol I, data not shown), release of ARF from nucleoli by RNase and low doses of actinomycin D (that sometimes causes degranulation of the GC), we concluded that p14ARF is a GC protein, but a small fraction might also reside in the DFC. As indicated, ARF did not to a large extent co-localize with fibrillarin although a partial overlap was seen in some cells. Moreover, we found that p14ARF was easily induced by overexpression of the E2F1 transcription factor in primary fibroblasts (and Saos-2, M Lindstrom unpublished data) and p14ARF was then mainly localized to the nucleolus, but p14ARF could also be seen in the nucleoplasm. p14ARF was also found in distinct aggregates scattered throughout the nucleoplasm. RNase treatment or selective inhibition of rRNA synthesis by low doses of actinomycin D resulted in nucleoplasmic translocation of p14ARF. This indicates that nucleolar localization of p14ARF is dependent on ongoing transcriptional activity in intact functional nucleoli.

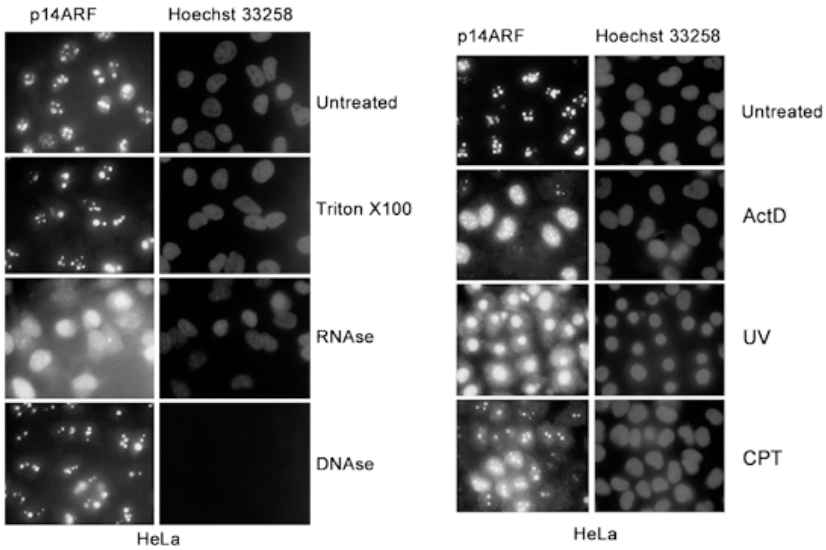

Fig 5. Localization properties of p14ARF. In these images, I try to illustrate the characteristics of nucleolar p14ARF in HeLa cells. As can be seen p14ARF is released from nucleoli after RNAse but not DNAse treatment and the localization of p14ARF is also altered in response to transcriptional inhibitors e g actinomycin D (Act D), camptothecin (CPT) and ultraviolet radiation (UV).

Table 3. p14ARF nucleolus-nucleoplasm shift in response to stress agents

	p14ARF	B23
Cisplatin	pos	pos
Campthothecin	pos	pos
Actinomycin D	pos	pos
DRB	pos	pos
Cycloheximide	neg	neg
Leptomycin B	neg	neg
MG 132	neg	neg
5-Fluorouracil	neg?	neg?
Mitomycin C	neg	neg
Puromycin	neg	neg
DMSO	neg	neg
EtOH	neg	neg
UV	pos	pos
Heat	neg?	neg?

Furthermore, p14ARF was not expressed or induced in senescent human primary fibroblasts, as was later shown and confirmed in more detail by others [288]. We could not find any evidence for p14ARF mediated nucleolar sequestration of MDM2 in human tumor cell lines also in agreement with later studies [127]. This could in part be explained by the fact that human tumor cell lines contain variants of MDM2 that do not contain a cryptic nucleolar localization motif necessary for nucleolar localization and that is exposed after ARF binding [347]. However, we did not find any evidence for sequestration of MDM2 in primary human cells either. We also observed that p14ARF was localized in some cases to nuclear bodies when overexpressed. Triple labeling should be performed together with phase contrast, or Hoechst 33258 (recognize peripheral nucleolar heterochromatin) to conclude "nucleolar sequestration" of a certain protein rather than accumulation in nuclear bodies. *We have also noted that small inclusions can be formed within the nucleolus itself.*

7.2 PAPER II

Disturbances in nucleolar function induced by unrestricted cell growth, overexpression of certain nucleolar proteins, and transcriptional inhibition could directly (or indirectly) activate p53 dependent (and independent) checkpoints [207]. B23 also known as nucleophosmin (NPM) is a multifunctional nuclear/nucleolar protein with chaperone

properties [348]. It has been found to interact with a number of nucleolar proteins for example nucleolin [349], Tat [350], Rb [351] and also p53 [352]. A key role of B23 is to participate in centrosome regulation in a process controlled by the cyclin E/CDK2 complex [353]. B23 was shown to stabilize p53 and cause premature senescence when overexpressed in primary fibroblasts [352]. From the previous work, we learnt that B23 and p14ARF have similar distribution in interphase cells. We found that p14ARF/p19ARF strongly interacts with B23 *in vitro* and *in vivo*. The B23-p14ARF/p19ARF association requires motifs in p14ARF/ p19ARF shown to be important for its nucleolar localization and inhibition of MDM2. The B23 protein was found to modulate the localization of ARF and *vice versa*. The ARF-B23 interaction increases in growth-arrested cells and decreases during S phase entry, suggesting that it may contribute to ARF-mediated growth inhibition. In this work, we sought to characterize the properties of p14ARF in relation to B23 during the cell cycle and in response to different types of cellular stress. During mitosis p14ARF and B23 were found dispersed in the cytoplasm, at the perichromosomal layer, nucleolus derived foci (NDFs) and later on also in pre-nucleolar bodies (PNBs). Similar B23 and p14ARF distribution and translocation patterns were also seen after nucleolar stress induced by transcriptional inhibitors (e g Act D, CPT, cisplatin and DRB). Levels of p14ARF protein (but not B23) were markedly reduced during mitosis (metaphase) in some but not all cell lines ([354] and M Lindstrom, unpublished data). B23 and p14ARF showed identical extraction properties *in situ* and *in vitro*. B23 was also found at the interphase centrosome and at the mitotic spindle, but p14ARF was not detected at these locations (M Lindstrom, unpublished data). Thus, we conclude from this work that B23 is a *bona fide* ARF-associated protein. Using siRNA to deplete B23 we are studying if B23 has additional roles affecting the levels, localizations and functions of p53 and MDM2. We are also studying the effect on p14ARF localization and stability and on nucleolar structure and function in general after B23 depletion.

I find it very likely that ARF inhibition of rRNA processing creates disturbances in the nucleolar machinery eventually leading to indirect p53 stabilization in addition to direct inhibition of MDM2 by ARF. Some clues to what might be going on came from studies on a dominant negative mutant of Bop-1DN (block of proliferation) [355]. Bop-1 is a Myc target gene and is involved in rRNA processing and ribosome assembly [356,357]. Bop-1DN was shown to block formation of the mature 28S and 5.8S rRNA species and

the mutant also reduced the levels of the 60S ribosome subunit [356]. Interestingly, Bop1DN overexpression led to cell cycle arrest in G1 dependent on p53 [355].

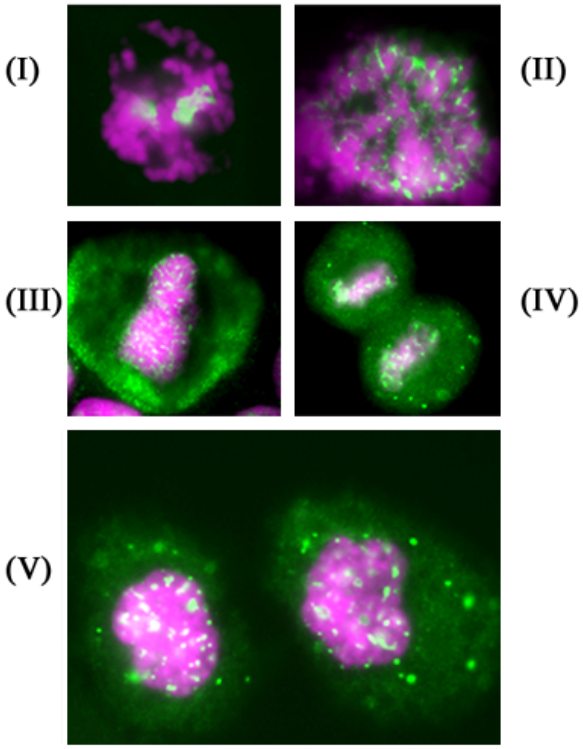

Fig 6. Localization of p14ARF (in green) in mitotic SW480 cells as detected and visualized with mAb 14P02/DCS240. *I*) p14ARF was associated with nucleolar remnants during early prophase. *II*) In late prophase, p14ARF became dispersed in the nucleoplasm in the condensing nucleus before the nuclear envelope breakdown. III) p14ARF decorated the condensed chromosomes in metaphase but was mainly dispersed in the cytoplasm. IV) During anaphase, p14ARF was seen in the chromosomal areas and in nucleolus derived foci (NDFs). V) In telophase, p14ARF starts to accumulate into prenucleolar bodies but was still localized to cytoplasmic structures.

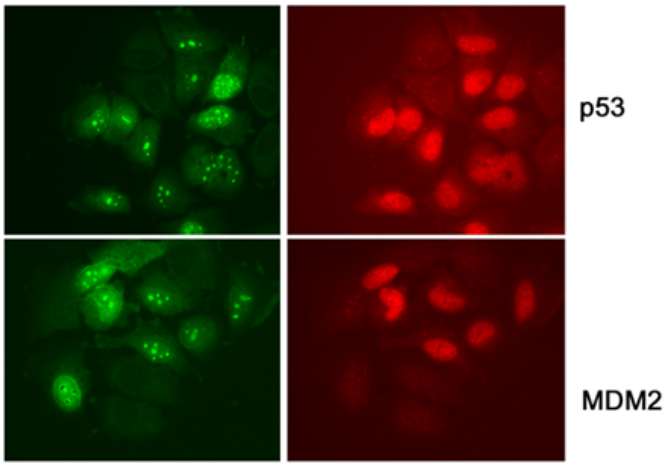

Fig 7. p14ARF was transfected into wt p53 MCF7 breast carcinoma cells. Cells were double stained for p14ARF using antiserum 271 (green) and p53 (mAb DO7) or

MDM2 (mAb SMP14). Stabilization of p53 and MDM2 was observed in a majority of transfectants after 24 hours.

7.3 PAPER III

In this article, we investigated the "p14ARF-MDM2-p53" pathway in Burkitt´s lymphoma (BL) cell lines. BL is a childhood tumor being characterized by reciprocal translocations between the c-*Myc* gene on chromosome 8 and one of the immunoglobulin loci on chromosome 2, 14 or 22 [358-360]. Thus, the hallmark of BL is a constitutively activated c-myc gene that drives tumor cell growth [361-363]. Mutations in p53 have been identified in 30-40% of BL biopsies and in a significantly higher proportion of BL cell lines [364,365]. In addition to defects in p53, the *p16INK4a* gene promoter is hypermethylated in some BL biopsies and in majority of BL cell lines, leading to silencing of this gene [366].

We found that the p14ARF protein was expressed and localized to nucleoli in all BL cell lines carrying mutant p53 included in this study. We had previously failed to detect p14ARF in BL cell lines using a polyclonal antiserum but re-screening with a monoclonal did reveal quite significant levels of p14ARF in for example DG75 and BL41 cell lines. This is a very clear example of how different antibodies work. Both poyclonal 271 and monoclonal 14P02/DCS240 give rather identical results when looking at p14ARF expressed from plasmids, but when going down on lower levels only 14P02 efficiently recognized p14ARF in BL cell lines despite using high concentration of 271.

Several lines of evidence indicate that disruption of p19ARF-p53-dependent apoptosis is essential for immortalization and tumor development in rodents. Deregulated Myc expression selects for inactivation of either p19ARF or p53 in mouse embryo fibroblasts, leading to the emergence of apoptosis-resistant cell clones [237]. Similarly, mice expressing a *Myc* transgene under the control of the Ig heavy chain enhancer develop clonal pre-B and B-cell lymphoma, in analogy with human BL [367]. The emerging tumors in these mice carried p53 mutation (28%), had *INK4a/ARF* deletion (24%), or overexpressed MDM2 [185]. Lack of both p16INK4a and p19ARF abrogates B-cell apoptosis in Eμ-*Myc* transgenic mice, and greatly accelerates lymphomagenesis [368]. These lymphomas displayed characteristics of p53 null lymphomas, including deficient apoptosis, resistance to chemotherapy, and rapid/invasive growth, and reduced p53 activity despite the presence of wt p53 alleles [368].

In our panel of more than 46 cell lines, we choose to study a subset in more detail (those with known p53 status). We found that three out of seven BL cell lines carrying wt p53 had a homozygous deletion of *INK4a/ARF* whereas three BL cell lines carrying wild type p53 retained the *INK4a/ARF* locus and had elevated levels of MDM2. DNA sequencing revealed a point mutation in exon 2 in one of these BL (Seraphine). However, this point mutation did not affect p14ARF´s nucleolar localization or ability to activate p53, when overexpressed as a GFP fusion protein.

Our results (and others) with MDM2 must be interpreted with caution. Most MDM2 antibodies cross react with other proteins in some settings, for instance the 2A10 monclonal antibody. Most of the MDM2 antibodies do not recognize commonly found isoforms. In our study, we used the p90 MDM2 product as an indicator of MDM2 levels and it was detected with both 2A10 and SMP14.

Studies in Eμ-*Myc* mice indicate important roles for p53, MDM2, p16INK4a and also p19ARF. How much of the results obtained in mouse models that can be translated to humans and in particular to BL remains unclear. Anyhow, our results indicate that inactivation of the p14ARF-MDM2-p53 pathway is not infrequent during the development of BL presumably as a mechanism to escape Myc induced apoptosis. I envisage that in the early stages of tumor development, the increased expression of p14ARF and MDM2 (that is quite common) indicates deregulated and increased cell growth. Some cases will probably display *p16INK4A* gene methylation quite early during tumor development and some of the tumors will also suffer p53 mutation. Later on, in the fully malignant tumor and during establishment of *in vitro* growing cell lines total loss of p14ARF/p16INK4A will occur more frequent and p53 mutation frequency rise. This scenario is further supported by results from other laboratories. For example, findings in primary BL tumors indicate that mutation in p53 is quite frequent and occurs mutually exclusive to *p16INK4A* promoter methylation or loss of *INK4a/ARF* by homozygous deletion [369]. Studies in other primary BL indicate that tumors have increased expression of p14ARF and MDM2, but they did not see deletion of p14ARF [370]. Another study of human non-Hodgkin lymphomas revealed that *INK4a/ARF* locus alterations mainly occur in tumors carrying wild type p53 [371]. Collectively, these results suggest that the p53 tumor suppressor pathway is quite often inactivated in human lymphomas, mostly through inactivation of p53 or

more seldom by “overexpression” of MDM2 [372], loss of p14ARF [373], mutation in Bax [374] or p21 [375]. Decreased expression of p73 and DAP kinase [307] through promoter methylation or deregulation of BCL-6 [376,377] could further interfere with p53 dependent and independent apoptosis mechanisms in BL and in other lymphomas. The presence of Epstein-Barr virus (EBV) in BL provides an apparent survival factor [378], and it is noteworthy that loss of p14ARF/p16INK4a by deletion was seen in EBV negative lines only.

A fraction of Eμ-*Myc* lymphomas and BL cell lines and primary BL tumors do not display any obvious alteration in the p19ARF–MDM2–p53 pathway, suggesting the existence of other genetic changes that could modify the activity of p53 or p14ARF/p19ARF. Examples of such "modifiers" could include DMP1 [379]. Lack of DMP1 reduces the rate of p19ARF inactivation and p53 mutation in Eμ-*Myc* lymphomas [379]. Another candidate "modifier" is BMI-1 that co-operates with Myc in lymphomagenesis in mice by repression of p19ARF and p16INK4a [302,303]. In paper III, we investigated the expression of the polycomb group (PcG) protein BMI-1 protein using the F6 monoclonal antibody. PcG proteins in general are involved and play very crucial roles in many important embryonic, hematopoetic and cell cycle related processes. We found no correlation with p16INK4A or p14ARF expression and levels of BMI-1. In fact, BMI-1 levels seemed to be higher in those with high expression of p14ARF. This was also confirmed on RNA levels (unpublished data), and not only assessed by immunostainings and western blotting. Since our analysis was restricted to *in vitro* cultured BL cell lines, the conclusions that can be drawn is limited. In the literature, there are both reports showing a correlation between BMI-1 expression and p14ARF/p16INK4A expression, see e g [380], but in other studies correlation of p16INK4A/ p14ARF and BMI-1 is not evident, see e g [381] and [382]. It will be interesting to look at p14ARF, p16INK4A and BMI-1 expression dynamics in primary BL.

Mouse plasmacytoma (MPC), a pristane oil-induced tumor in the susceptible Balb/c mouse strain, represents an analogy to human BL. MPC carries Ig–Myc translocations corresponding to those in BL. In contrast to BL, however, MPC does not carry p53 mutations [383]. The Balb/c strain harbors two functional polymorphisms in the *INK4a/ARF* locus, one in exon 1α and one in exon 2, that give raise to single amino

acid substitutions in p16Ink4a (His-18 to Pro and Val-51 to Ile) [384,385]. The p16Ink4a protein encoded by the Balb/c allele has reduced ability to induce cell cycle arrest [384,385]. The polymorphism in exon 2 also causes an amino acid substitution in p19ARF (Arg-72 to His) but this does affect the function of p19ARF [384,385]. In our screen of MPC lines and primary tumors we did not detect *p16Ink4a* or *p19ARF* deletions. Thus, MPC development occurs without frequent changes in p19ARF or p53 genes that are more common in BL and most frequent in Eμ-*Myc* lymphomas. It should be emphasized that BL, MPC and Eμ-*Myc* lymphomas are not equivalent entities but represents cells at various differentiation stages.

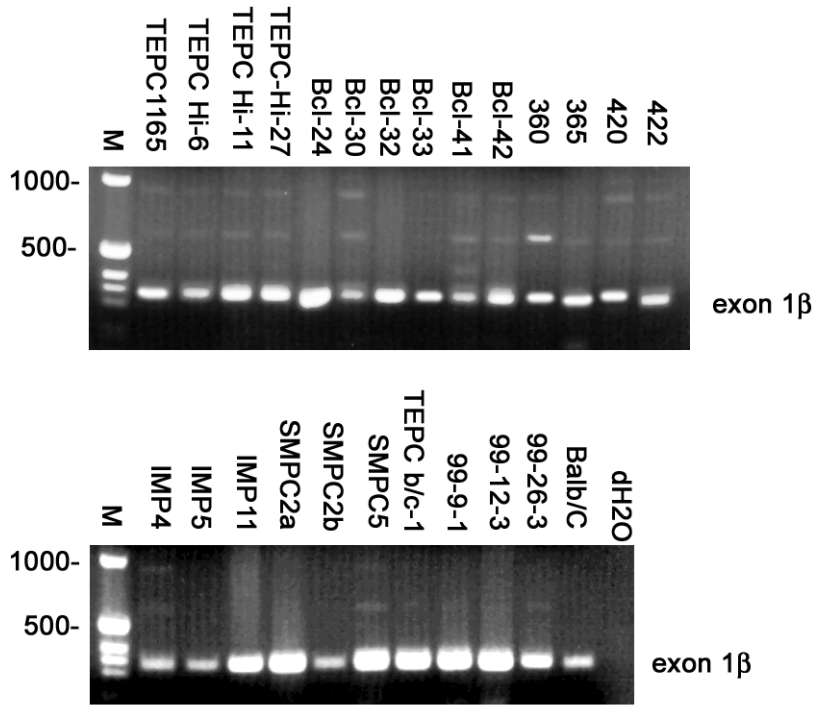

Fig 8. No loss of p19ARF gene in MPC. Genomic DNA was isolated from a selection of MPC cell lines and primary tumors, kindly provided by Dr Santiago Silva, and used for PCR with a p19ARF exon 1β specific primer pair. In this selection of MPC, some lines would be expected not to have loss of ARF in particular those harboring Bcl 2 protection (upper panel, middle).

In summary, we found that loss of p14ARF in BL cell lines did occur in wt p53 containing cell lines but was less frequent then in mouse models of B-cell lymphomas. Since loss of p16INK4a also occurs simultaneously it is difficult to know whether it is p14ARF, p16INK4a or both that is selected against. On basis of mouse models, we speculate that loss of p14ARF could be of a selective growth advantage in a subset of human BL.

7.4 PAPER IV, V

Experimental findings strongly support the idea that Myc is an activator of both cell proliferation and apoptosis where the outcome depends on the cell environment [386]. Ectopic overexpression of Myc induces p53 and sensitizes cells to both p53-dependent and independent apoptosis in low serum or after growth factor deprivation [228]. Zindy and coworkers (1998) showed that enforced expression of Myc induces p19ARF, p53 and its downstream targets MDM2 and p21 [237]. The findings in MEFs implicated that Myc induces p19ARF, via p53 and MDM2 independent upstream pathways, but p53 up-regulation in response to Myc is also likely to involve both p19ARF dependent and independent pathways, reviewed in [387]. Myc induction of MDM2 and p21 is also strictly dependent on p53, and furthermore Myc immortalization of wt MEFs is accompanied by either p19ARF or p53 loss and this confers resistance to Myc-induced apoptosis [237]. Myc can induce p53 in *p19ARF* null cells, demonstrating that p53 accumulation caused is not entirely p19ARF dependent [237].

We wanted to study the involvement of p14ARF as a regulator of p53 activity in normal human skin fibroblasts (NHFs) or WI38 lung embryonic fibroblasts expressing conditional Myc or E2F-1 fused to a modified estrogen receptor domain [388-390]. To summarize, both Myc and E2F-1 activation rapidly induced p53 phosphorylation at serine-15, p53 protein accumulation, and upregulation of the p53 target genes MDM2 and p21. Activation of E2F-1 also induced p14ARF levels. In contrast, Myc activation did not induce any significant increase in p14ARF mRNA or protein levels in neither NHFs nor WI38 fibroblasts within 48 hours. Treatment with the ATM/ATR kinase inhibitor caffeine prevented p53 accumulation upon activation of Myc or E2F-1, as well as p53 phosphorylation at serine-15. E2F-1 activation resulted in a clear induction of p14ARF mRNA and protein. Both Myc and E2F-1 induced p53 and cell cycle arrest also after depleting cells with p14ARF siRNA.

If p14ARF is not involved in stabilization of p53 upon Myc and E2F-1 activation in human fibroblasts, then what is the mechanism? p53 is phosphorylated at several N-terminal serine residues upon DNA damage including serine-15 and serine-20. Several kinases are known to phosphorylate p53 upon DNA damage either directly or indirectly, including ATM, ATR, Chk1, and Chk2 [391]. Phosphorylation of serine-

15 and serine-20 seem to lead to increased transcriptional transactivation efficiency, decreased MDM2 binding, and reduced nuclear export [392-394]. A novel nuclear export signal (NES) is located in the p53 N-terminus. Serine-15 phosphorylation blocks this NES, allowing nuclear p53 accumulation [395]. Thus, N-teminal phosphorylation could trigger p53 accumulation through multiple mechanisms. My results demonstrate that particularly serine-15 is phosphorylated after Myc activation, but I also detected increased phosphorylation of serine-37. In addition, I also noted that E2F-1 induced p53 phosphorylation of serine-15, consistent with previous studies by others [396,397].

Phosphorylation could be due to several events. Myc induces genes involved in cell growth and metabolism [260]. This in turn causes an overload on the respiratory chain in mitochondria. The extent of **oxidative stress induced DNA damage** might depend on cell type and is potentially confounded by *in vitro* cultivation cells that also suffer from oxidative stress. Oxidative stress and generation of reactive oxygen species (ROS) is considered to be a leading cause of DNA damage in cultured cells and is increased after serum deprivation. Evidently, Myc activation leads to an increase in ROS in human fibroblasts [398]. As a consequence, DNA damage checkpoint pathways become activated and key mediators could be ATM or ATR kinases. This in turn could phosphorylate multiple downstream targets p53 and Chk2. Second, **replicative stress** induced by inappropriate activation of Myc and E2F-1 could lead to errors in DNA replication that subsequently triggers p53 phosphorylation. This could occur by stalled replication forks in turn activating ATR and related kinases. Caffeine was used in some experiments since it blocks PI3 kinases such as ATM/ATR [399,400], but caffeine is not the best choice. Caffeine could also act as an antioxidant that could scavenge ROS and thus indirectly reduce oxidative stress-induced DNA damage [401,402]. Moreover, continuous exposure to caffeine was shown to inhibit the proliferation of NHFs in a dose-dependent manner suggesting that caffeine also could interfere with Myc and E2F-1-induced DNA replication [403,404]. Finally, caffeine could act specifically to enhance degradation and nuclear export of p53 as indicated by Renton and co-workers [405]. Third, a recent study indicated that ATM could be activated by dramatic **changes in chromatin** induced by chloroquine or salt concentration changes and this in the absence of DNA damage (i e strand breaks) [406]. Thus, ATM could become active in the absence of DNA damage, alternatively, Myc induced chromatin changes could

facilitate the occurrence of DNA strand breaks. Karlsson et al. found that Myc inhibits DNA repair mechanism in the same cell system [407]. One could also raise the question about stress imposed by the MycER protein itself, massively flooding the nucleus with this fusion protein, what will that mean?

Somewhat unrelated to the above scenarios, unrestrained Myc-induced cell growth (**hyper-growth**) could result in a "**nucleolar stress**" response leading to activation of p53 through upregulation of nucleolar proteins [207]. Rubbi and Milner provided strong evidence suggesting that p53 levels is controlled by the nucleolus and that up-regulation of certain proteins and p53 phosphorylation is not required for p53 stabilization in response to nucleolar dysfunction [31]. Phosphorylation could be used for fine-tuning the p53 response but is not critical for its accumulation.

As described in paper V, we found that activation of Myc resulted in a marked induction and accumulation of p16INK4a, but without cells adopting a classical senescence like morphology in agreement with other studies [169,267]. Co-expression of HPV oncoprotein E6 allowed for continued proliferation of Myc activated cells despite very high levels of p16INK4a.

In summary, the regulation of the *INK4a/ARF* locus is highly dependent on species, cell type and experimental set-up. For instance, the role of p19ARF in some well established mouse models have been dissected in detail with some surprising and interesting conclusions [397,408-411]. Most often but not always, p19ARF is not required for activation of the p53 pathway! Evidently, in human fibroblasts, p16INK4a plays the most important role as a regulator of oncogene induced senescence and growth arrest [169,412]. Still, p14ARF could play a role in modulation of oncogenic induced apoptosis, controlling and enhancing p53 inhibition of cell besides regulating nucleolar function [169,412].

8 CONCLUSIONS

The discovery of ribosomal protein L11 as an inhibitor of MDM2 actually strengthens the role of ARF as a modifier of MDM2 activity. Our understanding of why, when and how MDM2 is affected by these nucleolar/ribosomal proteins remains rudimental. Does this mean that MDM2 have p53 independent nucleolar or ribosomal functions? That ARF is a nucleolar protein strongly suggests that it functions in the nucleolus, but it does not exclude that it acts elsewhere in the cell as was shown to be the case for p53 that also localizes to mitochondria. The odd properties of ARF originating from its relatively small size, charge distribution, disordered structure, low level of expression, use of alternative reading frame, the resemblance to certain viral nucleolar protein and its apparent interaction with important cellular proteins makes this protein all together a true and interesting challenge for future research, and herein lies most of ARF´s attraction. But ARF is also a difficult protein to work with. Clear interspecies and intercellular differences in the regulation of ARF and p16INK4a add to the level of complexity. With regard to ARF, research should aim at identifying physiologically important targets using relevant methodology. Studies relying only on supra-physiological expression must be complemented with other approaches. Although the role of p19ARF in certain mouse cell types is well established there is certainly a need in understanding the functionality of p14ARF in humans, as well as further investigation of p19ARF functions in mice. The reason for this is obvious! This locus is so frequently targeted in malignant human cancers and a precise dissection of p14ARF/p19ARF and p16INK4a functions is necessary.

In brief

*p14ARF is a predominantly nucleolar protein but can also localize to other cellular compartments, and is mainly expressed in cells with loss of p53 function

*p14ARF interacts directly with nucleophosmin/B23, and B23 can modulate the localization of p14ARF

*The p53 pathway is frequently inactivated in BL cell lines

*Myc and E2F-1 mediated induction of p53 is not critically dependent on p14ARF in human fibroblasts

9 ACKNOWLEDGEMENTS

Cancerfonden, Karolinska Institutet and Robert Lundbergs minnesstiftelse are acknowledged for personal research and travel grants.

I would like to apologize to all scientists whose work was not cited, or cited incorrectly, inadvertently misunderstood or misused in this thesis. I also apologize to all my colleagues, for my inability for lunchroom chats about trivial matters.

My main supervisor, **Klas Wiman**, for continuous scientific and financial support, help with my chaotic manuscripts and for holding my horses when to many ideas popped up at the same time. For your interest about my projects, and your discrete criticism when something was not good enough.

I would like to thank all members of the group. **Magdalena** and **Nina**, for all scientific and contra-scientific discussions and for indirectly providing me with the positive energy necessary to complete this thesis. I wish you the best of luck in your future research and in your family life. **Nicole**, for always being friendly and positive towards me. **Fredrik**, for your careful scientific work and that you already have accomplished the most important thing here in life, two times actually. **Vladimir**, I am impressed by your broad scientific skills (especially statistics) and discipline. Keep up the good work! **Tao**, you are a very friendly, hard-working, polite, helpful and social person. Don´t let "big biology" knock you down! **Margaretha**, you had and still have the toughest projects of us all that I would never have managed, and for sure, you have learnt so much from it. **Cristina, Ruby** and **Helene** for bringing law and order (it is needed!), laughters and tittle-tattle, as well as deep knowledge and scientific skills in the areas of RNA, telomerase and apoptosis to the group. **Masoud,** for your true scientific interest combined with clinical skills and understanding! Former member **Wang Qian,** for your friendly company during weekends and late evenings at MTC. All project and exam workers, especially **Anna Vihlborg**!

Charlotte Asker and **Ulf Klangby**, without you no ARF project. Thanks Lotta for taking care of me the fist time making it possible for me to continue work with this exciting protein. This thesis also belongs to you.

Galina Selivanova and members of your group for all help discussions and advice as well as sharing reagents.

Then I would like to thank **Jan-Åke Gustafsson** for introducing me to **Eckart Treuter** and **Lotta Bourgeous,** who taught me protein related techniques during summer 1997. Also, **Katarina Nordqvist** for letting me explore the power of PCR during 1998 and **Erna Möller** for a key advice in 1995. **George Klein** for interest in my work and for asking all those brilliant questions in my early years working at MTC. I also acknowledge the privilege to have been working in the organization headed by the present and former MTC/CCK prefects **Ingemar Ernberg**, **Stefan Einhorn and Tina Dalianis .**

Anders Zetterberg with kind help from **Yvonne Lindell** for providing me with fibroblasts from the very beginning. Special thanks also to **Hanna-Stina, Fredrik E. Susanne** and **Olivia** for being friendly and helpful. **Jamileh Hashemi** for a smooth and efficient collaboration as well as all the other members of the "melanoma group". **Keng-Ling Wallin** for interesting collaboration with true clinical relevance as well as your help in other matters. **Katja** and **Mira** for always being so friendly and positive, and all members of the **Olle Larsson, Berta Brodin, Lars Holmgren, Dan Grander, Stig Linder, Marie Henriksson** and **Laszlo Szekelys** groups at CCK and MTC. My oversea collaborators **Steven Berberich, Moshe Oren** and co-workers for interest in my work.

I would like to thank all administrative and technical personal at CCK, MTC and Radiumhemmet for all help. In particular **Ann-Britt Spåre, Evi-Gustavsson Kadaka, Joe Lawrence, Sören Linden** and **Anders Eklöf.**

My friends and colleagues from KI Biomed –95 in particular: **Vendela, Tony, Ola W, Emma E, Anna DG, Marianne, Andrea, Petronella, Malin, Pontus, Kristina E and George**, and my friends from N3: **Ola W, Linda E** and **Kristina A** for being so friendly and keeping in touch now and then.

Pia, for sharing my interest for mountain hiking, for spending hours with me in the microscope room at very odd hours looking at cells and for spending some cold hours (at even more odd timepoints) outside looking at stars in the telescope. For pointing out some trivial facts such as "you have only written about what others have done!" and "don´t you have a normal relation to anyone in the group?" That you are my friend, girlfriend, fiancé and providing me with a new family (kiitos paljon Klas ja Eeva!).

eno **Matti** ja Aili, paljon kiitoksia!

Pappa för att du trots allt har varit där!

Tack världens bästa **Farmor** för alla brev och samtal!

Tack snälla rara **Mamma** för all hjälp. Även om tiderna inte alltid varit lätta har vi klarat det!

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