Role of the tumor suppressor ARF and the p53-pathway in retinoblastoma development

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Molecular Genetics
University of Toronto

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Master of Science, 2009
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Abstract

Retinoblastoma development is a multistep process, and inactivation of the RB1 gene is not sufficient for tumorigenesis. Previous studies suggest that the p53-tumor suppressor is inactivated due to overexpression of p53-antagonists MDM4 and MDM2. This thesis evaluates the importance of ARF, a p53-activator that inhibits MDM2. In retinoblastomas, ARF protein is nearly undetectable despite robust mRNA expression. Chemical inhibition of the proteasome, which regulates ARF protein-turnover, did not result in ARF accumulation in retinoblastoma cells, indicating that ARF protein was not aberrantly degraded by the proteasome. During mouse retinoblastoma development, Arf protein was expressed at low level, and p53-target genes involved in cell cycle arrest and autoregulation were not activated. Overexpression of ARF in retinoblastoma cells led to growth inhibition, accompanied by increased expression of p53 and p53-transcriptional targets. Taken together, our data suggests that low ARF protein is an important factor in silencing of the p53-pathway during retinoblastoma development.
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternate reading frame</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>BCL2-like 1</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>Bmi1 polycomb ring finger oncogene</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CBX</td>
<td>Chromobox homolog</td>
</tr>
<tr>
<td>CDC6</td>
<td>Cell division cycle 6 homolog</td>
</tr>
<tr>
<td>CDH11</td>
<td>Cadherin-11</td>
</tr>
<tr>
<td>CDH13</td>
<td>Cadherin-13</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>Chx10</td>
<td>C elegans ceh-10 homeo domain-containing homolog</td>
</tr>
<tr>
<td>Cre</td>
<td>Cyclization recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAP-kinase</td>
<td>Death-associated protein kinase</td>
</tr>
<tr>
<td>DEK</td>
<td>DEK oncogene (DNA binding)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagles media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR5/KILLER</td>
<td>Tumor necrosis factor receptor superfamily, member 10b</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>E2F</td>
<td>E2F transcription factor</td>
</tr>
<tr>
<td>Egr1</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>Eμ</td>
<td>Immunoglobulin heavy chain enhancer</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>Gadd45</td>
<td>Growth arrest and DNA-damage-inducible alpha</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>gfp</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>H3</td>
<td>H3 histone</td>
</tr>
<tr>
<td>hnRNP K</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
</tr>
<tr>
<td>H-RAS</td>
<td>v-Ha-ras Harvey rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>JunD</td>
<td>Jun proto-oncogene related gene d</td>
</tr>
<tr>
<td>KIF14</td>
<td>Kinesin family member 14</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>M1</td>
<td>Mutation 1</td>
</tr>
<tr>
<td>M2</td>
<td>Mutation 2</td>
</tr>
<tr>
<td>MDM</td>
<td>Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Transformed mouse 3T3 cell double minute 2</td>
</tr>
<tr>
<td>Mdm4</td>
<td>Transformed mouse 3T3 cell double minute 4</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEL-6</td>
<td>Polycomb group ring finger 2 (PCGF2)</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro-blue tetrazolium chloride</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells</td>
</tr>
</tbody>
</table>
Noxa  Phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1)
NPM    Nucleophosmin (B23)
ONL    Outer nuclear layer
P    Postnatal
p107    Retinoblastoma-like 1
p130    Retinoblastoma-like 2
p15^{INK4b}    Cyclin-dependent kinase inhibitor 2B
p16^{INK4a}    Cyclin-dependent kinase inhibitor 2A
p21    Cyclin-dependent kinase inhibitor 1A
p53    Tumor suppressor protein p53
Pax-6    Paired box gene 6
PBS    Phosphate buffered saline
PcG    Polycomb group
PHC2    Polyhomeotic homolog 2
PMH/OCI    Princess Margaret Hospital/Ontario Cancer Institute
pRB    Retinoblastoma protein
Puma    BCL2 binding component 3 (BCC3)
RB    Retinoblastoma
RB1    Retinoblastoma tumor suppressor gene
RBL2    Retinoblastoma-like 2 (p130)
RING1b    Ring finger protein 2 (RNF2)
RITA    Reactivation of p53 and induction of tumor cell apoptosis
RNA    Ribonucleic acid
RNAi    RNA-interference
RNU6B    RNA, U6 small nuclear 2
RPMI    Roswell Park Memorial Institute
RT-PCR    Reverse transcriptase-polymerase chain reaction
SDS-PAGE    Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA    Short-hairpin RNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA</td>
<td>Short-interfering RNA</td>
</tr>
<tr>
<td>smARF</td>
<td>Short mitochondrial ARF</td>
</tr>
<tr>
<td>STS</td>
<td>Sequence tagged site</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBX</td>
<td>T-box</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranscribed region</td>
</tr>
<tr>
<td>WERI</td>
<td>Wills Eye Research Institute</td>
</tr>
<tr>
<td>WRN</td>
<td>Werner Syndrome helicase</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZBTB7A</td>
<td>Zinc finger and BTB domain containing 7A, Pokemon</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1. Retinoblastoma: Disease manifestation

Retinoblastoma, affecting approximately 1 in 18,000 live births (Devesa, 1975), is a pediatric ocular cancer that originates from the developing retina. The disease is initiated by two mutational events (M1 and M2), as originally postulated by Alfred Knudson in his Two-Hit hypothesis (Knudson, 1971). Subsequent seminal discoveries showed that the two hits consisted of inactivation of both alleles of the tumor suppressor gene, RB1 (Cavenee et al., 1983; Comings, 1973; Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Patients with retinoblastoma may have the first hit as a germline mutation in one allele of RB1, either inherited from one of the parents or arising sporadically during early development, and subsequent somatic mutation inactivates the second allele in the retina. Alternatively, both hits may occur sporadically in the same cell within the developing retina. Patients with germline RB1 mutation carry one mutant RB1 allele in every cell of the body, and are predisposed to heritable retinoblastoma in both eyes (bilateral retinoblastoma) early in life and second malignancies in other tissues later in life (Marees et al., 2008). Affected individuals without germline mutation manifest nonheritable retinoblastoma in only one eye (unilateral retinoblastoma).

1.2. Current treatment for retinoblastoma

Treatment for retinoblastoma varies depending on the laterality of the disease. In developed countries, unilateral retinoblastoma is cured at a high rate (>95%) by enucleation of the affected eye, while bilateral retinoblastoma requires multimodality therapy consisting of local treatments, chemotherapy or external beam radiation therapy, in order to save vision
Current treatment can be effective in curing retinoblastoma, but has considerable side effects. In addition to compromised vision as a result of enucleation and local treatments, retinoblastoma patients have to withstand the toxic effects of systemic chemotherapy and in some cases, external beam radiation. Radiation markedly increases the chance of second malignancies, especially in patients carrying germline \textit{RB1} mutation (Chan et al., 2005). Therefore, treatment with minimal side effects is urgently needed in order to maximize vision preservation, and to minimize the chance of inducing second malignancies in retinoblastoma patients. The development of such targeted therapy requires a thorough understanding of the genetic and molecular mechanisms governing retinoblastoma survival and growth, and the identification of tumor specific markers that can be targeted by therapy.

Moreover, the early onset of retinoblastoma indicates that tumorigenesis is intertwined with retinal development; therefore it is important to understand the role of the protein encoded by \textit{RB1}, pRB, in the developing retina, and to dissect the molecular response to \textit{RB1} inactivation.

\subsection*{1.3. Retinal development}

Mammalian eye development begins with the bilateral evagination of the neural tube, forming a pair of optic vesicles that induce the surface ectoderm to form the lens placodes. Subsequent invagination of the lens placode and the optic vesicle results in the formation of the lens vesicle and a double-layered optic cup. The inner layer of the optic cup eventually forms the neural retina, while the outer layer of the optic cup gives rise to the retinal pigment epithelium (Chow and Lang, 2001).

The early neural retina consists of a neuroblastic layer, in which retinal progenitor cells divide and give rise to the seven main classes of retinal cells in a specific birth order that is evolutionarily conserved. In the mature retina, cells are organized into three distinct layers.
The outer nuclear layer consists of rod and cone photoreceptors, while the inner nuclear layer is made up of bipolar, Müller glia, amacrine and horizontal cells. The ganglion cell layer is home for ganglion and amacrine cells (Dyer and Cepko, 2001).

1.4. pRB molecular function

The best characterized function of the retinoblastoma protein, pRB, is to control cell cycle progression by interacting with and inhibiting the E2F-family of transcription factors, which regulate the expression of genes required for cell proliferation (Cobrinik, 2005; Dyson, 1994). The affinity of pRB for E2Fs (E2F-1, -2, -3a, -3b) depends on its phosphorylation status, which is modulated by cyclins and cyclin-dependent kinases (CDK). In the presence of proliferative signals, elevated activity of cyclin-CDK complexes phosphorylates pRB, leading to the dissociation of E2Fs from pRB. Consequently, E2Fs activate their transcriptional targets, thereby promoting cell cycle entry and cell division. Given the pivotal role of pRB in cell cycle control, RB1 mutations confer on cells the capacity to enter the cell cycle and divide inappropriately, or allow cells destined for cell cycle exit to remain proliferative.

1.5. Retinal specific Rb-knockout in mice

The development of a valid mouse model for retinoblastoma has proven to be difficult. In mice, germline homozygous deletion of Rb led to embryonic lethality (Jacks et al., 1992), and it was necessary to circumvent the developmental defect by the generation of Rb-mutant chimeric mice (Robanus-Maandag et al., 1998). However, generation of chimeric Rb-mutant mice was laborious and low yield, leading to the demand for a mouse model with heritable retinal specific Rb-inactivation. This demand was finally met in 2004, when several groups independently developed mouse models for retinoblastoma using the Cre-lox technology (Macpherson, 2008; Pacal and Bremner, 2006). Various promoters, including those of Nestin, Chx-10 and Pax-6,
were employed, with each promoter driving Cre expression that excises Rb in a unique spatial and temporal pattern in the developing retina (Chen et al., 2004; MacPherson et al., 2004; Zhang et al., 2004). Nevertheless, inactivation of Rb driven by the different promoters give rise to common phenotypes during retinal development, consisting of ectopic cell division, increased apoptosis and defects in differentiation. Chen et al later confirmed that E2f1 and E2f3a are the main effectors of the aberrant phenotypes induced by Rb-inactivation, as the deletion of E2f1 rescues the abnormalities in proliferation and apoptosis of Rb-deficient retinas. Although ablation of E2f3a does not rescue aberrant proliferation or apoptosis, it reverses the differentiation defect of a subset of amacrine cells (Chen et al., 2007).

Despite causing ectopic division during mouse retinal development, Rb-inactivation does not lead to retinoblastoma formation. In fact, all Rb-deficient cells eventually exit the cell cycle and differentiate into one of the seven cell types in the mature retina (Chen et al., 2004; MacPherson et al., 2004). Retinoblastoma development in mice requires the simultaneous inactivation of Rb and either one of its homologs, p107 or p130 (Chen et al., 2004; MacPherson et al., 2007; MacPherson et al., 2004; Robanus-Maandag et al., 1998).

1.6. Rb-p107 and Rb-p130 compound mutants

Collectively, pRb, p107 and p130 are known as the pocket proteins owing to the conserved pocket domain they share, which is important for E2fs binding. Like pRb, p107 and p130 are important cell cycle regulators, whose activities are modulated by cyclin-CDK complexes (Classon and Dyson, 2001; Cobrinik, 2005). In mice with p107 homozygous deletion, retinal specific inactivation of Rb leads to exacerbated ectopic division and apoptosis compared to that in mice with wild-type p107; about 60% of the compound mutant animals develops retinoblastoma (Chen et al., 2004; MacPherson et al., 2004). Mice with Rb-
inactivation in a \( p130 \) mutant background are even more tumor-prone, with all compound mutant animals developing retinoblastoma (MacPherson et al., 2007).

1.7. Multistep model of retinoblastoma development

Data from transgenic mice indicates that \( Rb \)-inactivation alone is not sufficient for retinoblastoma development. Similar evidence from the characterization of the precursor to human retinoblastoma, retinoma, also supports that \( RB1 \) inactivation alone is not sufficient for malignant tumorigenesis (Dimaras et al., 2008). Retinoma is a benign lesion that can be found adjacent to some malignant retinoblastomas. Dimaras et al showed that despite the presence of mutations in both alleles of \( RB1 \), retinoma remains non-proliferative and expresses markers of senescent cells. In addition, compared to its adjacent malignant counterpart, retinoma possesses fewer genomic alterations, suggesting that these additional changes are necessary for malignant transformation (Dimaras et al., 2008).

Therefore, data from transgenic mouse studies and retinoma supports that, like other cancers, retinoblastoma development is a multistep process that requires additional molecular alterations subsequent to the inactivation of \( RB1 \) (Vogelstein and Kinzler, 1993). Evidence suggests that with pRB inactivation alone, tumor development does not progress beyond the benign retinoma stage, perhaps due to the presence of additional tumor suppressors that prevent malignant transformation. This model suggests that malignant retinoblastoma develops only when these secondary tumor surveillance systems are abrogated due to their inactivation (Figure 1).

A major goal in retinoblastoma research is to identify the alterations subsequent to \( RB1 \) mutations that promote malignant development. Retinoblastomas exhibit high level of genomic instability presented as recurrent chromosomal gains and losses. Genomic losses
Figure 1. Multistep model of retinoblastoma development
A. Inactivation of both alleles of $RB1$ alone is not sufficient for malignant retinoblastoma development due to the presence of other tumor suppressors, which prevent tumor progression beyond the benign retinoma stage. B. Malignant retinoblastoma develops only when these tumor suppressors are inactivated, or when gain of oncogenic function allows $RB1$-mutant cells to evade tumor surveillance. The exact number of oncogene and tumor suppressor involved, and the order in which the gene modifications occurs are not known. C. Retrospective review of archived paraffin-embedded sections of eyes enucleated for retinoblastoma revealed the presence of retinoma contiguous with retina and retinoblastoma in 15.6% (20/128) of cases examined (Dimaras et al, 2008). In the cases where no retinoma was detected, retinoblastoma development might have bypassed the benign retinoma stage.
may confer growth advantages to corrupted cells by deletions of tumor suppressor genes. Alternatively, copy number gain of oncogenes due to genomic instability may enhance proliferative capacity of corrupted cells, or enable mutant cells to evade clearance by tumor surveillance mechanisms.

1.8. Genomic changes in retinoblastoma

Early karyotype analyses of retinoblastomas reported frequent gain of chromosome 1 and 6, and the loss of chromosome 16 (Benedict et al., 1983; Chaum et al., 1984; Kusnetsova et al., 1982; Pogosianz and Kuznetsova, 1986; Squire et al., 1985). With the advent of the comparative genomic hybridization (CGH) methodology, several groups were able to characterize genomic changes in retinoblastomas at higher resolution (Chen et al., 2001; Herzog et al., 2001; Lillington et al., 2003; Mairal et al., 2000; van der Wal et al., 2003; Zielinski et al., 2005). Based on the six published CGH studies, the most frequently gained regions in retinoblastomas are 1q (53%) and 6p (54%), with the most frequently lost region being 16q (32%) (Corson and Gallie, 2007). Genomic changes in retinoblastomas often encompass multiple genes, making it challenging to deduce the true target that drives the gain or loss of a particular chromosomal region. Nevertheless, based on the premise that the true target gene(s) is differentially expressed in retinoblastoma compared to normal tissue, genes that showed similar levels of expression in both tumor and normal tissues can be excluded and considered “passengers” because of their genomic proximity to the true cancer genes. This approach of identifying tumor suppressors and oncogenes has yielded several interesting candidates, which may be useful for future therapeutic development.
1.8.1. 16q loss

1.8.1.1. CDH11

Marchong et al identified CDH11 and CDH13 as the most frequently lost genes on 16q based on published CGH results and microsatellite markers on 16q (Marchong et al., 2004). Expression analysis showed a reduction in protein expression for CDH11, but not CDH13 in retinoblastomas compared to normal adult retinas, suggesting that CDH11 is a target for 16q loss in retinoblastomas (Marchong et al., 2004). CDH11 belongs to the cadherin family of membrane bound glycoproteins involved in cell-cell adhesion. Homozygous deletion of Cdh11 led to faster growing retinoblastomas in the TAg-RB murine model (Marchong and Yurkowski, personal communication).

1.8.1.2. RBL2

Although not as frequently lost as CDH11, another interesting gene located on 16q is RBL2, encoding p130, which strongly cooperated with pRb in suppressing mouse retinoblastoma development (MacPherson et al., 2007; MacPherson et al., 2004). MacPherson et al showed that deletion of p130 in concert with Rb led to aggressive bilateral retinoblastoma, while no tumor was observed with Rb inactivation alone (MacPherson et al., 2007; MacPherson et al., 2004). In human, Dimaras et al showed that p130 protein was strongly expressed in the benign retinoma, but its expression was decreased in adjacent malignant retinoblastoma (Dimaras et al., 2008). Using similar techniques, Bellan et al also showed a reduction of p130 in their retinoblastoma cohort (Bellan et al., 2002). Cautions have to be taken while interpreting these data since p130 protein is cell cycle regulated and is normally not present in proliferating cells (Tedesco et al., 2002). Further studies are required
to confirm that the reduced p130 expression observed in retinoblastomas is tumor specific due to genomic loss, and not the result of proliferation.

1.8.2. 6p gain

1.8.2.1. DEK & E2F3

*DEK* and *E2F3* are implicated to be the target genes of 6p gain in retinoblastomas (Grasemann et al., 2005; Orlic et al., 2006). *DEK* is a phosphoprotein with suggested roles in transcriptional regulation, chromatin architecture and mRNA splicing (Waldmann et al., 2004). Its level of expression correlates with histological grade, aggressiveness or invasiveness in several cancer types (Kondoh et al., 1999; Lu et al., 2005; Sanchez-Carbayo et al., 2003; Savli et al., 2002). *E2F3* encodes a transcription factor that regulates the cell cycle and proliferation, and has been shown to be the target of 6p gain in bladder cancer (Oeggerli et al., 2006; Olsson et al., 2007; Veltman et al., 2003). In a retinoblastoma cell line with extra copies of *DEK* and *E2F3*, shRNA-knockdown of *DEK* or *E2F3* led to decreased proliferation, while *DEK* knockdown also led to cell death (Orlic et al, submitted). This data indicates that *DEK* and *E2F3* play important roles in proliferation, and survival in the case of *DEK*, and may serve as useful therapeutic targets in retinoblastomas.

1.8.3. 1q gain

1.8.3.1. KIF14

Based on CGH results and analysis of sequence tagged site (STS) markers on 1q, Corson et al identified the most frequently gained region to be within the chromosomal band 1q32 (Corson et al., 2005). Among the genes within that region, *KIF14* is the only gene up-
regulated in retinoblastomas compared to normal adult retinas (Corson et al., 2005). *KIF14* encodes a mitotic kinesin involved in cytokinesis (Carleton et al., 2006; Gruneberg et al., 2006). *KIF14* knockdown using siRNA in cervical cancer and non-small cell lung cancer cell lines leads to reduced proliferation and colony formation on soft agar (Corson et al., 2007). Similar results were also observed in retinoblastoma and ovarian cancer cell lines (Brigitte Theriault, personal communication).

1.8.3.2. MDM4

Laurie et al identified *MDM4* as another candidate oncogene of 1q gain in retinoblastomas (Laurie et al., 2006). MDM4 is a negative regulator of the p53-tumor suppressor, by inhibiting p53-transcriptional activity and stabilizing MDM2, which is an E3-ligase that ubiquitinylates p53 for proteasomal degradation (Marine and Jochemsen, 2004). Therefore, overexpression of MDM4 is one way to inactivate the p53-tumor suppressor pathway in cancer development (Toledo and Wahl, 2006). In mice, inactivation of p53 accelerates the kinetics of retinoblastoma development in *Rb-p107* compound mutants (Zhang et al., 2004). In human, Laurie et al reported copy number gain of *MDM4*, using fluorescence in situ hybridization (FISH), in 63% of retinoblastomas examined (Laurie et al., 2006). *MDM4* mRNA and protein are overexpressed in human retinoblastomas compared to normal fetal retinas, and ectopic expression of *Mdm4* leads to more aggressive tumors in mice with *Rb* and *p107* deletions (Laurie et al., 2006). Guo et al examined *MDM4* expression in another cohort of retinoblastomas using normal adult retinas as controls, and found no differential expression in *MDM4* mRNA or protein (Guo et al., 2008). This suggests that *MDM4* may be up-regulated during normal retinal development, which should be taken into account when targeting *MDM4* for therapy, since the therapeutic window for cancer-specific
killing may narrow as the patients age. Further analyses of MDM4 expression in normal retinas of different ages relevant to retinoblastoma (eg. at birth and early childhood) are required.

In another study, Dimaras et al examined both KIF14 and MDM4 copy number changes on chromosome 1q simultaneously in retinoblastomas using FISH (Dimaras et al., 2008). They found that MDM4 gain was observed only in combination with gain of KIF14, while KIF14 showed genomic gain even when MDM4 copy number normal. This suggests that KIF14 may be the more important 1q oncogene in retinoblastoma development.

1.9. ARF and the p53-pathway in retinoblastoma

The p53-pathway serves as an important tumor surveillance mechanism that responds to oncogenic signals by triggering cell death or cell cycle arrest, and its inactivation is an obligatory step in many cancers (Whibley et al., 2009). Retinoblastoma was believed to be an exception since no mutation in TP53 has ever been reported. Laurie et al reported MDM4 and MDM2 copy number gain in 63% and 10% of retinoblastomas, respectively, inferring that the p53-pathway is functionally inactivated in these tumors (Laurie et al., 2006). A key component of the p53-pathway is the upstream activator, ARF, which transmits oncogenic signals to activate p53 by inhibiting the p53-antagonist MDM2. ARF is frequently inactivated in cancers, resulting in a compromised p53-tumor surveillance pathway. The role of ARF in retinoblastoma was dismissed by Laurie et al., who reported an elevation of ARF mRNA in retinoblastomas compared to normal fetal retinas, and proposed that high ARF expression is functionally irrelevant due to overexpression of MDM4 and MDM2 (Laurie et al., 2006). However, Guo et al showed that despite robust mRNA expression, ARF protein was undetectable in retinoblastomas (Guo et al., 2008). This observation raises the possibility that
low level of ARF protein may prevent p53 activation, and contribute to silencing of the p53-pathway in retinoblastoma.

1.10. Role of ARF in the crosstalk between the pRB- and p53-pathway

*In vitro* and *in vivo* studies suggest that ARF is important in conveying the signal initiated by pRB inactivation to trigger p53-mediated tumor surveillance during cancer development. The immediate consequence of pRB inactivation is the derepression of E2F1 transcription factor, which, in certain contexts, is capable of inducing ARF transcription. E2F1 regulates ARF through a novel E2F-responsive element that varies from the typical E2F site (Komori et al., 2005). This element responds to the aberrant E2F1 activity resulting from pRB inactivation or ectopic E2F1 expression, but not to the normal physiological fluctuation of E2F1 during the cell cycle. Therefore, upon pRB inactivation, aberrant E2F1 activity may activate ARF, which in turn activates p53 to suppress tumorigenesis. Indeed, ARF-mediated tumor suppression was demonstrated in a mouse pituitary tumor model initiated by Rb mutation, in which Arf is activated upon Rb-inactivation (Tsai et al., 2002). Moreover, Arf-knockout animals with Rb mutations developed more aggressive pituitary tumors than those carrying normal copies of Arf.

1.11. INK4a/ARF in cancer development

ARF is encoded by the INK4a/ARF locus, which also codes for the important tumor suppressor, p16\textsuperscript{INK4a} (Figure 2). ARF and p16\textsuperscript{INK4a} share common exon-2 and -3 while having unique promoters and exon-1. The two proteins have no sequence homology, resulting in entirely different molecular functions (Sherr, 2001). p16\textsuperscript{INK4a} prevents phosphorylation of the pocket proteins by inhibiting CDK4/6, thereby blocking cell cycle progression. Interestingly,
**Figure 2. Schematic of the INK4a/ARF locus**

ARF and p16\(^{\text{INK4a}}\) share exon-2 (E2) and exon-3 (E3), while having unique promoters and exon-1 (E1) that recruit different sets of transcription factors. ARF and p16\(^{\text{INK4a}}\) proteins have no sequence homology, since translation of the two genes utilizes different reading frames. Expression of ARF and p16\(^{\text{INK4a}}\) can be repressed coordinately by PcG proteins and CDC6 through chromatin remodeling.
it is highly expressed in the benign retinoma, but not in malignant retinoblastoma, suggesting a potential tumor suppressor role of p16\(^{INK4a}\) in retinoblastoma development (Dimaras et al., 2008). However, ectopic expression of p16\(^{INK4a}\) in \(RB1^{−}\) retinoblastoma cells did not induce cell cycle arrest (unpublished data). This result is consistent with previous report, which shows that p16\(^{INK4a}\) function requires intact pRB (Bruce et al., 2000). Therefore, p16\(^{INK4a}\) is unlikely to play an important role in retinoblastoma development due to the lack of functional pRB.

In many cancers, \(ARF\) is inactivated through co-deletion with \(p16^{INK4a}\) (Rocco and Sidransky, 2001). Deletions affecting only exon-1\(β\) (specific to \(ARF\)) have also been observed, but not as frequently as co-deletion with \(p16^{INK4a}\) (Freedberg et al., 2008). The function of \(ARF\) can also be abolished through promoter methylation that shuts down \(ARF\) transcription (Esteller et al., 2000; Freedberg et al., 2008). In some cancers, transcriptional repressors of \(ARF\), including BMI-1, CBX-7, POKEMON, TBX-2 and -3, are overexpressed compared to their normal cellular counterparts, thereby preventing \(ARF\) activation during tumor development (Bernard et al., 2005; Maeda et al., 2005; Scott et al., 2007; Silva et al., 2006; Sinclair et al., 2002; Vance et al., 2005; Vonlanthen et al., 2001; Yarosh et al., 2008).

The functional relevance of \(ARF\) inactivation observed in human cancers has been validated using transgenic mouse models. Mice with \(Arf\)-specific deletion in exon-1\(β\), which spares \(p16^{Ink4a}\) function, are highly prone to developing lymphoma, soft tissue sarcoma, lung carcinoma and osteosarcoma (Kamijo et al., 1997; Sharpless et al., 2004). Mouse embryonic fibroblasts (MEFs) derived from \(Arf^{−}\) mice exhibit defects in oncogenic- and culture-induced growth arrest \textit{in vitro}, and are highly susceptible to oncogenic transformation compared to wild-type MEFs.
The roles of Arf in specific tissues were addressed by deleting Arf in various mouse cancer models. In a melanoma model induced by expression of the activated H-RAS oncogene, Arf deletion significantly decreased the latency of melanoma development (Sharpless et al., 2003). In a mouse model of B-cell lymphoma initiated by expression of the c-Myc oncogene driven by the immunoglobulin heavy chain enhancer (Eμ), Arf inactivation drastically reduced the mean survival time of Eμ-Myc animals. Moreover, tumors in Eμ-Myc animals that were Arf+/+ and Arf+/- sustained spontaneous deletions of Arf, mutations in p53, or overexpression of Mdm2, indicating the strong selection for tumor cells with loss of function in the Arf-p53-tumor surveillance pathway (Eischen et al., 1999).

1.12. ARF protein and functions

1.12.1. ARF protein

ARF is a small protein comprised of 173 amino acids in human and 169 amino acids in mouse. The protein is highly basic, made up of 20% arginine residues that are widely spread throughout the molecule. ARF primarily localizes to the nucleolus, an intranuclear organelle mainly implicated in ribosome biosynthesis, where it interacts and forms complexes with nucleophosmin (NPM, also known as B23) (Bertwistle et al., 2004; Brady et al., 2004; Itahana et al., 2003). Despite the lack of lysine residue, a common substrate for polyubiquitylation, ARF protein-turnover is regulated by the ubiquitin-proteasome pathway through ubiquitinylation at its N-terminus (Kuo et al., 2004). The stability of ARF is highly dependent on its interaction with NPM, as the disruption of ARF-NPM binding by the introduction of ARF mutations, or by the reduction in NPM level resulting from RNAi-knockdown, significantly accelerates the degradation of ARF by the proteasome (Kuo et al.,
The protein segment encoded by exon-1β is required and sufficient for its binding to NPM (Bertwistle et al., 2004), and also for its p53-dependent function, as enforced expression of a synthetic ARF exon-1β mini-gene, excluding exon-2, was sufficient to elicit a p53-dependent response (Weber et al., 2000).

Interestingly, translation of ARF mRNA initiated from an internal start site produces an N-terminus truncated isoform. When overexpressed in vitro, this short form of ARF, lacking all the amino acid residues required for NPM binding and p53-dependent activities, triggers autophagy, a process usually initiated in response to nutrient deprivation in order to digest cellular components for energy derivation (Reef et al., 2006).

1.12.2. p53-dependent functions

The best characterized function of ARF is to activate p53 in response to various oncogenic signals (Gil and Peters, 2006). Once activated, ARF localizes to the nucleolus, where it binds and sequesters MDM2, thereby enabling p53 to accumulate in the nucleoplasm (Tao and Levine, 1999). An important function of p53 is to induce expression of target genes by associating into homotetrameric transcription factor (Levine et al., 2006). The functions of p53-target genes fall into several categories. A set of genes are involved in cell cycle arrest, including p21, 14-3-3σ and GADD45 (Levine et al., 2006). Another set of genes important for tumor suppression are those involved in apoptosis, which can be subdivided into the intrinsic and the extrinsic apoptotic pathways. In the intrinsic pathway, p53 activates the expression of pro-apoptotic genes BAX, NOXA and PUMA (Haupt et al., 2003). In the extrinsic pathway, p53 up-regulates the cell surface transmembrane receptors Fas (Muller et al., 1998) and DR5/KILLER (Wu et al., 1997), which transmit extrinsic death signals to activate intracellular apoptotic machineries (Nicholson and Thornberry, 2003). In addition,
p53 can exit the nucleus to act upon the mitochondria and its associated proteins, BCL-2 and BCL-XL, in order to promote apoptosis (Chipuk et al., 2004; Moll et al., 2005).

**1.12.3. p53-independent functions**

**1.12.3.1. Evidence from compound mutant studies**

Studies using transgenic mice establish that Arf has additional functions outside the p53-pathway. In the pituitary tumor model driven by Rb mutation, either Arf or p53 cooperated with Rb in tumor suppression; however, the types of lesions in Arf-null and p53-null mice did not completely overlap, providing the first hint that Arf and p53 might not function in a strict linear manner (Tsai et al., 2002). To address the dependency of Arf function on p53, mice nullizygous for Arf, mdm2 and p53 (TKO) were generated, and these animals developed multiple tumors at a frequency greater than those lacking both p53 and Mdm2 or p53 alone. Moreover, ectopic expression of Arf in MEFs lacking p53 and Mdm2 was able to induce cell cycle arrest, further supporting p53-independent functions of Arf (Weber et al., 2000). In addition to its role in tumor suppression, Arf is also implicated in normal eye development, independent of p53. Deletion of Arf leads to defects in developmental regression of the hyaloid vascular system in the eye, and the condition persists even when p53 is co-deleted with Arf (McKeller et al., 2002).

In addition to MDM2, ARF physically interacts with more than 25 other proteins, and some of these interactions have been suggested to convey p53-independent functions (Sherr, 2006). Nonetheless, these studies were performed *in vitro* using supra-physiological levels of ARF, and these findings, briefly discussed in the following sections, remain to be validated *in vivo* under physiological conditions.
1.12.3.2. Regulation of transcription factors

Evidence suggests that ARF can directly regulate the activity of the proto-oncogene E2F1, independent of p53. In p53-null cells, ARF binds and inhibits E2F1, and DP1, a dimerization partner of E2F1 that is essential for transactivation of E2F1-target genes (Datta et al., 2002; Datta et al., 2005; Eymin et al., 2001; Martelli et al., 2001). In addition, ARF can also antagonize the proto-oncogene c-MYC in a number of ways. ARF binds and inhibits HECTH9, an E3 ubiquitin ligase known to catalyze c-MYC polyubiquitinylation, a process that enhances c-MYC transactivating activity (Chen et al., 2005). ARF has also been shown to bind directly to the MYC BOX II domain of c-MYC (Amente et al., 2006), thereby preventing c-MYC association with TIP60, a histone acetyl transferase essential for transactivation mediated by c-MYC (Frank et al., 2003). ARF was also found to antagonize other transcription factors implicated in cancers, including the forkhead box family member FOXM1b (Costa et al., 2005; Kalinichenko et al., 2004), B-cell lymphoma-6 (BCL6) (Suzuki et al., 2005) and MYCN (Amente et al., 2007).

1.12.3.3. Ribosome biogenesis

When activated, ARF protein primarily localizes to the nucleolus, an intranuclear organelle mainly implicated in ribosome biosynthesis. *In vitro*, enforced expression of ARF inhibits rRNA transcription (Ayrault et al., 2004) and processing (Itahana et al., 2003; Sugimoto et al., 2003) in p53-null cells, and disrupts ribosome export from the nucleus to the cytoplasm (Brady et al., 2004; Yu et al., 2006).
1.12.3.4. Link to ATM and ATR

Several studies suggest that ARF can influence the activity of ATR- and ATM- kinase through mechanisms independent of the MDM2-p53 axis. Overexpression of ARF in cells lacking functional p53 activates the ATR- and ATM-signaling cascade (Eymin et al., 2006). In addition, expression of ARF triggers ATR and leads to phosphorylation of NFκB. This promotes the association of NFκB with histone deacetylase 1, thereby inhibiting the transactivating function of NFκB (Rocha et al., 2003; Rocha et al., 2005).

1.12.3.5. Sumoylation

When overexpressed, ARF promotes sumoylation of several of its binding partners (Chen and Chen, 2003; Rizos et al., 2005; Tago et al., 2005; Woods et al., 2004; Xirodimas et al., 2002). Although the functional relevance of ARF-induced sumoylation has not been fully elucidated, sumoylation of the Werner Syndrome Helicase (WRN) by ARF is associated with the relocalization of WRN from the nucleolus to the nucleoplasm (Woods et al., 2004).

1.12.3.6. smARF and autophagy

Initiation of ARF translation from an internal start site produces a short truncated form that lacks the N-terminus, termed short mitochondrial ARF (smARF). Overexpression of smARF in p53-null cells leads to its localization in the mitochondria, and the reduction of mitochondrial membrane potential, thereby triggering autophagy and cell death (Reef et al., 2006).
1.13. Regulation of ARF expression

1.13.1. Transcriptional repressors

ARF expression is tightly regulated and its deregulation may lead to detrimental physiological defects. For instance, Arf is normally repressed by Bmi-1 in the stem cell compartment within the nervous system. Deletion of Bmi-1 leads to aberrant activation of Arf, causing defects in stem cell renewal, forebrain proliferation and gut neurogenesis. These phenotypes are in part rescued by deletion of Arf in Bmi-1− mice (Molofsky et al., 2005).

BMI-1 is a member of the Polycomb group (PcG) of transcriptional repressors. PcG proteins silence transcription by participating in multi-component complexes, PRC1 and PRC2, which generate and recognize histone modifications in order to form Polycomb bodies at specific chromosomal locations (Gil and Peters, 2006; Lund and van Lohuizen, 2004; Otte and Kwaks, 2003). Given their mode of action at the chromatin level, gene silencing by PcG proteins often has a wide spread effect, affecting both ARF and p16INK4a. In addition to BMI-1, other PcG proteins, CBX2, CBX7, MEL18, PHC2 and RING1b, are also implicated in INK4a/ARF repression (Core et al., 2004; Gil et al., 2004; Isono et al., 2005; Voncken et al., 2003). Besides repression by PcG proteins, INK4a/ARF and neighboring gene p15INK4b are regulated coordinately through a DNA replication origin upstream of p15INK4b. Loading of CDC6 to this origin recruits histone deacetylases and increases methylation of histone H3, thereby promoting heterochromatin formation and gene silencing (Gonzalez et al., 2006).

In addition to PcG proteins and CDC6, ARF transcription is regulated by a number of other molecules, demonstrated mainly by knockout studies in mice. MEFs derived from mice deficient of Atm (Kamijo et al., 1999), JunD (Weitzman et al., 2000), Egr1 (Krones-Herzig et al., 2003), Twist (Maestro et al., 1999) or E2f3 (Aslanian et al., 2004) exhibit elevated
expression of Arf compared to wild-type MEFs. In addition, Pokemon, encoded by the Zbtb7 gene, represses Arf promoter directly. MEFs lacking Zbtb7 are refractory to oncogene-mediated cellular transformation, in part attributed to Arf hyperactivity in the absence of Pokemon (Maeda et al., 2005). Other direct repressors of ARF include the T-box proteins TBX2 and TBX3, which bind the T-box element within the ARF promoter. Ectopic expression TBX2 or TBX3 in cultured cells enables the bypass of senescence in vitro, in part by suppressing ARF activation (Brummelkamp et al., 2002; Jacobs et al., 2000; Lingbeek et al., 2002).

1.13.2. Translational repressor

In addition to transcriptional regulation, ARF expression is also modulated at the translational level by the microRNA miR-24. MicroRNAs are endogenous short RNAs (approximately 23 nucleotides) that play important gene-regulatory roles in animals and plants by pairing to the mRNAs of protein-coding genes in order to direct their post-transcriptional repression (Bartel, 2009). Enforced expression of miR-24 in HeLa cells down-regulates ARF protein production; conversely, knockdown of miR-24 using antisense oligonucleotides leads to the accumulation of ARF protein (Lal et al., 2008).

1.13.3. Transcriptional activators

ARF expression can be activated by several upstream pathways with oncogenic properties. Ectopic expression of c-Myc in MEFs activates Arf gene expression and apoptosis (Zindy et al., 1998). Ras-signaling induces Arf expression through the activation of transcription factor Dmp1 (Inoue et al., 1999; Palmero et al., 1998; Sreeramaneni et al., 2005). Alternatively, Ras can activate the AP-1 family members, c-Jun and Fra-1, which
assemble into heterodimeric transcription factors that drive Arf gene expression (Ameyar-Zazoua et al., 2005). β-catenin is also implicated in Arf activation; its ectopic expression in MEFs leads to Arf accumulation, in part through E2f1, given that Arf induction is significantly weakened in E2f1-null MEFs (Damalas et al., 2001). DAP-kinase, a pro-apoptotic serine/threonine kinase, seems to link oncogenic signals to Arf activation. Ectopic expression of DAP-kinase induces Arf-dependent apoptosis, while its inactivation significantly weakens c-Myc or E2f1 induced apoptosis (Raveh et al., 2001).
Chapter 2: Role of ARF and the p53-pathway in retinoblastoma development

2.1. Introduction

The p53-pathway is corrupted in many cancers, either by mutation in ARF or TP53, or by overexpression of the p53-antagonists MDM2 or MDM4 due to genomic gain. In retinoblastomas, mutation in TP53 has never been observed. Recent study suggests that the p53-pathway is disrupted by genomic gain of MDM4 or MDM2 in a subset of retinoblastomas (Laurie et al., 2006). However, the role of ARF, an upstream activator of the p53-pathway, in retinoblastoma development remains unclear. ARF mRNA expression is highly induced in retinoblastomas compared to normal retinas (Guo et al., 2008; Laurie et al., 2006), but ARF protein is undetectable in neither tumors nor retinas (Guo et al., 2008).

2.2. Hypotheses

ARF mRNA transcription is activated in response to RB1 mutation; however, inhibition of ARF translation, or instability of the ARF protein, prevents ARF-mediated activation of the p53-tumor surveillance pathway during retinoblastoma development.

2.3. Thesis aims & rationales

2.3.1. Determine the cause of low ARF protein in retinoblastomas

Translation of ARF mRNA is known to be regulated by a microRNA, miR-24 (Lal et al., 2008), and ARF protein-turnover is controlled by the ubiquitin-proteasome pathway (Kuo et
al., 2004). These mechanisms were assessed in order to determine the cause of low ARF protein expression despite the abundance of ARF mRNA.

2.3.2. Examine the expression of Arf, p53 & p53-transcriptional targets during early retinoblastoma development in a mouse retinoblastoma model

It is unclear whether ARF expression is consistently low during the entire course of retinoblastoma development, or ARF expression is induced during early tumor development but subsequently extinguished due to oncogenic insults. The expression of Arf, p53 and p53-transcriptional targets were determined in a mouse model of retinoblastoma, in order to evaluate the activity of this pathway during early retinoblastoma development.

2.3.3. Determine the effect of exogenous ARF expression in retinoblastoma cells

Expression studies show that MDM2 and MDM4 are expressed in retinoblastomas and may even be overexpressed in a subset of the tumors, while the upstream activator of the pathway, ARF, was undetectable at the protein level (Guo et al., 2008; Laurie et al., 2006). It is unclear whether increased level of ARF protein could activate p53 despite high level of MDM2 and MDM4. The effect of ARF overexpression on cell growth and gene expression of retinoblastoma cells was determined.

2.4. Material and methods

2.4.1. Tissue culture

All cell lines were grown in a humidified 37°C incubator with 5% CO$_2$ in their respective tissue culture media (Tissue Culture Media Facility, PMH/OCI, ON, Canada) with the addition of 100 U/ml penicillin and 0.1 mg/ml streptomycin (Wisent Bioproducts). Media
and supplements used for each cell line were as follows. Retinoblastoma cell lines: Iscove's MDM with 15% FetalClone III (HyClone), 10 mg/L insulin (Sigma-Aldrich) and 0.0004% (v/v) β-mercaptoethanol; HEK-293: alpha-MEM with 10% FBS (Wisent Bioproducts); HEK-293T and HeLa: DMEM-H16 with 10% FBS; Saos-2 and U2OS: DMEM-H21 with 10% FBS; OVCAR-3: RPMI-1640 with 20% FBS and 10 mg/L insulin; MEFs: DMEM-H16 with 15% FBS and 4 mM L-glutamine (Wisent Bioproducts); NIH-3T3: DMEM-H16 with 10% calf serum (Wisent Bioproducts).

To study the role of proteasome-mediated protein degradation in retinoblastoma cell lines, cells were incubated in culture media containing 50 µM of the proteasome inhibitor MG132 (Sigma-Aldrich) for 10 hours and then harvested for Western Blot analysis.

2.4.2. Mouse model of retinoblastoma

Mice with retinal specific Rb-inactivation were used for studying early retinoblastoma development. Rb<sub>loxp/loxp</sub>, α-Cre; Rb<sub>loxp/loxp</sub> and α-Cre; Rb<sub>loxp/loxp</sub>; p107<sup>−/−</sup> mice were kind gifts from Dr. Rod Bremner (Toronto Western Research Institute, ON, Canada). For simplicity, animals with the genotype Rb<sub>loxp/loxp</sub> and α-Cre; Rb<sub>loxp/loxp</sub> were referred to as wild-type and Rb-knockout, respectively. In Rb-knockout animals, Rb is flanked by loxp sites and is excised by a Cre-recombinase driven by the α-enhancer of Pax-6 beginning from embryonic day 10 in the peripheral retina. Inactivation of Rb in the p107<sup>−/−</sup> background leads to retinoblastoma with 60% penetrance, while Rb-mutant retina in the p107<sup>−/+</sup> background is not tumor prone (Chen et al., 2004). Mice were maintained, handled and sacrificed using protocols approved by the University Health Network Animal Resource Centre (ON, Canada). For genotyping, genomic DNA was extracted from mouse tail. PCR was performed in a RoboCycler Gradient 96 thermal cycler (Stratagene). Unless otherwise stated, PCR
began with 2 minutes at 94°C, followed by 30 cycles of amplification and ended with 10 minutes at 72°C, and the reaction mixture consisted of 200 µM dNTPs, 2.5 mM MgSO₄, 0.5 µM each of forward and reverse primers, homemade Taq polymerase and reaction buffer, in a final volume of 25 µl (Table 1).

**Table 1. Primer sequences and PCR conditions for genotyping**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>PCR condition</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>5'-CCTGATGGACATGTTCAGGG-3' 5'-CTTCAGGTTCTGCGGGAAAC-3'</td>
<td>94°C 50 s, 55°C 30s, 72°C 50 s</td>
<td>120</td>
</tr>
<tr>
<td>p107</td>
<td>5'-TCGTGAGCGGATAGAAAG-3' 5'-GTGTCCAGCAGAAGTTA-3' 5'-CCGCTTCCATTGCTCAGCGG-3'</td>
<td>94°C 50 s, 54°C 50s, 72°C 1 min</td>
<td>386 (Knockout) 513 (Wild-type)</td>
</tr>
<tr>
<td>Rb&lt;sup&gt;loxP&lt;/sup&gt;</td>
<td>5'-GGCGGTGTCGCGATCAATG-3' 5'-CTCAAGAGCTCAGACTCATGG-3'</td>
<td>94°C 50 s, 54°C 50s, 72°C 1 min</td>
<td>235 (Wild-type) 283 (loxP)</td>
</tr>
</tbody>
</table>

**2.4.3. Tissue harvesting and fixation**

To generate tissue sections for immunofluorescence microscopy, heads of euthanized neonatal mice were fixed overnight in 4% paraformaldehyde at 4°C and subsequently stored in 70% ethanol at 4°C. For mice over the age of five days, fixed heads were decalcified in 8% formic acid before transferring into 70% ethanol. Fixed mouse heads were embedded in paraffin blocks and cut into 5 µm sections (Pathology, Hospital for Sick Children, ON, Canada). To examine the histology of tissue sections, slides were stained with hemotoxylin and eosin, and scanned using the Aperio ScanScope XT.

For extraction of retinal total RNA and protein, eyes were removed from euthanized mice at different ages, and dissected to extract the retinas under a dissecting microscope. Retinas from 2 to 3 mice of the same genotype were pooled and frozen in dry ice immediately and subsequently stored at -70°C. Littermate controls were used for expression analyses.
To embed MEFs in paraffin block, cells were first harvested from tissue culture flasks using trypsin and then fixed in 4% paraformaldehyde for 15 minutes at room temperature, followed by three washes in PBS. Fixed cells were resuspended in 1% agarose and allowed to solidify. The agarose block was subsequently paraffin embedded and sectioned as previously described.

To fix the retinoblastoma cell line WERI for immunofluorescence staining, cells grown on a coverslip were incubated in 4% paraformaldehyde for 15 minutes at room temperature, followed by three washes in PBS.

2.4.4. RT-PCR

Total RNA was extracted from cell lines and mouse retinas using TRIzol (Invitrogen) according to the manufacturer’s instructions. Mouse retinas resuspended in TRIzol were dissociated by passage through a gauge 20.5 needle prior to the addition of chloroform. The concentration and quality of total RNA were determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using random primers (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen). For gene expression analyses, PCR was performed using a RoboCycler Gradient 96 thermal cycler (Stratagene). Thermal cycling conditions for the genes examined were listed in Table 2. Unless otherwise stated, PCR began with 2 minutes at 94°C, followed by 30 cycles of amplification and ended with 10 minutes at 72°C, and the reaction mixture consisted of 200 µM dNTPs, 2.5 mM MgSO₄, 0.5 µM each of forward and reverse primers, 0.5 U KOD hot start DNA polymerase (Novagen), 1x PCR buffer (Novagen) and 1 µl of product from cDNA synthesis, in a final volume of 25 µl.
### Table 2. Primer sequences and PCR conditions for human and mouse gene expression analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>PCR condition</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>5'-TGGGTCCAGTCGCAGTTA-3' 5'-CCTGTAGGACCTTCCGGTGAC-3'</td>
<td>94°C 50 s, 56°C 30 s, 72°C 1 min, 0.2 M betaine</td>
<td>683</td>
</tr>
<tr>
<td>GADD45</td>
<td>5'-GAGAGCAGAAGACCGAAAGG-3' 5'-CCTGTAGGACCTTCCGGTGAC-3'</td>
<td>94°C 50 s, 58°C 30 s, 72°C 1 min</td>
<td>591</td>
</tr>
<tr>
<td>NOXA</td>
<td>5'-CACCCTGTAGGACCTTCCGGTGAC-3' 5'-TTCCATCTCTCCTCCCAAG-3'</td>
<td>94°C 50 s, 58°C 30 s, 72°C 1 min</td>
<td>474</td>
</tr>
<tr>
<td>TBP-1</td>
<td>5'-ACACACCCTGCACCCTTCCTCG-3' 5'-GCTGGAAAACCCAACTCTC-3'</td>
<td>94°C 50 s, 60°C 30 s, 72°C 1 min</td>
<td>743</td>
</tr>
<tr>
<td><strong>Mouse genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-3-3σ</td>
<td>5'-GAAGGCTCGACCTGCAAGAAGG-3' 5'-GTAGGGAGGATGCTCCTG-3'</td>
<td>94°C 50 s, 63°C 30 s, 72°C 1 min</td>
<td>581</td>
</tr>
<tr>
<td>Arf</td>
<td>5'-GTGCGAGCTTTCCGGTGAC-3' 5'-AGGGCTCGACCTGCAAGAAGG-3'</td>
<td>94°C 50 s, 60°C 30 s, 72°C 1 min</td>
<td>484</td>
</tr>
<tr>
<td>Bax</td>
<td>5'-GCACTCCAAAGGTCGACCTG-3' 5'-ATGCTCAGCTGCTGCAAGAAGG-3'</td>
<td>94°C 50 s, 59°C 30 s, 72°C 1 min, 0.2 M betaine</td>
<td>599</td>
</tr>
<tr>
<td>Gadd45</td>
<td>5'-AGGGGAGGATGCTCCTG-3' 5'-TGAGGGAGGATGCTCCTG-3'</td>
<td>94°C 50 s, 57°C 30 s, 72°C 1 min</td>
<td>598</td>
</tr>
<tr>
<td>Mdm2</td>
<td>5'-TGCTCAAGCCTCAGACTTAC-3' 5'-CAGGCTCGACCTGCAAGAAGG-3'</td>
<td>94°C 50 s, 57°C 30 s, 72°C 1 min</td>
<td>555</td>
</tr>
<tr>
<td>Noxa</td>
<td>5'-GGTGCTCAGCTGCTGCAAGAAGG-3' 5'-TGAGGGAGGATGCTCCTG-3'</td>
<td>94°C 50 s, 59°C 30 s, 72°C 1 min</td>
<td>562</td>
</tr>
<tr>
<td>p21</td>
<td>5'-CGAGCGTTCATCAGCTGCTGCAAGAAGG-3' 5'-TGAGGGAGGATGCTCCTG-3'</td>
<td>94°C 50 s, 57°C 30 s, 72°C 1 min</td>
<td>558</td>
</tr>
<tr>
<td>Puma</td>
<td>5'-GCGCTTAATGCAAGCAAGACACACTC-3' 5'-GGTGCTCAGCTGCTGCAAGAAGG-3'</td>
<td>94°C 50 s, 59°C 30 s, 72°C 1 min, 0.2 M betaine</td>
<td>599</td>
</tr>
<tr>
<td>Tbp-1</td>
<td>5'-GGGAGAATCATGAGACCAGAC-3' 5'-ATGATGACTGCTGCAAGAAGG-3'</td>
<td>94°C 50 s, 60°C 30 s, 72°C 1 min, 28 cycles</td>
<td>544</td>
</tr>
</tbody>
</table>

#### 2.4.5. Real-time PCR

RNA extraction and cDNA synthesis were performed as described in the previous section. Real-time PCR was performed in a 7900HT Fast Real-Time PCR system using the Universal PCR Master Mix in a 384-wells plate (Applied Biosystem). Taqman gene
expression assays (Applied Biosystem) were employed to measure the mRNA expression of ARF (Hs99999189_m1), GAPDH (Hs99999905_m1), HPRT-1 (Hs99999909_m1) and TBP-1 (Hs99999910_m1) in triplicate. Mean relative gene expression and standard deviation were determined using the ΔΔCt method built-in to the SDS 2.2 software (Applied Biosystem). GAPDH was used as the endogenous control gene and HeLa was used as the calibrator sample. For expression analysis of microRNAs, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer’s instructions (Applied Biosystem). Taqman gene expression assays (Applied Biosystem) were used for the detection of miR-24 (hsa-miR-24), RNU6B (Part # 4373381) and U18 (Part # 4380904) in triplicate. Mean relative gene expression and standard deviation were determined by the ΔΔCt method using RNU6B or U18 as the endogenous control and HeLa as the calibrator sample.

2.4.6. Western Blotting

Cell lines and mouse retinas were resuspended in lysis buffer consisting of phosphate buffered saline, 1% Nonidet P40, 5% sodium deoxycholate, 0.1% SDS, 1.0 µg/ml leupeptin, 0.1 mM PMSF, 1.0 µg/ml aprotinin and 100 µM sodium orthovanadate. Cells and tissues were dissociated mechanically and lysed by three cycles of freezing and thawing, followed by a 30 minutes incubation on ice, and centrifugation to remove cellular debris. Protein concentration was determined using the BioRad Protein Assay (BioRad) in a Beckman Coulter DU640B spectrophotometer. For SDS-PAGE, 50 µg of protein lysate were resolved in 4-20% Tris-Glycine gradient gels (Lonza) and then transferred onto PVDF membranes (BioRad). Membrane was blocked in 5% blotto (BioRad) in TBS overnight at 4°C and subsequently probed with primary antibody in TBS with 1% BSA and 0.05% Tween-20 at room temperature for one hour, followed by three washes in TBS with 0.1% BSA and 0.05%
Tween-20. The primary antibodies and the respective dilutions used are as follows: ARF (1:100, Abcam, ab3642), p21 (1:200, BD-Pharminen, Cat. # 556430), p53 (1:200, Santa Cruz, sc6243-G), BAX (1:200, Santa Cruz, sc7480), MDM2 (1:200, Calbiochem, Cat. # OP115) and β-tubulin (1:1000, Sigma-Aldrich, T0198). After primary antibody incubation and washes, blot was then probed with the appropriate secondary antibodies at the specified dilution in TBS with 1% BSA and 0.05% Tween-20 at room temperature for one hour, followed by three washes. Secondary antibodies used are as follows: anti-rabbit-HRP (1:10000, Santa Cruz, sc2004), anti-goat-HRP (1:10000, Santa Cruz, sc2020) and anti-mouse-AP (1:10000, Santa Cruz, sc2008). HyGLO Chemiluminescence Detection Reagent (Denville) and HyBlot autoradiography film (Denville) were used to detect protein of interest. Alternatively, proteins probed with AP-conjugated secondary antibodies were detected using NBT/BCIP (Denville).

2.4.7. Immunofluorescence staining

For paraffin embedded sections, paraffin removal was performed as follows: two times of 10 minutes each in xylene, two times of 5 minutes each in 100% ethanol, once for 2 minutes in 95%, 70%, and 50% ethanol, followed by a 5 minutes incubation in TBS. Heat-mediated antigen retrieval was performed by heating microscope slides immersed in PBS-citrate using a microwave pressure cooker. Slides were then incubated in 5% Triton-X for 10 minutes at room temperature. Blocking was carried out for 30 minutes at room temperature in TBS with 10% DAKO Protein Block (DAKO-Cytomation), 1% BSA and 0.05% Tween-20. Slides were then incubated with primary antibody against ARF (1:200, Abcam, ab3642) in TBS with 1% BSA, 0.05% Tween-20 and 10% Antibody Diluent (DAKO-Cytomation), followed by three washes in TBS with 0.1% BSA and 0.05% Tween-20. Subsequently, slides
were incubated in goat biotinylated anti-rabbit IgG secondary antibodies (1:200, Vector Laboratories, BA-1000) at room temperature for 1 hour, followed by three washes. Streptavidin-Alexa-594 was used for the detection of ARF. DAPI was used to visualize nuclei of cells. Slides were mounted using the DAKO-Cytomation Fluorescent Mounting Medium.

Staining of fixed cells on a cover-slip was performed as described above, with the omission of paraffin removal and heat-mediated antigen retrieval.

2.4.8. Exogenous gene expression using adenoviruses

E1 and E3 early regions deleted adenovirus encoding gfp (adgfp) or human ARF (adARF) (Huang et al., 2003) under the control of a CMV promoter are kind gifts from Dr. Erik Knudsen (Kimmel Cancer Center, PA, USA) and Dr. Ruth Gjerset (Sidney Kimmel Cancer Center, CA, USA), respectively. Adenoviruses were amplified in HEK-293 cells. Infected HEK-293 cells were resuspended in PBS with 10% glycerol, and viral particles were released by three cycles of freezing and thawing. Cellular debris was removed by centrifugation. Viral titers were determined using the QuickTiter Adenovirus Titer Immunoassay Kit (Cell Biolabs) according to the manufacturer’s instructions. Adenoviruses were stored at -72°C in aliquots.

To assess the effect of ARF overexpression on gene expression and cell growth, WERI cells were seeded at 200,000 cells per well in 6-well plates pretreated with poly-D-lysine and incubated overnight under normal culture condition. For immunostaining, cells were seeded on poly-D-lysine treated coverslips placed within 6-well plates. After the overnight incubation, culture media was removed, and cells were rinsed once with PBS. Cells were then incubated in 500 µl of serum-free Iscove’s media containing adgfp or adARF at 20 multiplicity of infection (MOI) for 1 hour in a 37°C incubator with 5% CO₂. Complete
growth media (2 ml) was then added to each well and incubated for four days, after which cells were harvested for gene expression analyses. Cell viability was determined by trypan blue exclusion. Means and standard deviations were calculated based on three replicates per treatment. Statistical significance was determined using the two-tailed t-test.

2.5. Results

2.5.1. Cause of low level of ARF protein in retinoblastomas

2.5.1.1. Analysis of ARF expression in retinoblastoma cell lines

Guo et al previously showed that ARF protein in primary retinoblastoma tumors is disproportionally low compared to the amount of mRNA detected (Guo et al., 2008). To confirm this result, mRNA and protein expression in four control cell lines (HeLa, HEK-293T, OVCAR-3, Saos-2) with high ARF protein were compared to that in retinoblastoma cell lines. All retinoblastoma cell lines examined expressed very low levels of ARF protein relative to the control cell lines (Figure 3), yet three of the cell lines (RB381, RB383, RB247) expressed comparable amount of mRNA relative to that in HeLa (Figure 4B). GAPDH was used as the endogenous control for real-time PCR analysis; since compared to other housekeeping genes, it exhibited the least variation between the different cell lines examined (Figure 4A). Real-time PCR amplifies only 72 base pairs of the full length ARF mRNA and therefore could detect fragmented mRNA. To ensure that ARF mRNA is intact in retinoblastoma cell lines, RT-PCR analysis spanning all three ARF exons was performed, confirming the presence of full length ARF mRNA (Figure 4C). Therefore, in three of six retinoblastoma cell lines examined (RB381, RB383, RB247), the level of ARF protein was very low despite high mRNA expression, suggesting attenuation of ARF protein synthesis.
Figure 3. Western Blot analysis of ARF and p21 in retinoblastoma and control cell lines

Cell lines expressing high level of ARF protein were used as controls (HeLa, HEK-293T, OVCAR-3, Saos-2). Retinoblastoma cell lines (RB247, RB381, RB383, RB1021) expressed very low level of ARF protein. In control cell lines, p53 and pRB are inactivated by mutations or expression of viral-oncproteins, while p53 in the retinoblastoma cell lines appears normal (low mRNA levels, no mutations by PCR-SSCP, as shown in these and 25 other retinoblastomas, Ducket-Brown & Gallie, unpublished data). p21, a transcriptional target of p53, was not expressed, or expressed at low level in control cell lines (first four lanes), consistent with the p53 status in these cells. In contrast, p21 was highly expressed in retinoblastoma cell lines, further suggesting that p53 is intact in these cells. β-tubulin was used as a loading control. Intensity of the p21 bands were quantified using ImageJ, and normalized to that of β-tubulin.
Figure 4. *ARF* mRNA expression in retinoblastoma and control cell lines
Real-time PCR analysis was performed to determine if low ARF protein in retinoblastoma (RB) cell lines is due to low *ARF* mRNA. A. Expression of three housekeeping genes, *GAPDH*, *HPRT-1* and *TBP-1*, was evaluated in order to identify the most suitable endogenous control for data analysis. Ct (y-axis) represents the cycle when a predetermined fluorescence signal was reached. *GAPDH* had the most consistent Ct-values in different cell lines, and was therefore used as the endogenous control for data analysis. B. Real-time PCR measurement of *ARF* mRNA in control and retinoblastoma cell lines. Normalized *ARF*
expression was presented relative to that of HeLa. The level of ARF mRNA in RB381, RB383 and RB247 was comparable to that found in HeLa, although ARF protein was very low (Figure 3), suggesting that ARF protein synthesis is attenuated, or ARF protein is unstable in these RB cell lines. Error bars represent the standard deviations of a triplicate experiment. C. To determine if full length ARF mRNA was expressed, RT-PCR was performed in control and retinoblastoma cell lines using PCR primers spanning the entire coding region of ARF. TBP-1 was used as an endogenous control. C33A, a cervical cancer cell line, and HeLa were used as positive controls, while normal human retina (HR) was used as a negative control.
or instability of the ARF protein. For the remaining cell lines (RB1021, WERI, Y79), low ARF protein was at least in part due to low ARF mRNA.

In the four control cell lines (HeLa, HEK-293T, OVCAR-3, Saos-2), p53 is inactivated either by mutation or by the expression of viral oncoproteins. This p53-status correlated with the absence of p21, which is a p53-transcriptional target (Figure 3). In contrast, all examined retinoblastoma cell lines expressed p21 protein, indicating that p53 may be intact in these cells, as suggested by the lack of TP53 mutation found in these cell lines and 25 other retinoblastomas (Duckett-Brown & Gallie, unpublished data).

2.5.1.2. Role of the proteasome pathway in ARF protein degradation in retinoblastoma cell lines

ARF protein turnover is normally regulated by the ubiquitin-proteasome pathway (Kuo et al., 2004). To determine if ARF protein is aberrantly degraded by this pathway, which leads to low ARF expression, retinoblastoma cells were treated with a proteasome inhibitor, MG132. After 10 hours of MG132 treatment, all retinoblastoma cell lines responded with the accumulation of p21, a positive control whose turnover is regulated by the proteasome (Figure 5). However, no accumulation of ARF protein was observed in response to MG132 treatment (Figure 5). This indicated that low ARF protein in retinoblastoma cells was not the result of aberrant turnover by the ubiquitin-proteasome pathway.
Figure 5. Western Blot analysis for ARF and p21 in retinoblastoma cell lines after MG132 treatment
To determine if low ARF protein is the result of aberrant degradation by the ubiquitin-proteasome pathway, retinoblastoma cell lines were treated with the proteasome inhibitor MG132 for 10 hours. After treatment, retinoblastoma cell lines responded with the accumulation of the positive control, p21, which is known to be regulated by the ubiquitin-proteasome pathway. In contrast, ARF protein did not accumulate in retinoblastoma cells after MG132 treatment, indicating that low ARF protein in retinoblastomas is not due to aberrant degradation by the proteasome pathway. Untreated HeLa was used as a positive control for ARF expression.
2.5.1.3. Analysis of miR-24 expression in retinoblastoma cell lines

Translation of ARF mRNA is negatively regulated by the microRNA, miR-24 (Lal et al., 2008). Expression of miR-24 was measured to determine if this microRNA is expressed at a high level in retinoblastoma cell lines, thereby preventing efficient ARF protein synthesis. Real-time PCR analysis showed that miR-24 expression was significantly lower in retinoblastoma cell lines than that in HeLa or OVCAR-3, when using U18 or RNU6B as the endogenous control for data analysis (Figure 6).

![Graph showing expression analysis of miR-24 in control and retinoblastoma cell lines.](image)

**Figure 6. Expression analysis of miR-24 in control and retinoblastoma cell lines**
The expression of miR-24 was significantly lower in retinoblastoma cell lines (RB247, RB381, RB383) relative to HeLa or OVCAR-3 when using either U18 or RNU6B as endogenous control. Error bars represent the standard deviations of a triplicate experiment.
2.5.2. Expression of Arf, p53 & p53-downstream targets during early retinoblastoma development in a mouse retinoblastoma model

2.5.2.1. Expression of Arf during early mouse retinoblastoma development

To study the expression of Arf, p53 and p53-target genes in response to Rb-inactivation, transgenic mice with retinal specific Rb-inactivation (termed Rb-knockout for simplicity) were used. In this model, the Rb gene is excised in the peripheral retina by Cre-recombinase under the control of the Pax-6 α-enhancer beginning from embryonic day 10. Rb-deficient cells exhibit apoptosis and ectopic division during retinal development. These cells eventually cease to divide and differentiate into mature retinal cells, and they are not prone to retinoblastoma development (Figure 7B) (Chen et al., 2004; MacPherson et al., 2004). In contrast, Rb-knockout animals in the p107−/− background develop retinoblastoma with incomplete penetrance (Chen et al., 2004; MacPherson et al., 2004). During postnatal development, Rb-p107 compound mutant retina exhibits retinal dysplasia (Figure 7C), which progresses into tumor in approximately 60% of mutant animals (Chen et al., 2004; MacPherson et al., 2007; MacPherson et al., 2004). In the remaining animals, the mutant retinas eventually cease to proliferate and terminally differentiate by postnatal day 30 (P30) without any tumor development (Chen et al., 2004). For this study, mutant retinas from p107+/+ and p107−/− animals were collected at postnatal day 0 (P0), P5, P10 and P15 for RNA and protein extractions.

RT-PCR analysis showed that Arf mRNA was increased in Rb-knockout retinas compared to retinas with wild-type (Wt) Rb in both the p107+/+ (Figure 8A) and p107−/− (Figure 8B) backgrounds, indicating that Arf transcription was activated in response to Rb-
Figure 7. Hematoxylin and eosin staining of Rb-knockout retinas in p107+/+ or p107−/− background at P9

In Rb-knockout retina, the Rb gene is excised by Cre-recombinase controlled by the α-Pax6 enhancer in the peripheral retina beginning from embryonic day 10 of development. A & a. At P9, the formation of the three distinct retinal layers has already completed throughout the wild-type retina (R). B & b. This process is delayed in the peripheries of Rb-knockout retina where Rb is inactivated (KO region); the inner and outer nuclear layers have not yet completely separated. Nevertheless, in the p107+/+ background, cells in the developing Rb-knockout retina are not tumor prone, and eventually exit the cell cycle (Chen et al., 2004). C & c. In the p107−/− background, Rb-inactivation in the peripheral retina (KO region) leads to retinal dysplasia. In approximately 60% of Rb-knockout; p107−/− animals, the dysplastic retina progresses into tumor, while retinas of the remaining 40% undergo substantial apoptosis and terminally differentiate by P30 (Chen et al., 2004). R= Retina. L = Lens. ON = Optic nerve. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer.
Figure 8. RT-PCR analysis for Arf in Rb-knockout retinas from P0 to P15
A. Expression of Arf mRNA in Wt (+) and Rb-knockout (-) retinas in p107+/+ background. Tbp-1 was used as an endogenous control. Late passage MEFs were used as the positive control, while NIH-3T3, in which the Ink4a/Arf locus is deleted, was used as the negative control. NTC = no cDNA control. B. Arf expression in Wt (+) and Rb-knockout (-) retinas in p107/- background. The low signal at P5 in the Rb+ retinas might be related to the resorbing hyaloid system.
inactivation. In Wt-retinas, Arf mRNA was detected at P5 but declined from P10 and beyond. This was likely due to contamination during dissection by the resorbing hyaloid vascular system, where Arf is normally expressed (McKeller et al., 2002).

To assess Arf protein expression, immunofluorescence staining was performed on tissue sections of Rb-knockout retinas in p107+/+ and p107−/− backgrounds at P9. Rb is inactivated in the peripheral retina, while the central retinal region possesses intact Rb, and therefore was used as an internal control. In both the central and peripheral retinas of p107+/+ animal, positive signal in the Arf channel was only detected in the outer plexiform layer (between the inner and outer nuclear layer of the retina, Figure 9A and 9B). This signal was likely due to non-specific staining since it did not overlap with nuclei, where Arf normally localizes. In the Rb-deficient peripheral retina of p107−/− animal, in which the outer plexiform layer was disrupted due to retinal dysplasia, it was clear that specific Arf staining was completely absent (Figure 9C and 9D). Late passage wild-type MEFs, which express high level of Arf (Krimpenfort et al., 2001; Sharpless et al., 2001), were used as the positive control.

To further assess Arf protein expression, Western Blotting was performed using Wt- and Rb-knockout retinas in both p107 backgrounds. Arf was readily detected in MEFs (Figure 10A), but not in Wt- nor Rb-knockout retinas in either background (Figure 10A). To confirm the presence or absence of Arf protein, increased amount of protein lysate and prolonged chemilluminescence exposure were used. Under this condition, an elevation of Arf protein was detected in Rb-knockout retina relative to Wt-retina at P5 and P10 in the p107+/+ background (Figure 10B). Taken together, Arf transcription was activated upon Rb-inactivation in the developing retina, but the protein was expressed at very low level.
Figure 9. Immunostaining for Arf protein in Rb-knockout retinas in p107<sup>+/+</sup> or p107<sup>-/-</sup> background at P9

Central retina (CR) possesses wild-type Rb, and was used as an internal control. In both p107<sup>+/+</sup> (A & B) and p107<sup>-/-</sup> (C & D) backgrounds, positive Arf signal was present only in the outer plexiform layer, which lies between the INL and ONL in the Wt central retina (B & D, top panel). This signal was likely due to non-specific staining since it did not overlap with the nuclei, where Arf normally localizes, and Arf mRNA expression in Wt retinas at P9 was very low. A & B. In the p107<sup>+/+</sup> background, no nuclear staining was detected in the central Wt or peripheral Rb-knockout retina. As explained previously, staining between the INL and ONL in Wt retina was likely non-specific. C & D. In the p107<sup>-/-</sup> background, nuclear Arf staining was absent in both central and peripheral retinas. Late passage MEFs were used as
the positive control (bottom panel). DAPI was used to stain the nuclei of cells. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer.
Figure 10. Western Blot analysis of Arf in Rb-knockout retina in p107<sup>++</sup> or p107<sup>-/-</sup> background

A. Protein expression of Arf was undetectable in Wt (+) and Rb-knockout (-) retinas in p107<sup>++</sup> (top) or p107<sup>-/-</sup> (bottom) background. MEFs were used as the positive control. β-tubulin was used as a loading control. MW = molecular weight markers. B. Increased amount of protein extract and prolonged chemilluminesence exposure were used in order to detect Arf protein (100 µg of retinal protein extract, instead of 50 µg). Under this condition, Arf protein showed an increase in Rb-knockout compared to Wt retinas at P5 and P10. 3T3 = NIH-3T3.
2.5.2.2. Expression of p53 and p53-transcriptional targets during early mouse retinoblastoma development

The protein level of Arf was found to be low in Rb-knockout retinas, and therefore it was questionable whether the attained level of Arf was sufficient for p53 activation. To assess the activity of the p53 pathway in Rb-knockout retinas, Western Blotting was performed for p53 and its downstream targets p21 and Bax. Inactivation of Rb led to an increase in p53 and p21 protein in the p107+/+ or p107−/− background, but no increase in Bax protein was observed (Figure 1). To further examine the extent of p53-target gene activation, RT-PCR analysis was performed for genes involved in cell cycle arrest (p21, Gadd45, 14-3-3σ), apoptosis (Bax, Puma, Noxa), and autoregulation (Mdm2). For Gadd45, 14-3-3σ and Mdm2, no induction was observed upon Rb-inactivation in the p107−/− background (Figure 12). Increase in mRNA expression was observed for Bax, Puma, Noxa and p21 in response to Rb-inactivation. Similarly, in the p107+/+ background, p21 was up-regulated upon Rb-inactivation; however, no induction of Gadd45, 14-3-3σ and Mdm2 was observed (Figure 13).

2.5.3. Effect of exogenous ARF expression in a retinoblastoma cell line

To test whether enforced expression of ARF can activate p53 and p53-transcriptional targets, an adenovirus encoding the human ARF cDNA (adARF) (Huang et al., 2003) was employed to drive the overexpression of ARF in the retinoblastoma cell line WERI, which expresses both MDM2 and MDM4 (Guo et al., 2008), and possesses extra copies of MDM4 due to genomic gain (Laurie et al., 2006). After viral infection, ARF expression was detected by immunostaining as foci within the nucleus, characteristic of nucleolar localization where ARF normally resides. Approximately 60% of treated cells were positive for ARF (Figure
Figure 11. Western Blot analysis of p53, and p53-transcriptional targets p21 and Bax in Rb-knockout retinas in p107<sup>+/+</sup> or p107<sup>-/-</sup> background

Protein expression of p53 and its transcriptional target p21 was elevated in Rb-knockout retinas (-) compared to Wt-retinas (+) at P5, P10 and P15 in both p107<sup>+/+</sup> (A) and p107<sup>-/-</sup> (B) backgrounds. However, no increase of the proapoptotic protein Bax was observed in Rb-knockout retinas. Note that protein lysates were extracted from whole retinas. Therefore, protein extracted from Rb-knockout retinas contained a mixture of central (intact Rb) and peripheral (mutant Rb) retinal materials.
Figure 12. mRNA expression of p53-transcriptional targets in Rb-knockout retinas in p107−/− background

RT-PCR was performed to assess the expression of p53-target genes involved in autoregulation (Mdm2), cell cycle arrest (p21, 14-3-3σ, Gadd45) and apoptosis (Bax, Noxa, Puma) in Wt (+) and Rb-knockout (-) retinas at P5, P10 and P15. The response of p53-target genes to Rb-inactivation was heterogeneous. Mdm2, 14-3-3σ and Gadd45 were not induced upon Rb-inactivation, while the mRNA expression of p21, Bax, Noxa and Puma increased upon Rb-inactivation. Although Bax mRNA was elevated, its protein expression did not show an increase in Rb-knockout retinas (see Figure 11), suggesting that the magnitude of Bax mRNA induction was not sufficient to sustain detectable protein accumulation. Tbp-1 was used as an endogenous control. 0.1x = ten fold dilution of cDNA from 1.0x (to ensure that the log-phase of PCR amplification was captured)
**Figure 13. mRNA expression of p53-transcriptional targets in Rb-knockout retinas in p107+/+ background**

Consistent with results obtained from the p107−/− strain, RT-PCR analysis showed a lack of induction for *Mdm2*, 14-3-3σ and *Gadd45*, and an elevation for *p21* mRNA in Rb-knockout retinas (−) compared to Wt-retinas (+) at postnatal P5 and P15. *Tbp-1* was used as an endogenous control. 0.1x = ten fold dilution of cDNA from 1.0x
Cells treated with adARF showed accumulation of p53 protein and p53-downstream targets p21, MDM2 and BAX (Figure 15A). RT-PCR analysis showed that ARF overexpression also led to the induction of GADD45 and NOXA transcription (Figure 15B). The changes in gene expression were accompanied by inhibition of cell growth (Figure 15C), primarily due to inhibition of proliferation rather than cell death, given that no significant increase in the number of dead cells was observed after treatment of adARF (Figure 15D). Therefore, despite the presence of MDM2 and MDM4, enforced expression of ARF activated p53 and p53-transcriptional targets, including those that remained inactive upon Rb-inactivation during early mouse retinoblastoma development (Figure 11-13). This data suggests that weak ARF protein expression, as observed in human retinoblastomas and in pre-malignant Rb−/− retinas in mice, may prevent efficient activation of the p53-tumor surveillance pathway during retinoblastoma development.
Figure 14. Exogenous expression of ARF in the retinoblastoma cell line WERI
Cells were infected with adenovirus encoding the human ARF cDNA (adARF) or gfp (adgfp). Immunostaining for ARF was performed four days after infection. Punctated stainings of ARF in adARF treated cells were consistent with nucleolar localization (E), indicating that exogenous ARF protein was properly localized.
Figure 15. Effect of ARF overexpression on gene expression and cell growth
A. Western Blot analysis showing protein expression of p53 and p53-transcriptional targets in cells treated with adARF or adgfp four days after infection. Expression of p53, MDM2, BAX and p21 proteins was elevated in adARF treated cells. B. RT-PCR analysis showing elevation in mRNA expression of the p53-transcriptional targets GADD45 and NOXA in cells treated with adARF. C. The number of viable cells was significantly reduced in adARF treated cells four days after infection compared to adgfp treated cells. D. No significant difference in the number of dead cells was observed between adgfp and adARF treated cells, suggesting that the growth inhibitory effect observed in C was caused by inhibition of proliferation rather than the induction of cell death. C & D are data from a representative experiment performed in triplicate. Statistical significance was determined by the t-test.
2.6. Discussion

2.6.1. Cause of low ARF protein in retinoblastomas

2.6.1.1. Low ARF protein in retinoblastomas is unlikely due to aberrant protein degradation

Data from the present and previous studies (Guo et al., 2008) shows that ARF protein is low in retinoblastoma despite robust expression of full length mRNA, suggesting that ARF protein synthesis or stability may be deregulated. In this study, inhibition of the proteasome by MG132 treatment in retinoblastoma cells did not lead to accumulation of ARF protein, indicating that low ARF protein was not a result of aberrant turnover by this pathway. Besides the proteasomal pathway, proteins may also be subjected to lysosomal degradation. Studies in cell culture showed that chemical inhibition of the lysosome did not lead to ARF protein accumulation (Kuo et al., 2004; Pollice et al., 2007), indicating ARF protein turnover is primarily regulated by the proteasomal but not the lysosomal pathway.

Previous studies have shown that ARF protein stability is enhanced by its interaction with TBP-1 (Pollice et al., 2004; Pollice et al., 2007) or NPM (Itahana et al., 2003; Korgaonkar et al., 2005), therefore the absence of these proteins may shorten the half-life of ARF. If ARF is aberrantly degraded in retinoblastoma cells due to the absence of TBP-1 or NPM, inhibition of the proteasome by MG132 in this study would have resulted in ARF accumulation. However, the lack of ARF accumulation in retinoblastoma cell lines after MG132 treatment indicates that low ARF protein is not due to the absence of its binding partners TBP-1 and NPM. Therefore, in retinoblastomas with high ARF mRNA expression,
low ARF protein is unlikely due to aberrant protein degradation. Instead, attenuation of protein synthesis seems more plausible.

2.6.1.2. Role of microRNAs in regulation of ARF translation

Recently, much attention is focused on microRNAs and their role in regulation of gene expression. ARF mRNA has been shown to be regulated by the microRNA, miR-24 (Lal et al., 2008). In this study, we showed that miR-24 was expressed in retinoblastoma cell lines, but at a lower level than in cell lines highly expressing ARF protein. Nevertheless, the level of miR-24 attained in retinoblastoma cells may still be sufficient to repress ARF translation. Future studies could employ antisense oligonucleotides to knockdown miR-24 in retinoblastoma cells, and determine if cells respond by accumulating ARF protein and activation of the p53-pathway.

In addition to miR-24, ARF translation may be regulated by other microRNAs. Using the miRanda microRNA target detection software (Enright et al., 2003), ARF mRNA was predicated to interact with 136 microRNAs in addition to miR-24 (Guo, personal communication). In the future, expression analysis of candidate microRNAs could be performed in retinoblastomas, followed by knockdown experiments to assess microRNA functions.

2.6.1.3. Block in ARF protein synthesis may be overcome by elevation in ARF mRNA expression

Data from this study indicates that the p53-pathway may be activated in retinoblastoma cells by the infusion of extra copies of ARF mRNA, in this case, through the use of adenovirus-mediated gene transfer. The adenovirus used encodes an ARF cDNA that lacks
the 3’ untranslated region. Since miR-24 interacts with binding sites residing in this region (Lal et al., 2008), it is possible that the exogenous ARF mRNA escapes translational regulation due to the absence of this segment and the binding site. Future experiments could test ARF cDNA that encodes the full length mRNA containing the 3’ UTR, in order to confirm whether the block in protein synthesis is simply overcome by extra copies of ARF mRNA, or by the absence of the 3’ UTR in the exogenous cDNA.

A recent study reported that the chemotherapeutic agent, paclitaxel, could induce ARF protein accumulation in a retinoblastoma cell line (Drago-Ferrante et al., 2008). The expression of ARF mRNA was not measured in that study; therefore it is unclear whether ARF protein accumulation was caused by an elevation in ARF mRNA expression or by other mechanisms. Nevertheless, after paclitaxel treatment, increased E2F1 protein expression was detected, which might induce ARF transcription and led to ARF protein accumulation. Future studies could examine ARF mRNA expression in retinoblastoma cell lines after paclitaxel treatment.

2.6.2. Expression of Arf, p53 & p53-transcriptional targets during early retinoblastoma development in a mouse retinoblastoma model

2.6.2.1. ARF protein is expressed at low level during early mouse retinoblastoma development

To assess the activity of Arf and the p53-pathway in response to Rb-inactivation, expression analysis was performed in Rb-knockout retinas in p107+/+ and p107−/− mice during early postnatal development. Arf mRNA was weakly detectable at P0 but became prominent at P5, P10 and P15 in the Rb-knockout retinas. This pattern of Arf induction is parallel to the
*E2f1* activity in *Rb*-knockout retinas, in which *E2f1*-dependent apoptosis elevates from P0 to P8 (Chen et al., 2004; Chen et al., 2007), supporting that *Arf* transcription is *E2f1*-dependent. Despite the induction of mRNA synthesis, *Arf* protein expression remained weak throughout early retinoblastoma development. The functional relevance of *Arf* at this low level remains to be elucidated. Downstream of *Arf* in the p53-pathway, several p53-transcriptional targets remained inactive upon *Rb*-inactivation, suggesting that the attained *Arf* protein level might not be sufficient to effectively activate p53. Nevertheless, the possibility of p53-independent function of *Arf* cannot be excluded. The attained level of *Arf* protein during early retinoblastoma development might not be sufficient to activate the p53-pathway; however, it may be sufficient for *Arf*-functions outside the p53-pathway. This could be determined by analyzing *Rb* or *Rb-p107* mutant animals with *Arf* deletion. Precaution has to be taken when using this genetic approach to assess *Arf* function in the mouse retina. Germline *Arf* inactivation disrupts the regression of the hyaloid vascular system during postnatal development, indirectly causing retinal dysplasia and detachment (McKeller et al., 2002). These retinal phenotypes may confound tumor development, and it may be necessary to conditionally inactivate *Arf* specifically in the retina using the Cre-lox system.

**2.6.2.2. Despite increased p53 expression, some p53-target genes remained inactive upon Rb-inactivation**

The expression of p53 was elevated in *Rb*-knockout retinas (Figure 1). It is unclear whether this p53 accumulation was *Arf*-dependent, since *E2f1* has been shown to stabilize p53 by promoting p53-phosphorylation independent of *Arf* (Berkovich and Ginsberg, 2003; Powers et al., 2004; Rogoff et al., 2002; Rogoff et al., 2004). However, based on the low expression of *Arf* protein, it is likely that p53 accumulation in *Rb*-knockout retinas is
mediated by E2f1. This prediction could be confirmed by analyzing p53 expression upon Rb-inactivation in Arf\textsuperscript{-/-} mice. Nevertheless, despite p53 accumulation in Rb-knockout retinas, no increase in expression was observed for several of p53-transcriptional targets, including \textit{Mdm2, 14-3-3σ and Gadd45} (Figure 12 and 13). \textit{Bax} was induced at the mRNA level; however, an elevation at the protein level was not observed (Figure 11). This data suggests that the p53-pathway remains relatively inactive upon Rb-inactivation during early retinoblastoma development in the \textit{Rb-p107} knockout murine model. Although a subset of p53-targets, including \textit{p21, Bax, Noxa} and \textit{Puma}, were induced at the mRNA level, it is unclear whether their activations were p53-dependent, since these genes are also direct transcriptional targets of E2f1 (Gartel et al., 2000; Hershko and Ginsberg, 2004; Hiyama et al., 1998). Analysis of expression for these p53 target genes in Rb-knockout retina with p53 deletion will resolve whether induction of \textit{p21, Bax, Noxa} and \textit{Puma} transcriptions is p53-dependent.

\textit{14-3-3σ} and \textit{Gadd45} are important effectors for p53-dependent cell cycle arrest. Failure to activate these targets may compromise p53-mediated tumor surveillance and favor malignant tumorigenesis. Gadd45 is an important tumor suppressor that regulates the cell cycle through its interaction with the cyclin-CDK complex (Smith et al., 1994), and is also implicated in maintaining genomic stability (Hollander et al., 1999). The 14-3-3σ protein is a potent inhibitor of G2/M phase transition that sequesters cyclin B and CDC2 to prevent entry into the nucleus (Chan et al., 1999). Therefore, the lack of \textit{14-3-3σ} and \textit{Gadd45} activation in \textit{Rb}-knockout retinas may favor cell cycle progression and subsequent acquisition of genomic changes in \textit{Rb}-deficient cells.
2.6.2.3. Several factors might influence the transactivating activity of p53

The response of p53 to particular stresses has been found to be highly cell-, tissue- and stress-specific (Lu and Lane, 1993; MacCallum et al., 1996). p53 is at the hub of numerous signaling pathways that are triggered by particular stresses, all of which may leave their marks on p53 in the form of post-translational modifications, interactions with cofactors, relocalization, and alteration in expression level. These various factors, collectively, dictate the resulting cellular response to a particular stress (Murray-Zmijewski et al., 2008). In the Rb-knockout retinas, the cellular response to p53 accumulation is presumably dictated by a number of factors, including p53 localization, expression level, post-translational modifications, and the availability of cofactors. A possible explanation for the lack of activation of some p53-targets is that these factors are missing in the retina. The developing retina may lack the modifications or cofactors that p53 needs in order to transactivate Mdm2, 14-3-3σ and Gadd45. Or, as the present study suggests, the attained level of p53 expression is not sufficient for activation of those genes, perhaps due to the weak protein expression of its upstream activator, Arf. We showed that exogenous expression of ARF in human retinoblastoma cells led to p53 accumulation, and elevated protein expression of the p53-targets p21, MDM2 and BAX. Enforced ARF expression also induced the transcription of GADD45 and NOXA. This result suggests that during early retinoblastoma development, the p53-pathway stays relatively inactive in part due to weak Arf expression (Figure 16). Future experiments could validate this notion in vivo using a vector that overexpresses Arf in Rb-p107 mutant retinas to determine if exogenous Arf leads to the activation of p53-target genes, and reduce the frequency of malignant transformation.
Figure 16. Role of the Arf-p53 pathway in early mouse retinoblastoma development
Although p53 protein expression increased when Rb was inactivated, its transcriptional targets Gadd45, 14-3-3σ and Mdm2 were not activated. The lack of induction of Gadd45 and 14-3-3σ might compromise tumor surveillance, given their crucial roles in cell cycle arrest. This inactivity might be the result of weak Arf protein expression. In Rb heterozygote retinas, increased mRNA expression of p21, Bax, Puma and Noxa suggested that the p53-transactivating activity might be elevated. However, these genes are also targets of the E2f1 transcription factor, which is hyperactive in the absence of functional pRb. Further studies are required to determine whether the induction of these genes is p53- or E2f1-dependent.
2.6.3. Effect of exogenous ARF expression in retinoblastoma cell line

2.6.3.1. The p53-pathway can be activated by exogenous ARF in retinoblastoma cells

Data from this study suggests that the p53-pathway is not disrupted by MDM4 and MDM2 in retinoblastomas, as previously postulated by Laurie et al (Laurie et al., 2006). The retinoblastoma cell line used in this study, WERI, possesses extra copies of MDM4 due to genomic gain, and it overexpresses MDM4 relative to normal fetal retina (Laurie et al., 2006). Nevertheless, enforced expression of ARF was able to activate p53 and p53-target genes. ARF physically interacts with MDM2 but not MDM4, and activates the p53-pathway by relieving p53 from negative regulation by MDM2. Our data indicated that even in retinoblastoma with MDM4 gain, p53 was also bound and regulated by MDM2, and was available for activation by ARF. Therefore, in retinoblastoma, the lack of robust ARF protein expression was an important factor contributing to the silencing of the p53-pathway.
Chapter 3: Discussion

3.1. Tumor surveillance by the ARF-p53-pathway in retinoblastoma development

Data from the present and previous studies suggests that the p53-pathway does not play a prominent role during early retinoblastoma development. MacPherson et al showed that the deletion of p53 has no effect on apoptosis or ectopic division induced by Rb-inactivation during early retinoblastoma development (MacPherson et al., 2004). Consistent with previous observations in retinoblastoma, we now show that Rb-inactivation failed to induce expression of p53-target genes, evidenced by the lack of induction of Mdm2, 14-3-3σ and Gadd45. Our data suggests that weak Arf protein expression may contribute to the lack of robust p53-response during early retinoblastoma development.

Despite the apparent low p53-activity during early tumor development, p53 may play an important role later in retinoblastoma development. Inactivation of p53 in Rb-knockout retina does not affect apoptosis nor does it initiate tumor formation (MacPherson et al., 2004). But when inactivated in the tumor prone Rb-p107 mutant retina, p53 deletion leads to more aggressive retinoblastoma development (Zhang et al., 2004). In human, expression analysis by immunohistochemistry shows that p53 expression is only detectable in malignant retinoblastoma but not in the benign retinoma (Dimaras et al., 2008), further supporting a role of p53 in later stages of retinoblastoma development.

Genomic studies in mouse and human show that retinoblastomas exhibit genomic instability (Corson and Gallie, 2007; MacPherson et al., 2007). Therefore, p53 may be activated later during retinoblastoma development by the DNA damage and genomic
instability induced by loss of \textit{RB1}. In the present study, analysis of p53 expression in \textit{Rb}^{-/-} murine retinas only extended up to P15, at which point genomic instability might yet to manifest, and therefore robust p53 activation could not be observed. To test this in the future, retinas from older \textit{Rb-p107} mutant animals could be collected to examine the expression of DNA damage markers, and determine if DNA damage coincides with p53-activation.

\subsection*{3.2. ARF-MDM2/4-p53 pathway: Therapeutic target in retinoblastomas?}

\subsubsection*{3.2.1. Targeting MDM2 and MDM4}

Previous studies show that the p53-pathway can be activated in retinoblastoma cells using the small molecule Nutlin-3a (Vassilev et al., 2004), which inhibits MDM2 and MDM4 (Laurie et al., 2006), leading to the induction of apoptosis in retinoblastoma cell lines (Elison et al., 2006; Laurie et al., 2006). The specificity of this approach is questionable. \textit{MDM4} and \textit{MDM2} were reported to exhibit copy number gain on 1q and 12q in 63\% and 10\% of retinoblastomas, respectively (Laurie et al., 2006). However, it is unclear whether \textit{MDM4} and \textit{MDM2} are the target genes of the respective chromosomal gains. Dimaras et al showed that gain of \textit{MDM4} is not obligatory for gain of 1q (Dimaras et al., 2008), indicating that either 1q contains multiple oncogenes, or \textit{MDM4} is a “passenger” of 1q gain in retinoblastoma development. Future studies could determine the exact extent of genomic copy number gain of \textit{MDM4} and \textit{MDM2}, and their flanking genes on 1q and 12q, respectively.

\subsubsection*{3.2.2. Targeting ARF}

Data from the present and previous studies shows that the upstream activator of the p53-pathway, ARF protein, is expressed at low level in retinoblastomas (Guo et al., 2008), and
enforced expression of ARF can activate the p53-pathway and inhibit cell growth in retinoblastoma cell line. Therefore, ARF activation may be a feasible therapeutic strategy. ARF mRNA expression is much higher in retinoblastomas than in normal fetal or adult retinas (Guo et al., 2008; Laurie et al., 2006). Retinoblastomas possess a pool of ARF mRNA that is not efficiently translated into protein. When the mechanism governing ARF protein synthesis is elucidated, it may be modulated to utilize the ARF mRNA present in retinoblastomas in order to synthesize ARF protein. In retinoblastomas, ARF translation might be blocked by presently unknown microRNAs, which, when identified and characterized, could be targeted using synthetic antisense antagonist.

Alternatively, the block in ARF protein synthesis in retinoblastomas may be overcome simply by increasing ARF mRNA. In this case, modulating the upstream transcriptional regulators of ARF may be a feasible therapeutic strategy. E2F3, which exhibits copy number gain, and is overexpressed in retinoblastomas (Orlic et al., 2006), has been found to directly repress Arf transcription (Aslanian et al., 2004). In retinoblastoma cell line with extra copies of E2F3, shRNA-knockdown of E2F3 led to growth inhibition (Orlic-Milacic, submitted). Therefore, future studies could determine whether the growth inhibition induced by E2F3 knockdown is ARF-mediated, by measuring ARF mRNA and protein expression after E2F3-knockdown. In addition, Arf expression could be examined in Rb-knockout retinas with E2f3 inactivation, since E2f3 ablation may further activate Arf transcription. Future experiments could also determine if other known ARF transcriptional repressors (BMI-1, CBX-7, POKEMON, TBX-2 and -3), which are overexpressed in some cancers and prevent ARF activation, are overexpressed in retinoblastomas relative to normal retinas. Subsequently,
RNAi experiment targeting the overexpressed repressor(s) could be performed, followed by expression analysis of ARF, p53 and p53-target genes.

### 3.2.3. Targeting p53

A potential therapeutic strategy is to activate the endogenous wild-type p53 in order to trigger apoptosis in retinoblastomas. Small molecules that activate p53 have been identified and characterized. In addition to Nutlin-3a (Vassilev et al., 2004), RITA (reactivation of p53 and induction of tumor cell apoptosis) has been identified from a chemical screen that aimed to discover novel p53-activating compounds (Issaeva et al., 2004). In comparison to Nutlin-3a, which predominantly induced cell cycle arrest in tumor cells, RITA potently induced apoptosis in a p53-dependent manner (Enge et al., 2009). RITA promoted the degradation of p21, and hnRNP K, a cofactor that promotes p21 transcription, while Nutlin-3a did not down-regulate either protein (Enge et al., 2009). p21, which was highly expressed in retinoblastomas (Figure 3), possesses anti-apoptotic functions in addition to its role in cell cycle arrest (Han et al., 2002). Therefore, RITA may be the more effective cancer killing agent compared to Nutlin-3a, owing to its inhibitory effect on p21 (Enge et al., 2009). Future studies could examine and compare the effect of RITA and Nutlin-3a on gene expression and cell growth in retinoblastoma cell lines.

### 3.3. Future directions

This study has generated new insights into the role of ARF and the p53-pathway in retinoblastoma development. Future studies will focus on answering the important questions that remain, and validating the ideas and hypotheses that have arisen from this thesis. The pending research questions (Q) and the proposed experiments (E) are listed below.
Q: What is the cause for low ARF protein in retinoblastomas despite high ARF mRNA expression?

E: Elucidation of the functional role of miR-24, a known regulator of ARF translation (Lal et al., 2008): Antisense oligonucleotides could be used to knockdown miR-24 in retinoblastoma cell lines, and the response in ARF protein expression and p53-activity could be determined.

E: Identification of other microRNAs that inhibit ARF mRNA: The miRanda microRNA target prediction software predicted 136 potential microRNAs (Guo, personal communication) that bind the ARF mRNA. Expression analysis of these microRNAs could be performed in retinoblastomas, and the functional relevance of candidates could then be assessed using knockdown experiments in retinoblastoma cell lines.

Q: Can a further increase in ARF mRNA expression, which may lead to an excess amount of ARF mRNA relative to the factors that inhibit ARF translation (eg. microRNAs), overcome the blockade in protein synthesis in retinoblastomas?

E: Overexpression of ARF mRNA containing the 3’ UTR in retinoblastoma cell lines: The ARF cDNA employed to transduce retinoblastoma cells in this study lacked the 3’ UTR, therefore it is unclear whether the observed ARF protein accumulation was due to increased ARF mRNA expression or the evasion of exogenous ARF mRNA from endogenous translational regulation. To clarify the role of the 3’UTR, cells could be transduced with a vector containing an ARF cDNA that possesses the entire ARF mRNA sequence including the 3’ UTR, where many microRNAs are known to bind.
Q: Can inhibition of known ARF transcriptional repressors further increase ARF mRNA, and induce ARF protein accumulation in retinoblastomas?

E: Knockdown of known ARF transcriptional repressors: E2F3 is a known transcriptional repressor of ARF (Aslanian et al., 2004) and is overexpressed in human retinoblastomas (Orlic et al., 2006). RNAi knockdown of E2F3 may increase ARF transcription in retinoblastoma cells. In addition, expression of other known ARF transcriptional repressors could be examined, and their roles in regulating ARF could subsequently be assessed by knockdown experiments in retinoblastoma cell lines.

Q: Is the p53-pathway more active in malignant tumors, in which signals emanating from genomic instability or other factors may trigger p53, than in pre-malignant lesions, in which these signals have not yet manifested?

E: Determine the expression of p53 in concert with markers for genomic instability in malignant tumors versus pre-malignant lesions in a mouse model of retinoblastoma (eg. Rb-p107 knockout mice).

Q: Can the small molecule RITA activate p53, and lead to more potent cell death compared to the chemotherapeutic agents currently used for retinoblastoma treatment?

E: Determine and compare the effect of RITA, Nutlin-3a, and the chemotherapeutic agents currently used for retinoblastoma treatment, on cell proliferation and survival in retinoblastoma cell lines.
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