A NOVEL ROLE FOR THE $p16^{INK4A}$ TUMOR SUPPRESSOR IN REGULATING CELLULAR OXIDATIVE STRESS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Mutations, deletions, and epigenetic silencing of the cyclin-dependent kinase inhibitor $p16^{INK4A}$ are associated with several cancer types, but are more commonly associated with familial melanoma predisposition and melanoma tumors. $p16^{INK4A}$ functions as a tumor suppressor by negatively regulating the cell cycle, however several outstanding questions remain. It remains unclear why compromise of $p16^{INK4A}$ predisposes to melanoma over other cancers, and why several melanoma-associated $p16^{INK4A}$ mutations do not compromise CDK4-binding. This study describes a novel function of $p16^{INK4A}$ in regulating intracellular oxidative stress independently of its role in cell cycle inhibition, and analyzes these functions in several familial melanoma-associated $p16^{INK4A}$ point mutants. I also demonstrate that, due in part to the pro-oxidizing nature of melanogenesis, melanocytes have higher constitutive levels of intracellular reactive oxygen species (ROS) than other cell types, suggesting why genetic compromise of $p16^{INK4A}$ preferentially predisposes to melanoma.

This dissertation demonstrates that $p16^{INK4A}$ was rapidly upregulated following ultraviolet-irradiation and H_2O_2-induced oxidative stress (Chapter 2). Depletion of $p16^{INK4A}$ increased ROS and oxidative DNA damage in several cell types, which was exacerbated by H_2O_2. Aberrant ROS levels in *Cdkn2a*-deficient fibroblasts were elevated relative to controls and normalized by expression of exogenous $p16^{INK4A}$. Finally, $p16^{INK4A}$-mediated suppression of ROS could not be attributed to the potential effects of

p16 on cell cycle phase. I then constructed 12 different familial melanoma-associated point mutants and analyzed their capacity to restore normal cell-cycle phase and ROS levels in $p16^{INK4A}$-deficient fibroblasts (Chapter 3). Whereas wild-type $p16^{INK4A}$ fully restored both functions, various $p16^{INK4A}$ mutants showed different abilities to normalize ROS and cell cycle profiles. Different mutations were found to affect both, neither, or only one of the functions of $p16^{INK4A}$, indicating that these two regulatory functions can be uncoupled. Structural analysis indicated that these distinct functions may be mediated by distinct regions of the protein. Lastly, in normal melanocytes, inhibition of melanin was sufficient to decrease levels of intracellular ROS to levels constitutively observed in fibroblasts (Chapter 4), indicating that the unique process of melanin production may be responsible for high basal levels of ROS and preferential susceptibility to oncogenic transformation brought on by genetic compromise of $p16^{INK4A}$.

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CHAPTER 1

INTRODUCTION

Tumorigenesis Requires Several Enabling Characteristics

The transformation of normal tissue into neoplastic tumors is thought to include a progressive evolution involving the acquisition of several characteristics that enable normal cells to become tumorigenic, and eventually, migratory and malignant. The six original characteristics described as "hallmarks of cancer" (Hanahan and Weinberg 2000) included unregulated proliferative signaling, evasion from growth suppressors, resistance from cell death, and enabling replicative immortality, induction of angiogenesis, and activating invasion and metastasis. Recently, two more characteristics were added that are involved in the development of malignancy, reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011). While understanding the complexity of oncogenesis and the cross-talk existing between these various characteristics is daunting, understanding its integral factors in an organized and systematic fashion can potentially lead to the development of more effective and targeted therapies for different human malignancies.

Reactive Oxygen Species and Genomic Instability

The development of cancer is greatly aided by an environment that is conducive to the acquisition of the aforementioned characteristics. An intracellular environment that enables oncogenesis is one that promotes genomic instability, a situation involving the generation of numerous unrepaired random mutations to a cell's DNA. This genomic instability can result in several different mutagenic outcomes, including the inactivation of tumor suppressor genes or the activation of oncogenes. One potential factor that can lead to an environment of genomic instability is the presence of excess intracellular reactive oxygen species (ROS) (Waris and Ahsan 2006).

Any metabolism of molecular oxygen has the potential to generate ROS such as the highly reactive hydroxyl radical $^{\cdot}OH$, singlet oxygen $^1O_2$, superoxide radical O_2-, or hydrogen peroxide H_2O_2 (Halliwell 1999). ROS are produced in all aerobic cells, primarily during the natural process of mitochondrial respiration, and are normally tightly regulated by biochemical antioxidants (Nohl et al. 2003). A constitutively low level of intracellular ROS permits ROS intermediates to serve as second messengers in redox-dependent signal transduction and to function in proliferation, cell cycle arrest, and cell death (Klein and Ackerman 2003, Scandalios 2002, Martindale and Holbrook 2002). To prevent the prolonged presence of high levels of intracellular ROS, cells possess several antioxidant enzymes that convert highly reactive superoxide into hydrogen peroxide, such as mangenese superoxide dismutase (MnSOD) located in the mitochondria, and Copper/Zinc superoxide dismutase (Cu/ZnSOD) located in the cytoplasm (Weisiger and Fridovich 1973). Hydrogen peroxide itself can then lead to increased oxidative stress within a cell through participation in the "Fenton reaction" or reaction with copper or iron ions to produce the much more reactive hydroxyl radical (Aruoma et al. 1989, Halliwell and Gutteridge 1990, Halliwell and Gutteridge 1992, Halliwell 1993). The enzyme catalase therefore plays an important role in the decomposition of hydrogen peroxide to water (Waris and Ahsan 2006). These enzymatic antioxidant systems are aided by nonenzymatic antioxidants that function in the thiol/disulfide system such as glutathione (GSH) (Droge 2002, Misra et al 2009).

These ROS can potentially damage several different classes of cellular constituents. ROS can disrupt cellular membranes by directly reacting with lipids (Hunkar et al. 2002), can potentially cross-link ribonucleoprotiens (Waris and Alam

1998), and may modify sulphhydryl groups in proteins (Knight 1995). The accumulation of oxidative stress-induced damage to lipids, proteins and DNA is proposed to be a contributing factor in several classes of human disease, such as neurodegenerative, cardiovascular, metabolic, and inflammatory pathologies (Vurusaner et al. 2012). More specifically, ROS and organ damage contributes to Fanconi anemia, chronic hepatitis, cystic fibrosis, and numerous autoimmune diseases (Takeuchi and Morimoto 1993, Hagen et al. 1994, Shimoda et al. 1994, Waris et al. 2005). Oxidative stress-induced damage to DNA has now been widely accepted as a major contributor to the onset of cancer (Figure 1.1) (Ames 1983, Shimoda et al. 1994). This occurs as a result of DNA damage that leads to heritable changes in all cells downstream of the mutation event that impact the functions of the hallmarks of cancer that are believed to be crucial in the etiology of cancer.

Oxidative stress can induce several types of DNA damage, such as the oxidation of both purines and pyrimidines, creation of alkali labile sites, and development of single strand breaks (Breen and Murphy 1995, Wang et al. 1998, Dizdaroglu et al. 2002), (Cooke et al. 2003, Jaruga et al. 2004). DNA treated with ROS has also been observed to develop double tandem CC→TT substitutions, sometime refered to as thymine dimers (Reid and Loeb 1993). Some of these oxidative lesions have been shown to possess mutagenic properties, therefore oncogenesis can potentially follow if they are not quickly and correctly repaired (Waris and Ahsan 2006). Interestingly, while it has been shown that all four bases can potentially be modified by excessive ROS, the modification of GC base pairs has been shown to be much more mutagenic than AT base pair modification (Retel et al. 1993). Along those lines, the most common mutations in the p53 tumor

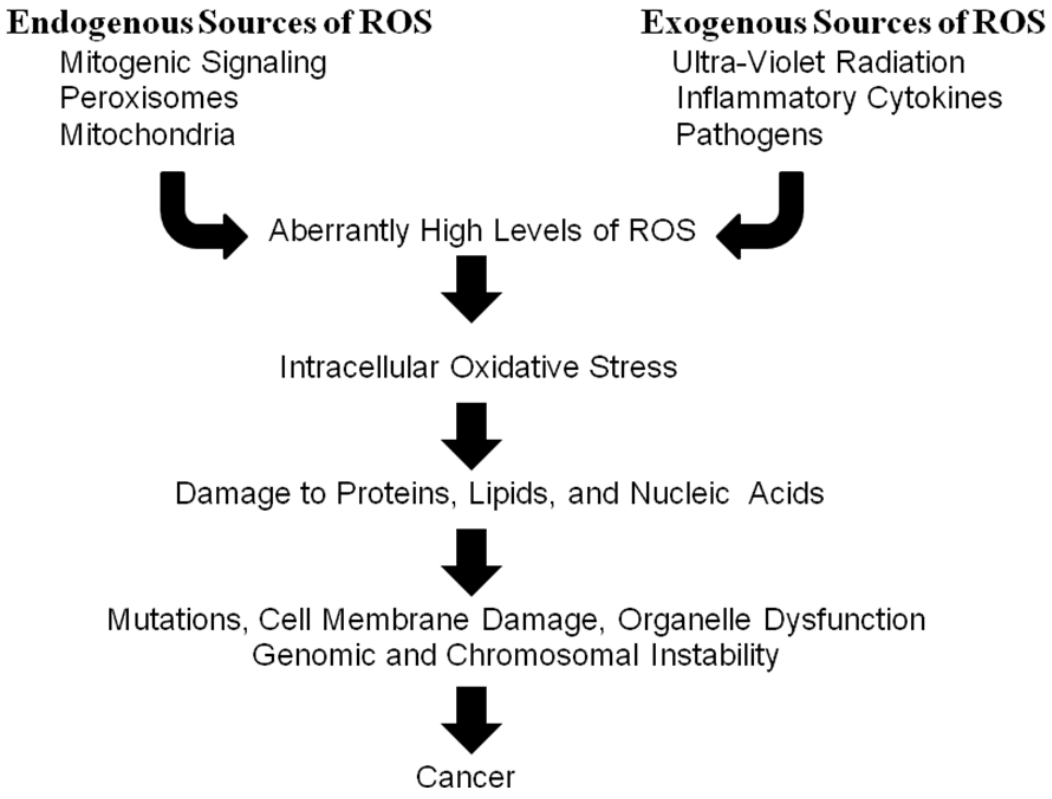


Figure 1.1 Aberrant intracellular ROS can lead to cancer. While ROS is produced during normal physiological and mitogenic processes, certain exogenous events can overwhelm the intracellular antioxidant system and create an environment conducive to the damaging of different cellular constituents. If excessive ROS damages DNA to the point of genomic instability, cancer may result.

suppressor gene are G to T transversions (Brash et al. 1991, Hollstein et al. 1991, Harris and Hollstein 1993). These observations have led the 8-hydroxydeoxyguanisine (8-OG) lesion to be the most well-studied oxidative DNA lesion.

If not efficiently repaired, the oxidative lesion 8-OG can persist until the cell replicates its DNA, a time at which the replication DNA polymerases α and δ frequently mismatch 8-OG with Adenine (A), instead of the usual (and proper) cytosine (C) (Shibutani et al. 1991). As replication of the first strand proceeds, the 8-OG then gets replaced by thymine (T) to properly match to the erroneous A during a subsequent round of DNA replication. Therefore the net result of 8-OG persisting until DNA replication is the aforementioned potentially highly mutagenic GC to AT transversion (David and Williams 1998). Also, high levels of intracellular oxidative stress may lead to a nucleotide pool that contains high levels of 8-OG that may be incorporated into DNA during replication (Maki and Sekiguchi 1992). As expected, several different cancers commonly exhibit one or more of the following: constitutively high intracellular ROS, high percentages of cells that are positive for 8-OG oxidative lesions, or genetic compromise of hOGG1 (8-oxoguanine glycosylase 1), one of the enzymes responsible for repairing the 8-OG lesion (Pashaei et al. 2008), Zyrek-Betts et al. 2008). However, much dispute remains in the field concerning whether this correlation is causative.

Mammalian cells have evolved several mechanisms to deal with the presence of 8-OG lesions, which underscores the hypothesis that this lesion poses a significant risk to the overall integrity of the genome (Cooke et al. 2003). The 8-OG:C pair is the substrate for hOGG1, which serves to free the 8-OG from double-stranded substrates using a

glycolytic mechanism, and relying on an internal lysine residue (Boiteux and Radicella 2000, Bruner et al. 2000, David-Cordonnier et al. 2001). If the 8-OG lesion has paired with adenine through its misincorporation into nascent DNA, it is predominantly removed by the glycolytic activity of hOGG2 (Hazra et al. 1998). The adenine itself in the mismatched 8-OG:A pair can be removed by the MutY homologue (MYH), which then liberates the 8-OG base to once again attempt to correctly pair with a cytosine (McGoldrick et al. 1995, Slupska et al. 1996). Nei-like glycosylase 1 (NEIL1) also removes 8-OG from mismatches with adenine guanine, relying on an amino-terminal prolyl residue rather than an internal lysyl residue like hOGG1 (Hazra et al. 2002).

Elevated levels of 8-OG adducts have been measured in the tissues from several different malignancies, including breast cancer (Malins and Haimanot 1991, Nagashima et al. 1995), melanoma (Meyskens et al. 2001), kidney (Okamoto et al. 1994), lung (Vulimiri et al. 2000, Inoue et al. 1998) and hepatocellular carcinoma (Schwarz et al. 2001). These elevated levels of oxidative damage are thought to be mainly a result of a tumor environment that is constitutively high in intracellular ROS and low in antioxidant enzyme production (Toyokuni et al. 1995). It has been observed that several tumor lines that produce high levels of intracellular H_2O_2, even in the absence of exogenous stimulation, exhibit permanent activation of transcription factors and their target genes. This constitutively high level of ROS coupled with aberrant transcription factor activity may contribute to a strong selective pressure for cells that are observed in a cancerous malignancy. However, to what the extent the formation of 8-OG lesions is playing a causative role in the development of cancer remains contested due to the existence of many pathologies that exhibit high levels of these oxidative lesions that do not involve

oncogenesis. The presence of high levels of oxidative DNA lesions may be (at least) partially the result of well-established characteristics of cancer cells and their intracellular environments themselves, such as increased metabolic activity and increases rates of DNA synthesis and cell division. More work will have to be done to mete out the details of the formation of oxidative lesions on the initiation and progression of carcinogenesis. However, it appears that oncogenesis may involve the positive selection of cells with constitutively high levels of intracellular ROS levels because of the acquisition of specific proliferative advantages and plasticity for malignant progression.

Cell Cycle Regulation as an Oncogenic Checkpoint

The most fundamental trait involved with oncogenic transformation is the capacity of tumor cells to chronically proliferate without succumbing to the normal tumor-suppressive effects of cell cycle regulation. During normal growth and development, the proliferative signals necessary for cell cycle progression and cell division are carefully regulated through cell cycle checkpoints in order to assure proper organismal development and tissue architecture. Regulation of cellular proliferation is also imperative for continued DNA integrity and to ensure that any mutations acquired in the course of the pre-mitotic life of the cell are not propagated into further generations. This unscheduled proliferation and propagation of potentially oncogenic chromosomal and genomic instability is to a large extent prevented by cyclin-dependent kinases (CDKs) and cyclins (Malumbres and Barbacid 2005).

The cell cycle is made up of four distinct phases. The first phase is called Gap 1, or the G1 phase. This phase is characterized by the cell increasing in size, and passing through the G1 checkpoint, a mechanism by which the cell confirms that it is ready to

replicate its DNA. The next phase is the synthesis, or S, phase, which involves the synthesis of replicated DNA. The Gap 2 or G2 phase follows, involving continued growth of the cell and the passing though the G2 checkpoint that ensures that the cell is ready for division. The final phase is mitosis, or the M phase, a time during which cell growth ceases and the majority of cellular energy is allocated to the precise division of the cell, a careful process mediated by the metaphase checkpoint. The progression of a cell through these phases is regulated by two classes of molecules, cyclins and CDKs, the discovery of which earned Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse the 2001 Nobel Prize in Physiology or Medicine (Lee and Nurse 1987) .

CDKs are relatively small proteins that contain little more than a kinase domain, and consistently weigh in the range of 30-40 kilodaltons (Lee and Nurse 1987). These evolutionarily conserved proteins are found in all known eukaryotes, and interestingly yeast can multiply when their CDK gene has been replaced by the human gene (Lee and Nurse 1987). For CDKs to impart their kinase activity and drive cell cycle progression they must bind to regulatory subunits called cyclins. Cyclins serve as the regulatory subunit and CDKs as the catalytic subunit of the activated heterodimer. Specific cyclin-CDK heterodimers then serve to activate or inactivate target proteins through phosphorylation and act as cell cycle phase checkpoints to progress the cell into proper entry into the next cell cycle phase. Cells regulate the activity of individual CDK-cyclin complexes through the controlled synthesis and degradation of specific cyclins during different phases of the cell cycle, whereas CDKs are expressed at relatively constant levels, with most of their regulation being post translational (Figure 1.2). CDKs are primarily regulated by four different mechanisms: cyclin binding, CDK-activating kinase

(CAK) phosphorylation, inhibitory phosphorylation, and the binding of CDK inhibitory proteins (CKIs) (Morgan 1995).

The ATP-binding site of CDKs lies in a cleft formed between an amino-terminal lobe and a carboxy-terminal lobe (Morgan 1995). A flexible structure called a T-loop blocks this cleft in the absence of cyclin binding, preventing ATP binding to the CDK. When a cyclin is bound to a CDK, a conformational change affects two α-helices that allows ATP binding and activation of the heterodimer (Morgan 1995). The phosphorylation of a threonine adjacent to the active site is performed by CAK, and is required for full CDK activation. Phosphorylation by CAK may occur before (as in yeast) or after (mammalian cells) cyclin binding. Since CAK is not regulated by any known cell-cycle pathways, the limiting step for CDK activation is thought to be cyclin binding (Morgan 1995). Various kinases also perform inhibitory phosphorylation on tyrosine and threonine residues within CDKs, and unlike activating phosphorylation, this level of regulation is essential for proper cell cycle regulation. One inhibitory kinase, Wee 1, phosphorylates a CDK tyrosine residue and is conserved in all eukaryotes. Vertebrate CDKs can have threonine and tyrosine phosphorylated by membrane-associated tyrosine/threonine protein kinase 1 (Myt1), while these residues are dephosphorylated by members of the cell division cycle 25 (Cdc25) family of phosphatases. Lastly, CDKs can be inhibited in response to toxic environmental insults or DNA damage through their binding to a class of proteins known as cyclin-dependent kinase inhibitors (CKIs). In the presence of a sufficiently damaging insult (most often during the G1 phase), intracellular signals induce the upregulation of CKIs that can bind to either a CDK or a CDK-cyclin complex to inhibit the cell cycle until the toxic insult is

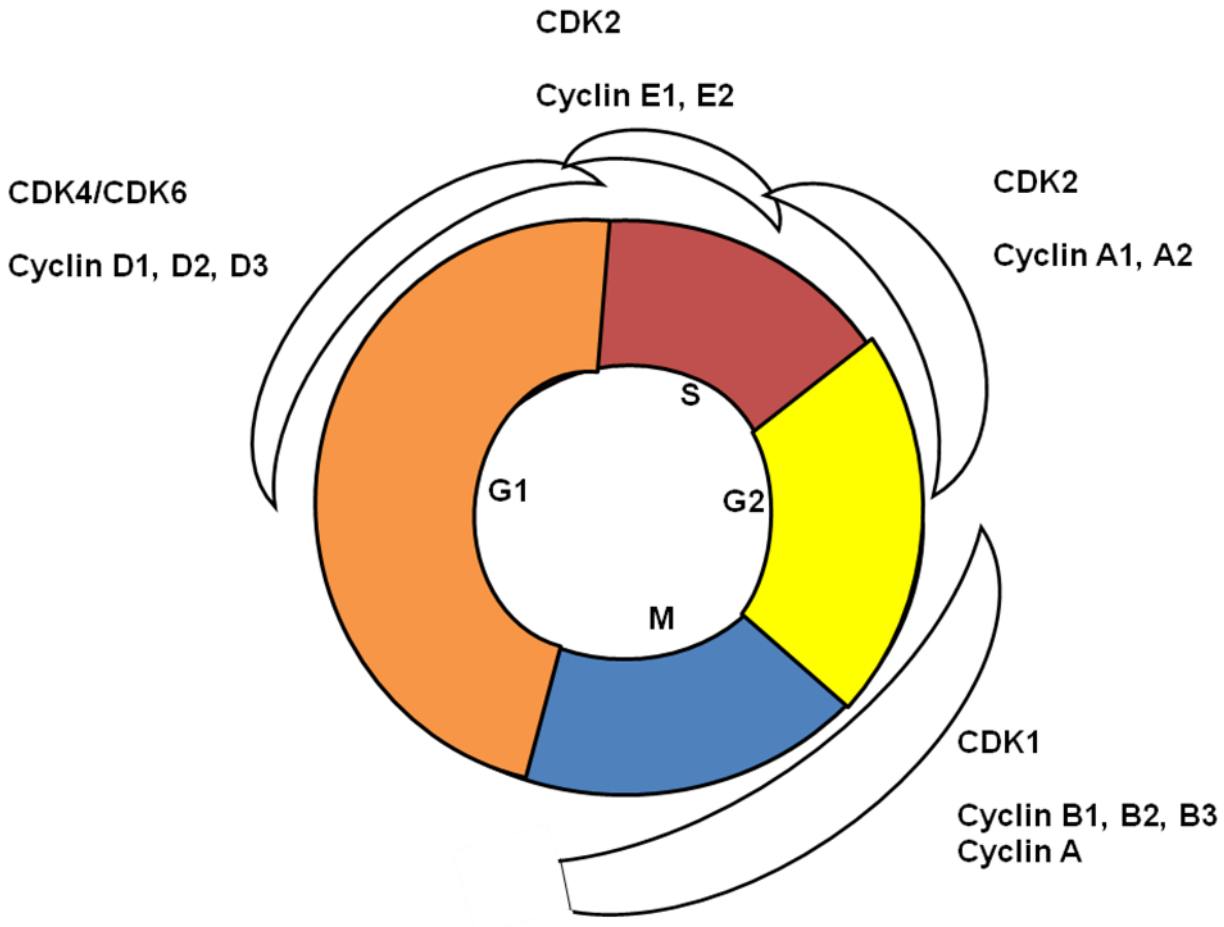


Figure 1.2 Simplified representation of the cell cycle. A typical (somatic) cell cycle, which is divided into four sequential phases: G_1, S, G_2 and M. Shapes outside the cycle indicate increase and reduction of corresponding CDK/cyclin activity.

alleviated. Animal cells contain two CKI families: the Cip/Kip family and the INK4 family (Sherr and Roberts 1999). Members of the INK4 family, such as p15, $p16^{INK4A}$, p18, and p19 specifically inhibit the activity of CDK4 and CDK6, whereas Cip/Kip members, such as, p21, p27, and p57 inhibit a wider spectrum of cyclin–CDK complexes (el-Deiry et al. 1993, Gu et al. 1993, Harper et al. 1993, Polyak et al. 1994). One of the INK4 proteins of particular interest to cancer researchers is $p16^{INK4A}$, as it is important in mediating a late G1 cell cycle checkpoint and is deleted, silenced, or mutated in many forms of cancer, including melanoma. Germline mutations in $p16^{INK4A}$ are also, for reasons that remain unclear, associated with a familial disposition to melanoma and pancreatic cancer.

The $p16^{INK4a}$ –Retinoblastoma (Rb) pathway is an important regulator of the G1/S transition. Under normal growth conditions, Cyclin-dependent kinase 4/6 binds to cyclin D and hyperphosphorylates Rb. While in a hypophosphorylated state, Rb is associated with transcription factor E2F1, keeping it inactive and thereby preventing transcription of E2F1 target genes necessary for transition into the S phase of the cell cycle. Rb also regulates $p16^{INK4A}$ expression through a negative feedback loop. CDK4/6-mediated hyperphosphorylation of Rb leads to increased $p16^{INK4A}$ expression. This increased $p16^{INK4A}$ expression in turn leads to the inhibition of any further hyperphosphorylation of Rb, thereby decreasing $p16^{INK4A}$ expression. Interestingly, despite the existence of this feedback loop the expression of $p16^{INK4A}$ has been observed to not dramatically change during the cell cycle to correlate with the phosphorylation status of Rb (Hara et al. 1996).

Another way by which $p16^{INK4A}$ provides a barrier to the emergence of tumorigenic cells is by inducing senescence. The senescent state is regarded as involving a permanent growth arrest but cells still remain metabolically active and stable. Senescence was first described upon observing the lifespan of human fibroblasts in cell culture, but is now regarded as a more common response to certain forms of stress. Common characteristics of senescence include expression of senescence-associated β-galactosidase activity, the appearance of senescence-associated heterochromatic foci, and an enlarged and flattened appearance of the cytoplasm (Gil and Peters 2006). Studies in human fibroblasts revealed that in this system the main trigger of the start of senescence was the erosion of telomeres that continues with every cell division (Zindy et al. 1997). Eventually this shortening is interpreted as DNA damage, cell cycle arrest is triggered, and the $p16^{INK4A}$ pathway is one of several pathways that is activated. This is evidenced by the observation that in many cell lines, $p16^{INK4A}$ expression increases with passage number (Zindy et al. 1997), whereas it has proven very difficult to detect during embryogenesis in vivo (McKeller et al. 2002, (Krishnamurthy et al. 2004, Zindy et al. 2003, Kim et al. 2006). Also, many immortalized cell lines show no $p16^{INK4A}$ expression due to methylation of its promoter (Ruas and Peters 1998, Sharpless and DePinho 1999).

Biology of Melanocytes and the Danger of Melanoma

As the largest organ of the human body, the skin is under constant bombardment of internal and external stimuli and therefore serves an imperative role as a barrier against microbial, chemical and physical exposures that could harm the body (Costin and Hearing 2007). In observing nature one can see the wide array of different colors and patterns that most species have evolved, a result of the modulation of the distribution of

pigments throughout the body. These different pigmentation patterns serve several purposes including heat regulation, camouflage, and cosmetic variation for mate acquisition. However from an oncological standpoint the most interesting use of these pigments is for the defense against ultraviolet radiation (UVR) (Costin and Hearing 2007). In humans, melanocytes produce melanin and subsequently pass the pigment into their dendrites and distribute it to keratinocytes. The melanin forms "caps" that shield the keratinocytes' nuclei from the UVR, reducing the overall level of DNA damage to the epidermis though the conversion of UVR to relatively harmless heat energy (Fitzpatrick and Breathnach 1963).

Melanocytes are highly specialized cells that produce the chemically inert and stable pigment melanin in membrane-bound organelles called melanosomes. One melanocyte may be in contact with an average of 40 keratinocytes via its dendrites to pass on these melanosomes, and this melanocyte-keratinocyte unit is known as an "epidermal melanin unit" (Fitzpatrick and Breathnach 1963). Melanocytes also reside in the middle layer (uvea) of the eye (Barden and Levine 1983), the inner ear (Markert and Silvers 1956), meninges (Mintz 1971), bones (Nichols et al. 1988), and heart (Theriault and Hurley 1970). Melanocytes are derived from the neural crest, which during the second month of embryonic life forms the melanocyte precursors known as melanoblasts. Melanoblasts migrate through the mesenchyme and reach their target sites (Boissy and Nordlund 1997). This migration and survival is contingent upon interactions between extracellular ligands and their corresponding receptors on the surface of the melanoblasts. For example, melanoblasts and melanocytes harbor the cell surface receptor KIT that binds to steel factor (formerly known as mast cell growth factor, KIT ligand, and/or stem

cell factor (SCF). At least one type of human piebaldism is caused by a mutation in the *KIT* gene, which results in a dysfunctional KIT receptor that exhibits a decreased ability to bind steel factor (Bolognia 1999). After the melanoblasts reach their destinations, they differentiate into melanocytes and become established at epidermal-dermal junction sites as early as the sixth month of fetal life (Costin and Hearing 2007).

One way through which melanocytes produce the pigment melanin is in response to DNA damage that has occurred from exposure to UVB radiation (Friedmann and Gilchrest 1987). Melanin pigments derive from tyrosine, and occur most commonly in the form of black-brown eumelanin, as well as the less common red-brown form called pheomelanin. Eumelanin occurs in two forms, black eumelanin and brown eumelanin, and both consist of a polymer of dihydroxyindole carboxylic acids and their reduced forms (Raper 1927). A small amount of black melanin in the absence of other pigments gives hair a grey color, whereas a small amount of brown eumelanin in the absence of other pigments causes blonde hair. Pheomelanin forms as a polymer of cysteine-containing benzothiazine units, and is responsible for the red-hair phenotype.

Melanin proves to be an excellent photoprotectant because it absorbs UV radiation and dissipates 99.9% of its energy as harmless heat through a process called "ultrafast internal conversion". This energy conversion is responsible for the protection against UV-induced skin cancer, including melanoma. Epidemiological studies have shown less skin cancer in individuals with higher levels of constitutive pigment and/or who tan well (Kollias et al. 1991, Weinstock 1993), although these studies failed to account for other factors important for development of skin cancer such as sensitivity to oxidative stress (Sander et al. 2004), sensitivity to UV-induced immunosuppression

(Kelly et al. 2000), and capacity to repair DNA photodamage (Gilchrest and Eller 1999, Sheehan et al. 2002).

Despite its classical view as a pure photoprotectant, several studies argue that some intermediates in the melanin biosynthesis pathway can be harmful (Kipp and Young 1999, Kvam and Tyrrell 1999, Kvam and Dahle 2004). Melanin itself can react with DNA and cause single-strand breaks (Marrot et al. 1999), and can produce intracellular ROS upon exposure to UVA radiation (Korytowski et al. 1987). The fact that certain intermediate products of melanin biogenesis, such as dihydroxyindole have been detected in plasma and urine indicate that the highly specialized melanosome has a propensity to leak or rupture (Agar and Young 2005). The tyrosinase reaction can produce quinones that have been found to mediate cell death at supraphysiological levels (Menon et al. 1983). There is a great deal of current research involved in learning more about the nature of melanin as a proverbial double-edged sword; a pigment nearly ubiquitous in nature and evolutionarily important for the photoprotection of mankind, the biogenesis of which exposes cells to toxic intermediates that can induce oxidative stress, DNA damage, and genomic instability.

Melanoma is the uncontrolled proliferation of the melanocytes, a specialized cell whose primary role is the production of melanin. Melanoma is the most aggressive form of skin cancer, being more metastatic than most solid tumors (it has the capability of becoming metastatic at a thickness as little as 1 mm), and while the rate of many cancers has either decreased or leveled off in recent years, melanoma occurrence continues to increase. Many countries have observed a doubling in the rate of melanoma in the last 20

years, and a person in the United States of America is now estimated to have a 1 in 55 lifetime chance of developing this insidious disease (Gray-Schopfer et al. 2007).

The problem of the increased rate of melanoma is compounded by the fact that there exist very few options for therapeutic intervention for the metastatic form of the disease. Distant metastases cause virtually all deaths from melanoma, and melanoma seems especially prone to quickly spread, a problem compounded by the fact that the only way to prevent metastases is through surgical excision of the original tumor, and many patients are not seen by a physician until the primary lesion has already metastasized.

Although the early diagnoses and surgical resection of localized cutaneous tumors usually cures melanoma, the original lesion has a tendency to quickly progress to an invasive state and metastasize to the lungs, liver and brain (Bastiaannet et al. 2005, Buzaid 2004, Danson and Lorigan 2005). At this point, patients generally have a five-year survival rate of less than 10%, with a mean survival time of 6 to 12 months, and are commonly presented with a treatment plan that is only palliative (Tsao et al. 2004, Gray-Schopfer et al. 2007, Balch et al. 2001, Francken et al. 2005, Bastiaannet et al. 2005). Patients with distant metastases respond poorly to the standard treatments with alkylating or cytotoxic agents, which usually results in drug resistance, melanoma relapse, and eventual death (Comis 1976, Hill et al. 1979, Tawbi and Buch 2010). The dramatic pace at which this disease progresses underscores the urgency in the field to identify and functionally characterize the molecular events that initiate the oncogenic transformation of melanocytes into melanoma cells, and to identify pertinent genetic events that predispose individuals to develop this disease.

Development of Melanoma

Melanoma commonly commences with the clinical presentation of benign or dysplastic nevi that have presented with some abnormality as defined by the "ABCDE" criteria (Asymmetry, irregular Border, uneven Color, Diameter greater than approximately 6 millimeters, and recent change or Evolution). Perhaps the factor the most indicative in oncogenic transformation is any recent evolution of a nevus, which includes a progression into the radial growth phase. Radial growth phase is typified by the lateral expansion of a nevus, while remaining localized to the epidermis (Ghosh and Chin 2009). The melanocytes then may become growth factor and anchorage independent and invade into the upper epidermis, as well as into the dermis and subcutaneous tissue (Ghosh and Chin 2009). This stage of progression is known as the vertical growth phase, and the thickness of this vertical growth is reported as the Breslow thickness (Balch et al. 2001). The Breslow thickness is currently regarded as the most important marker for prognosis. A high Breslow thickness indicates a high propensity for the lesion to become metastatic, first spreading to regional lymph nodes and then to distal sites (Ghosh and Chin 2009).

The tendency for a localized lesion to rapidly become invasive and spread to distant sites necessitates the earliest possible identification and eradication of a potentially cancerous lesion. A diagnosis of melanoma usually follows such imaging procedures as X-ray analysis, computed tomography, positron emission tomography, and magnetic resonance imaging (Algazi et al. 2010, Patnana et al. 2011). Moreover, biopsied tumor samples can be immunohistochemically stained with known melanocytic markers to improve the accuracy of the diagnosis. Such markers include S-100 and

melanoma-associated antigen recognized by T-cells (MART-1), also known as melanocyte antigen (Melan-A) (Nonaka et al. 2008, Mahmood et al. 2002, Ben-Izhak et al. 1994). Melanoma can also be more accurately identified using the monoclonal antibody gp100 that is highly sensitive and specific for melanoma, and does not cross-react with normal melanocytes, carcinomas or sarcomas; this allows clinicians to diagnose even very poorly differentiated melanoma subtypes (Mahmood et al. 2002, Gown et al. 1986). Despite the benefit of these markers, there exist few clinically validated markers for melanoma stem cells for early, noninvasive detection of the disease. It is therefore of great interest to continue to discover and validate biomarkers consistent with melanoma initiation (Mimeault and Batra 2012).

Targets Involved in Melanomagenesis for Therapeutical Intervention

The dismal response rates achieved while treating metastatic malignant melanoma reaffirm the need for continued investigation into the complex and unique signaling networks that are altered in this disease. The pace of scientific discovery and pathway elucidation has increased exponentially since RAS was discovered as the first melanoma oncogene in 1984 (Albino et al. 1984). The discovery of RAS in this context was achieved through the rather laborious efforts of functional and positional cloning. However, recent years have ushered in the use of microarrays and high-throughput sequencing, aided by the Human Genome Project (Hocker et al. 2008). Identifying specific genetic subsets of melanoma has and will continue to allow for more accurate diagnosis and selection of specific therapeutic interventions for individual patients. Examples of such altered pathways present in melanoma that have attracted and will continue to attract attention as putative therapeutic targets are the RAS-RAF-MAPK-

ERK signaling cascade (which can be further divided into the RAS-RAF-MAPK-ERK signaling cascade and the PI3K-AKT-mTOR cascade) and the CDKN2A/Cyclin D/CDK4/6 network.

The RAS-RAF-MAPK Signaling Cascade Is Altered and Activated in up to 90% of Melanomas

As is the case for many cancers, melanocyte transformation into melanoma involves alterations of the specific signaling pathways involved in normal cell differentiation, survival, and proliferation. Melanocytes utilize the complicated RAS signaling network to achieve these ends (Figure 1.3). In 1984, transforming mutations in RAS (specifically neuroblastoma ras viral oncogene homolog NRAS) were first identified in human melanoma cell lines (Albino et al. 1984), and subsequent studies have identified activating NRAS mutations in 26% of sporadic melanomas (Hocker et al. 2008).

An aberrant activation of the RAS pathway is common to many cancer types, and recent technological advances have allowed the more specific identification of very prominent, shared mutations. One such advance has been the description of the pathways of all relevant kinases involved in human cancer development, and the compilation of these data into the kinome. This kinome revealed that approximately 80% of pathogenic changes in *BRAF* are found in a single codon of its kinase domain, and that this same codon is affected in many cancers, including melanoma (Davies et al. 2002). This mutation is a 1799 T>A transition that results in a V600E amino acid change that activates this mitogenic pathway. Subsequent analysis have identified this BRAF V600E

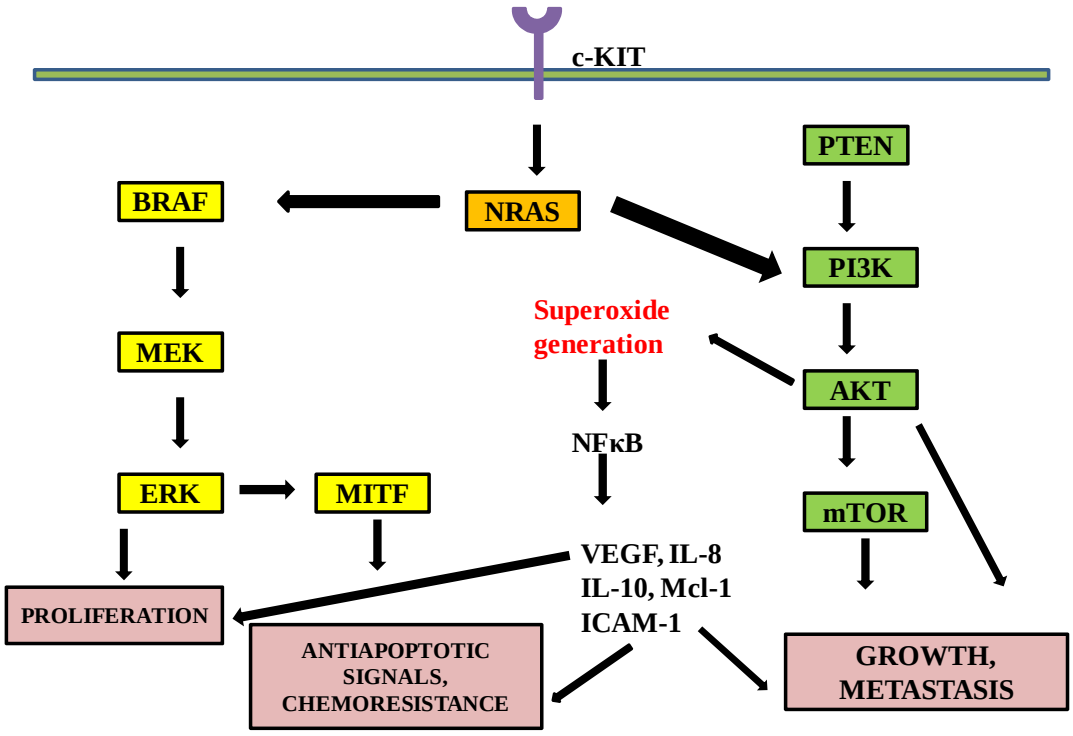


Figure 1. 3 NRAS signaling network in melanoma. Shown is a simplified diagram of the NRAS signaling network that is mutated in up to 90% of melanomas and benign melanocytic nevi, and commonly observed functional outcomes of pathway activation (pink boxes). Activated AKT can also lead to increased superoxide generation, thereby activating several pro-oncogenic genes in a NFκB manner.

mutation as the most commonly mutated gene in melanoma, found to be altered in 80% of short-term melanoma cultures and 66% of uncultured melanoma. The net result of these NRAS and BRAF mutations is a high constitutive activity of ERK, which can in turn lead to increased proliferation, invasiveness, angiogenesis, and metastasis (Cohen et al. 2002, Davies et al. 2002, Herlyn and Satyamoorthy 1996, Nikolaev et al. 2012).

Melanoma Progression Is Promoted Through the PI3K-AKT-PTEN Pathway

Another direct target of activated RAS that become constitutively activated is the phosphatidylinositol 3-kinase (PI3K) pathway (Figure 1.3), a signaling cascade that plays an integral role in regulating cellular proliferation, growth, migration and survival (Davies et al. 2008, Omholt et al. 2006). The PI3K pathway activates, via phosphorylation, the v-akt murine thymoma viral oncogene homologs, and is negatively regulated by lipid phosphatase phosphatidylinositol-3,4,5 triphosphate 3-phosphatase (PTEN). Common mutations therefore are seen in melanoma involving activation of the RAS-RAF-MAPK pathways in two different ways: either activation mutations are observed in NRAS or BRAF in a mutually-exclusive fashion, or the inactivation of the pathways negative regulator, PTEN (Tsao et al. 2000). Interestingly, PTEN inactivation is observed frequently in melanomas positive for activating mutations in *BRAF*, but not *NRAS*. This suggests that a synergy of activation could be occurring in the RAS pathway that involves the cooperation of these different types of mutations in the development of melanoma. Also, activated AKT has been associated with transition from radial growth phase to an aggressive vertical growth phase, and this transition has been associated with high levels of ROS, namely superoxide (Govindarajan et al. 2007). It is thought that

these high levels of superoxide are then sufficient to drive expression of NFκB, which then upregulates several genes thought to be essential for melanomagenesis (Figure 1.3) (Govindarajan et al. 2007). Also, increased levels of phospho-AKT has been measured in some melanomas that were negative for activating mutations in *NRAS* or loss of PTEN activity, suggesting that additional activating genetic alterations lie upstream of AKT that remain to be elucidated.

The CDKN2A/Cyclin D/CDK4/6 Tumor Suppressor Network Is Often Inactivated in Melanoma

Activating mutations in the *NRAS* pathway are observed in approximately 90% of melanoma cases, hence the emphasis that has been applied to discover and test therapeutic inhibitors of this pathway. However, activating mutations in *BRAF* or *NRAS* alone prove insufficient to drive melanomagenesis, and oncogenic BRAF activation leads to a senescent state (Mooi and Peeper 2006). This demonstrates that cells with these activating proliferative mutations must override the senescence program to fully achieve a transformed state. The senescence program primarily involves the tumor suppressors RB, $p16^{INK4a}$, $p14^{ARF}$, and p53, therefore it stands to reason that individuals that have genetic compromise in one or more of these genes have an increased lifetime risk of developing melanoma.

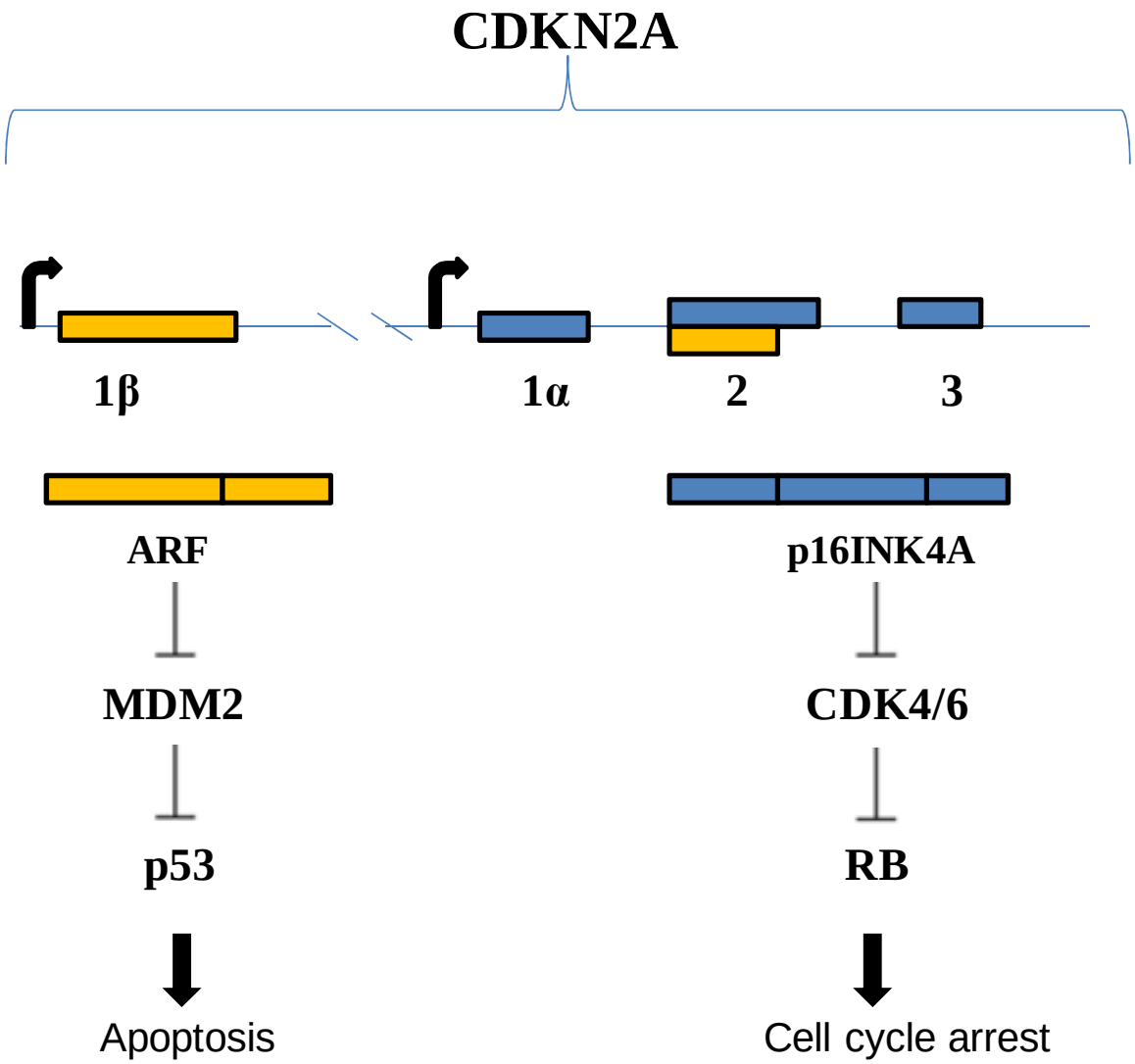
In the past it was hypothesized that critical information regarding melanoma susceptibility could be garnered through observing the karyotype of cultured melanocytes from different classes of melanocytic lesions, spanning from congenital nevi, dysplastic nevi, and melanoma (Cohen et al. 2002). Most congenital and dysplastic nevi showed normal karyotypes, whereas all melanomas observed showed aberrant chromosomal

profiles. The loss of one copy of chromosome 9 or the loss of its short arm 9p was observed to be the only chromosomal change common to the abnormal dysplastic karyotypes and the melanoma panels. This discovery over two decades ago was the first to implicate a melanoma tumor suppressor(s) on 9p to be involved in the progression from dysplastic nevi to melanoma (Kamb et al. 1994).

Regions of loss-of-heterozygosity in the genomes of dysplastic nevi and melanoma samples were then identified in the 9p21 region through deletional analysis, and genetic linkage studies showed that some cases of inherited melanoma predisposition could be attributed to genetic compromise of this region. Subsequent positional cloning efforts identified the *CDKN2A* locus as the area important for melanoma predisposition (Kamb et al. 1994, Nobori et al. 1994), as well as a locus that is deleted in pancreatic adenocarcinoma, glioblastoma, non-small cell lung cancer, bladder cancer, and certain leukemias (Kim and Sharpless 2006, Sherr 2000). This locus is unique in that it encodes two separate tumor suppressor proteins, $p16^{INK4A}$ and Alternate Reading Frame (ARF). These two proteins have different first exons that are spiced to a common second and third exon. Despite the sharing of the second and third exons, the $p16^{INK4A}$ and *ARF* are transcribed in alternate reading frames and share no amino acid homology (Figure 1.4).

Given the complex nature of this locus, it is of great interest to researchers to define which protein is the most relevant for tumor suppression, and under what circumstances mutations in these proteins lead to cancer development. The general consensus concerning human tumor development is that $p16^{INK4A}$ is more important for tumor development than ARF, as somatic loss of $p16^{INK4A}$ without compromise of *ARF* has been reported in thousands of human cancers (Forbes et al. 2006). At least 56

Figure 1.4 The CDKN2A locus at chromosome 9p21. The locus has a unique organization, coding for tumor suppressors $p16^{INK4A}$ and ARF. Two separate promoters drive the 1β (ARF) and 1α ($p16^{INK4A}$) exons, resulting in alternatively spliced transcripts that share exons 2 and 3. Although shared, different open reading frames within exon 2 give rise to two distinct protein products. $P16^{INK4A}$ inhibits CDK4/6-cyclin D-mediated hyperphosphorylation of RB; thereby, insuring that RB is in complex with the transcription factor E2F. RB-E2F complexes sequester factors that repress transcription, resulting in G1 cycle arrest. ARF blocks MDM2-mediated ubiquitylation and subsequent degradation of p53. This helps stabilize p53 and preserves its tumor suppressive activities. Loss of ARF activity can result in uncontrolled MDM2-mediated degradation of p53, resulting in loss of cell cycle control and compromised tumor suppression.


CDKN2A
1β
1α
2
3
ARF
p16INK4A
MDM2
CDK4/6
p53
RB
Apoptosis
Cell cycle arrest

different germline mutations in *p16*^{INK4A} that do not affect *ARF* have been observed in kindreds that present different forms of cancer in their families. (Greenblatt et al. 2003).

The Structure of p16 and the Significance of Ankyrin Repeats

The structure of p16^{INK4A} classifies it as a repeat protein, a very large group of proteins that consist of tandem repeating modular structures of high similarity that are found in many organisms that span a wide range of life forms. These repeat proteins are involved in a diverse range of physiological functions such as cell cycle control, apoptosis, development and differentiation, vesicular trafficking, cellular scaffolding, cytoskeleton integrity, transcriptional regulation and cell signaling (Forrer et al. 2003), (Main et al. 2003). The repeating motifs in these proteins consists of 20-40 amino acid residues that tend to organize themselves in a packed architecture that create a larger surface that forms an interface to participate in binding to other proteins (Li et al. 2006), (Binz and Pluckthun 2005). In fact, concerning proteins that are involved in protein-protein interactions only immunoglobulins are more abundant than the repeat protein class (Andrade et al. 2001). Over 20 different categories of repeat proteins have been classified, one of the most common of which are the ankyrin repeat proteins, which include the melanoma susceptibility gene p16^{INK4A}.

Ankyrin repeat proteins consist of repeated motifs of 30-34 amino acid residues that serve to mediate protein-protein interactions, some of which are highly involved in the pathogenesis of several human diseases (Sedgwick and Smerdon 1999). Genetic alterations including point mutations, methylation, and deletion of several different human tumors have been observed in genes encoding several different ankyrin proteins. While they have been understudied in the past compared to globular proteins, a recent

increase in the investigation of these proteins have been spurred on due to the increased availability of protein sequencing data that has described the commonality of these motifs For example, the nonredundant SMART protein database recently revealed the presence of 19,276 ankyrin repeat sequences in 3608 unique proteins (Mosavi et al. 2004).

The ankyrin repeat forms a helix-loop-helix structure, with the two α-helices being antiparallel to each other, giving the motif the appearance of the letter L. The β-hairpin loops project at roughly a 90◦ angle and form β-sheet structures with neighboring loops (Tevelev et al. 1996, Byeon et al. 1998). These repeated structures will stack in a nearly linear fashion, and this resulting helix bundle is stabilized through both intra- and interrepeated hydrophobic interactions (Mosavi et al. 2004, Michaely et al. 2002). $p16^{INK4A}$ contains four of these ankyrin repeats, flanked by flexible tails and the N- and C- termini (Figure 1.5).

Dissertation Overview

It has been well established that genetic compromise of $p16^{INK4A}$ is observed in a large numbers of tumors, with germline mutation leading to a higher predisposition to melanoma than other tumors. It is currently unknown why melanocytes seem especially sensitive to oncogenic transformation in the context of $p16^{INK4A}$ inactivation or mutation.

It has also been well established that the greatest environmental factor contributing to melanomagensis is exposure to excessive levels of UV radiation, and one of the main mechanisms by which this UV radiation induces its oncogenic effect is through the generation of ROS. These excessive ROS can then damage a number of cellular constituents, and seem especially harmful in that they can induce a state of genomic instability through specific forms of DNA damage. The work of this

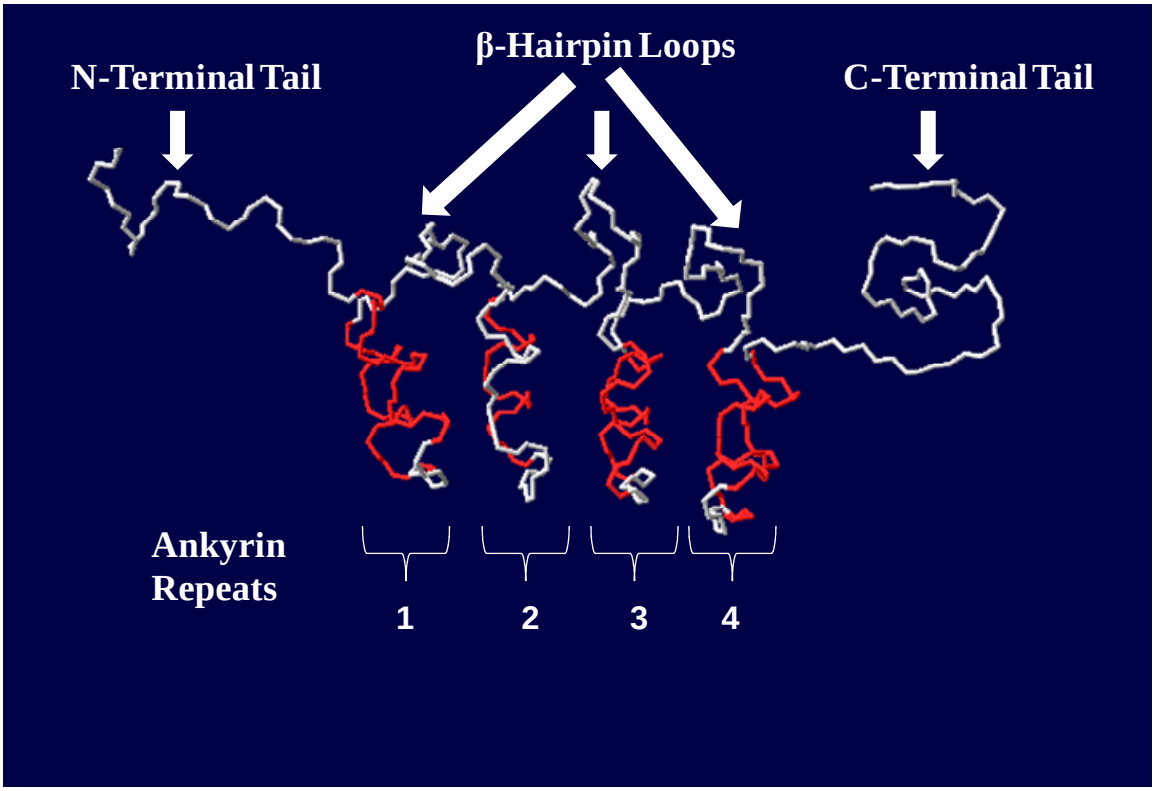


Figure 1.5 Diagram of the p16 structure. Flanked by flexible tails at the N and C termini, each ankyrin repeat exhibits a helix turn helix structure. The ankyrin repeats are designated as 1, 2, 3, and 4. The four ankyrin repeats are connected by three loops in β-hairpin turn structure. The individual ankyrin repeat is formed from a helix (red) loop (gray) helix (red).

dissertation demonstrates a novel functional link between the genetic melanoma predispositionary state of $p16^{INK4A}$ compromise and the environmental melanomagenic factor of excessive generation of intracellular oxidative stress. Specifically, this work describes a novel, potentially tumor-suppressive role of $p16^{INK4A}$ in the regulation of intracellular oxidative stress that is independent of its canonical tumor-suppressive role of a cell-cycle regulator. We report that this $p16^{INK4A}$-mediated regulation of oxidative stress is common to several cell types, but the higher constitutive levels of ROS we observed in melanocytes may indicate why genetic compromise of $p16^{INK4A}$ predisposes individuals to melanoma more often than other cancers.

Approximately 25-50% of familial melanoma kindreds and 10% of individuals with multiple primary melanomas show germline mutations in the *CDKN2A* locus (Soufir et al. 1998). Somatic alterations in CDKN2A have been reported in 30-70% of sporadic melanomas (Bartkova et al. 1996,Walker et al. 1998). Because this single locus codes for two separate proteins and mutational events can cause loss of function for either or both of these proteins, it can be difficult to assess the individual roles of either protein in promoting oncogenesis. Sporadic tumors have shown inactivation of $p16^{INK4A}$ and ARF through mutation (Pollock et al. 1996), promoter methylation (Merlo et al. 1995) or deletion (Cairns et al. 1995). Melanoma samples have exhibited point mutations in the $p16^{INK4A}$-specific exon 1α, and sequences shared by both $p16^{INK4A}$ and ARF, but not in the ARF-specific exon 1β. Also, most mutations that do occur in the shared sequences do not affect the function of ARF (Hewitt et al. 2002). Additionally, the CpG island that is methylation-silenced is in the $p16^{INK4A}$ promoter (Arap et al. 1997). It thus appears that the more important tumor suppressor protein at the CDKN2A locus is $p16^{INK4A}$.

Given my novel discovery that $p16^{INK4A}$ regulates intracellular oxidative stress independently of the Rb pathway and the Rb-mediated cell cycle regulation, I hypothesized that we may be able to functionally uncouple these two roles. Using mutagenic PCR, a panel of $p16^{INK4A}$ proteins harboring specific point mutations observed in familial melanoma was constructed. A lentiviral expression system was used to express these mutant proteins in a murine fibroblast line nullizygous for $p16^{INK4A}$ (Figure 1.6). Mutant proteins were classified according to their ability to restore cell cycle and/or oxidative stress regulation when compared to the re-expression of wild-type p16 expressed in these same $p16^{INK4A}$-deficient cells. Interestingly, several mutants showed a restoration of one, but not both, functions. Several mutants also showed an ability to functionally restore both oxidative and cell cycle regulation. It has been reported that the third ankyrin repeat is the region of $p16^{INK4A}$ that makes the most interactions with CDK 4/6 (Byeon et al. 1998, Mahajan et al. 2007, Russo et al. 1998), but several mutations lie in other regions of $p16^{INK4A}$. Interestingly, most mutations impairing oxidative but not cell-cycle function (A36P, A57V, P114S), or those not impairing either function (G35A, G35V, R24P), lie outside this repeat. Taken together, this work describes a novel function of the cell cycle regulator $p16^{INK4A}$ in regulating intracellular oxidative stress. We also demonstrate that these two putative tumor suppressor functions can be uncoupled in a subset of familial melanoma-associated point mutants and that these different functions are likely being regulated by different regions of the protein.

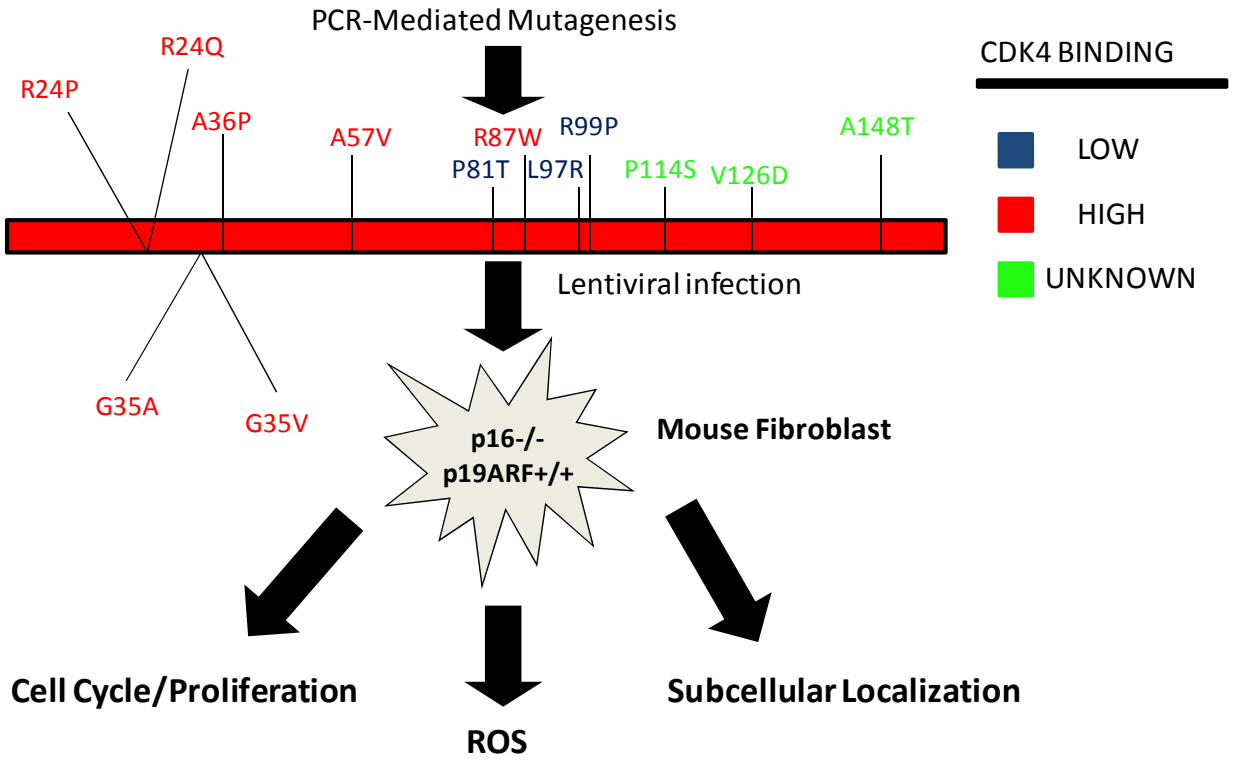


Figure 1.6 Schematic detailing mutant construction and analysis. Overlap PCR using mutagenic primers were used to construct a panel of 13 familial melanoma-associated $p16^{INK4A}$ point-mutants that spanned the coding region and have exhibited a range of CDK4/6 binding in previous literature. Constructs where expressed in $p16^{INK4A}$ deficient murine fibroblasts and analyzed for their capacity to correctly localize to the nucleus, restore cell cycle function, and restore intracellular ROS levels.

References

Agar N, Young AR (2005). Melanogenesis: a photoprotective response to DNA damage? *Mutat Res* **571:** 121-132.

Albino AP, Le Strange R, Oliff AI, Furth ME, Old LJ (1984). Transforming ras genes from human melanoma: a manifestation of tumour heterogeneity? *Nature* **308:** 69-72.

Algazi AP, Soon CW, Daud AI (2010). Treatment of cutaneous melanoma: current approaches and future prospects. *Cancer Manag Res* **2:** 197-211.

Ames BN (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science* **221:** 1256-1264.

Andrade MA, Perez-Iratxeta C, Ponting CP (2001). Protein repeats: structures, functions, and evolution. *J Struct Biol* **134:** 117-131.

Arap W, Knudsen E, Sewell DA, Sidransky D, Wang JY, Huang HJ *et al* (1997). Functional analysis of wild-type and malignant glioma derived CDKN2Abeta alleles: evidence for an RB-independent growth suppressive pathway. *Oncogene* **15:** 2013-2020.

Aruoma OI, Halliwell B, Dizdaroglu M (1989). Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. *The Journal of biological chemistry* **264:** 13024-13028.

Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG *et al* (2001). Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* **19:** 3635-3648.

Barden H, Levine S (1983). Histochemical observations on rodent brain melanin. *Brain Res Bull* **10:** 847-851.

Bartkova J, Lukas J, Guldberg P, Alsner J, Kirkin AF, Zeuthen J *et al* (1996). The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis. *Cancer research* **56:** 5475-5483.

Bastiaannet E, Beukema JC, Hoekstra HJ (2005). Radiation therapy following lymph node dissection in melanoma patients: treatment, outcome and complications. *Cancer Treat Rev* **31:** 18-26.

Ben-Izhak O, Stark P, Levy R, Bergman R, Lichtig C (1994). Epithelial markers in malignant melanoma. A study of primary lesions and their metastases. *Am J Dermatopathol* **16:** 241-246.

Binz HK, Pluckthun A (2005). Engineered proteins as specific binding reagents. *Curr Opin Biotechnol* **16:** 459-469.

Boissy RE, Nordlund JJ (1997). Molecular basis of congenital hypopigmentary disorders in humans: a review. *Pigment Cell Res* **10:** 12-24.

Boiteux S, Radicella JP (2000). The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Arch Biochem Biophys* **377:** 1-8.

Bolognia JL (1999). A clinical approach to leukoderma. *Int J Dermatol* **38:** 568-572.

Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP *et al* (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **88:** 10124-10128.

Breen AP, Murphy JA (1995). Reactions of oxyl radicals with DNA. *Free Radic Biol Med* **18:** 1033-1077.

Bruner SD, Norman DP, Verdine GL (2000). Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403:** 859-866.

Buzaid AC (2004). Management of metastatic cutaneous melanoma. *Oncology (Williston Park)* **18:** 1443-1450.

Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ *et al* (1998). Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. *Molecular cell* **1:** 421-431.

Cairns P, Polascik TJ, Eby Y, Tokino K, Califano J, Merlo A *et al* (1995). Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. *Nature genetics* **11:** 210-212.

Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G *et al* (2002). Mitogen-actived protein kinase activation is an early event in melanoma progression. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8:** 3728-3733.

Comis RL (1976). DTIC (NSC-45388) in malignant melanoma: a perspective. *Cancer Treat Rep* **60:** 165-176.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* **17:** 1195-1214.

Costin GE, Hearing VJ (2007). Human skin pigmentation: melanocytes modulate skin color in response to stress. *FASEB J* **21:** 976-994.

Danson S, Lorigan P (2005). Improving outcomes in advanced malignant melanoma: update on systemic therapy. *Drugs* **65:** 733-743.

David-Cordonnier MH, Boiteux S, O'Neill P (2001). Efficiency of excision of 8-oxo-guanine within DNA clustered damage by XRS5 nuclear extracts and purified human OGG1 protein. *Biochemistry* **40:** 11811-11818.

David SS, Williams SD (1998). Chemistry of glycosylases and endonucleases involved in base-excision repair. *Chem Rev* **98:** 1221-1262.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al* (2002). Mutations of the BRAF gene in human cancer. *Nature* **417:** 949-954.

Davies MA, Stemke-Hale K, Tellez C, Calderone TL, Deng W, Prieto VG *et al* (2008). A novel AKT3 mutation in melanoma tumours and cell lines. *Br J Cancer* **99:** 1265-1268.

Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H (2002). Free radical-induced damage to DNA: mechanisms and measurement. *Free Radic Biol Med* **32:** 1102-1115.

Droge W (2002). Free radicals in the physiological control of cell function. *Physiol Rev* **82:** 47-95.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM *et al* (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817-825.

Fitzpatrick TB, Breathnach AS (1963). [the Epidermal Melanin Unit System]. *Dermatol Wochenschr* **147:** 481-489.

Forbes S, Clements J, Dawson E, Bamford S, Webb T, Dogan A *et al* (2006). Cosmic 2005. *Br J Cancer* **94:** 318-322.

Forrer P, Stumpp MT, Binz HK, Pluckthun A (2003). A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett* **539:** 2-6.

Francken AB, Bastiaannet E, Hoekstra HJ (2005). Follow-up in patients with localised primary cutaneous melanoma. *Lancet Oncol* **6:** 608-621.

Friedmann PS, Gilchrest BA (1987). Ultraviolet radiation directly induces pigment production by cultured human melanocytes. *J Cell Physiol* **133:** 88-94.

Ghosh P, Chin L (2009). Genetics and genomics of melanoma. *Expert Rev Dermatol* **4:** 131-151.

Gil J, Peters G (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol* **7:** 667-677.

Gilchrest BA, Eller MS (1999). DNA photodamage stimulates melanogenesis and other photoprotective responses. *J Investig Dermatol Symp Proc* **4:** 35-40.

Govindarajan B, Sligh JE, Vincent BJ, Li M, Canter JA, Nickoloff BJ *et al* (2007). Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. *J Clin Invest* **117:** 719-729.

Gown AM, Vogel AM, Hoak D, Gough F, McNutt MA (1986). Monoclonal antibodies specific for melanocytic tumors distinguish subpopulations of melanocytes. *Am J Pathol* **123:** 195-203.

Gray-Schopfer V, Wellbrock C, Marais R (2007). Melanoma biology and new targeted therapy. *Nature* **445:** 851-857.

Greenblatt MS, Beaudet JG, Gump JR, Godin KS, Trombley L, Koh J *et al* (2003). Detailed computational study of p53 and p16: using evolutionary sequence analysis and disease-associated mutations to predict the functional consequences of allelic variants. *Oncogene* **22:** 1150-1163.

Gu Y, Turck CW, Morgan DO (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* **366:** 707-710.

Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM *et al* (1994). Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 12808-12812.

Halliwell B, Gutteridge JM (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* **186:** 1-85.

Halliwell B, Gutteridge JM (1992). Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* **307:** 108-112.

Halliwell B (1993). The chemistry of free radicals. *Toxicol Ind Health* **9:** 1-21.

Halliwell B (1999). Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition. *Mutat Res* **443:** 37-52.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell* **100:** 57-70.

Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* **144:** 646-674.

Hara E, Smith R, Parry D, Tahara H, Stone S, Peters G (1996). Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Molecular and cellular biology* **16:** 859-867.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75:** 805-816.

Harris CC, Hollstein M (1993). Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* **329:** 1318-1327.

Hazra TK, Izumi T, Maidt L, Floyd RA, Mitra S (1998). The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. *Nucleic Acids Res* **26:** 5116-5122.

Hazra TK, Izumi T, Boldogh I, Imhoff B, Kow YW, Jaruga P *et al* (2002). Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 3523-3528.

Herlyn M, Satyamoorthy K (1996). Activated ras. Yet another player in melanoma? *Am J Pathol* **149:** 739-744.

Hewitt C, Lee Wu C, Evans G, Howell A, Elles RG, Jordan R *et al* (2002). Germline mutation of ARF in a melanoma kindred. *Human molecular genetics* **11:** 1273-1279.

Hill GJ, 2nd, Metter GE, Krementz ET, Fletcher WS, Golomb FM, Ramirez G *et al* (1979). DTIC and combination therapy for melanoma. II. Escalating schedules of DTIC with BCNU, CCNU, and vincristine. *Cancer Treat Rep* **63:** 1989-1992.

Hocker TL, Singh MK, Tsao H (2008). Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. *J Invest Dermatol* **128:** 2575-2595.

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991). p53 mutations in human cancers. *Science* **253:** 49-53.

Hunkar T, Aktan F, Ceylan A, Karasu C (2002). Effects of cod liver oil on tissue antioxidant pathways in normal and streptozotocin-diabetic rats. *Cell Biochem Funct* **20:** 297-302.

Inoue M, Osaki T, Noguchi M, Hirohashi S, Yasumoto K, Kasai H (1998). Lung cancer patients have increased 8-hydroxydeoxyguanosine levels in peripheral lung tissue DNA. *Jpn J Cancer Res* **89:** 691-695.

Jaruga P, Theruvathu J, Dizdaroglu M, Brooks PJ (2004). Complete release of (5'S)-8,5'-cyclo-2'-deoxyadenosine from dinucleotides, oligodeoxynucleotides and DNA, and direct comparison of its levels in cellular DNA with other oxidatively induced DNA lesions. *Nucleic Acids Res* **32:** e87-e96.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV *et al* (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264:** 436-440.

Kelly DA, Young AR, McGregor JM, Seed PT, Potten CS, Walker SL (2000). Sensitivity to sunburn is associated with susceptibility to ultraviolet radiation-induced suppression of cutaneous cell-mediated immunity. *J Exp Med* **191:** 561-566.

Kim SH, Rowe J, Fujii H, Jones R, Schmierer B, Kong BW *et al* (2006). Upregulation of chicken p15INK4b at senescence and in the developing brain. *J Cell Sci* **119:** 2435-2443.

Kim WY, Sharpless NE (2006). The regulation of INK4/ARF in cancer and aging. *Cell* **127:** 265-275.

Kipp C, Young AR (1999). The soluble eumelanin precursor 5,6-dihydroxyindole-2-carboxylic acid enhances oxidative damage in human keratinocyte DNA after UVA irradiation. *Photochem Photobiol* **70:** 191-198.

Klein JA, Ackerman SL (2003). Oxidative stress, cell cycle, and neurodegeneration. *J Clin Invest* **111:** 785-793.

Knight JA (1995). Diseases related to oxygen-derived free radicals. *Ann Clin Lab Sci* **25:** 111-121.

Kollias N, Sayre RM, Zeise L, Chedekel MR (1991). Photoprotection by melanin. *J Photochem Photobiol B* **9:** 135-160.

Korytowski W, Pilas B, Sarna T, Kalyanaraman B (1987). Photoinduced generation of hydrogen peroxide and hydroxyl radicals in melanins. *Photochem Photobiol* **45:** 185-190.

Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L *et al* (2004). Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* **114:** 1299-1307.

Kvam E, Tyrrell RM (1999). The role of melanin in the induction of oxidative DNA base damage by ultraviolet A irradiation of DNA or melanoma cells. *J Invest Dermatol* **113:** 209-213.

Kvam E, Dahle J (2004). Melanin synthesis may sensitize melanocytes to oxidative DNA damage by ultraviolet A radiation and protect melanocytes from direct DNA damage by ultraviolet B radiation. *Pigment Cell Res* **17:** 549-550.

Lee MG, Nurse P (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature* **327:** 31-35.

Li J, Mahajan A, Tsai MD (2006). Ankyrin repeat: a unique motif mediating protein-protein interactions. *Biochemistry* **45:** 15168-15178.

Mahmood MN, Lee MW, Linden MD, Nathanson SD, Hornyak TJ, Zarbo RJ (2002). Diagnostic value of HMB-45 and anti-Melan A staining of sentinel lymph nodes with isolated positive cells. *Mod Pathol* **15:** 1288-1293.

Main ER, Jackson SE, Regan L (2003). The folding and design of repeat proteins: reaching a consensus. *Curr Opin Struct Biol* **13:** 482-489.

Maki H, Sekiguchi M (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355:** 273-275.

Malins DC, Haimanot R (1991). Major alterations in the nucleotide structure of DNA in cancer of the female breast. *Cancer research* **51:** 5430-5432.

Malumbres M, Barbacid M (2005). Mammalian cyclin-dependent kinases. *Trends Biochem Sci* **30:** 630-641.

Markert CL, Silvers WK (1956). The effects of genotype and cell environment on melanoblast differentiation in the house mouse. *Genetics* **41:** 429-450.

Marrot L, Belaidi JP, Meunier JR, Perez P, Agapakis-Causse C (1999). The human melanocyte as a particular target for UVA radiation and an endpoint for photoprotection assessment. *Photochem Photobiol* **69:** 686-693.

Martindale JL, Holbrook NJ (2002). Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* **192:** 1-15.

McGoldrick JP, Yeh YC, Solomon M, Essigmann JM, Lu AL (1995). Characterization of a mammalian homolog of the Escherichia coli MutY mismatch repair protein. *Molecular and cellular biology* **15:** 989-996.

McKeller RN, Fowler JL, Cunningham JJ, Warner N, Smeyne RJ, Zindy F *et al* (2002). The Arf tumor suppressor gene promotes hyaloid vascular regression during mouse eye development. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 3848-3853.

Menon IA, Persad S, Ranadive NS, Haberman HF (1983). Effects of ultraviolet-visible irradiation in the presence of melanin isolated from human black or red hair upon Ehrlich ascites carcinoma cells. *Cancer research* **43:** 3165-3169.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC *et al* (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* **1:** 686-692.

Meyskens FL, Jr., Farmer P, Fruehauf JP (2001). Redox regulation in human melanocytes and melanoma. *Pigment Cell Res* **14:** 148-154.

Michaely P, Tomchick DR, Machius M, Anderson RG (2002). Crystal structure of a 12 ANK repeat stack from human ankyrinR. *The EMBO journal* **21:** 6387-6396.

Mimeault M, Batra SK (2012). Novel biomarkers and therapeutic targets for optimizing the therapeutic management of melanomas. *World J Clin Oncol* **3:** 32-42.

Mintz B (1971). Clonal basis of mammalian differentiation. *Symp Soc Exp Biol* **25:** 345-370.

Misra MK, Sarwat M, Bhakuni P, Tuteja R, Tuteja N (2009). Oxidative stress and ischemic myocardial syndromes. *Med Sci Monit* **15:** RA209-219.

Mooi WJ, Peeper DS (2006). Oncogene-induced cell senescence--halting on the road to cancer. *N Engl J Med* **355:** 1037-1046.

Morgan DO (1995). Principles of CDK regulation. *Nature* **374:** 131-134.

Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY (2004). The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* **13:** 1435-1448.

Nagashima M, Tsuda H, Takenoshita S, Nagamachi Y, Hirohashi S, Yokota J *et al* (1995). 8-hydroxydeoxyguanosine levels in DNA of human breast cancer are not significantly different from those of non-cancerous breast tissues by the HPLC-ECD method. *Cancer Lett* **90:** 157-162.

Nichols PF, Charles HC, Read JS, Goldberg MF (1988). Evaluation of choroidal neovascular membranes by Octopus perimetry. *Retina* **8:** 24-29.

Nikolaev SI, Rimoldi D, Iseli C, Valsesia A, Robyr D, Gehrig C *et al* (2012). Exome sequencing identifies recurrent somatic MAP2K1 and MAP2K2 mutations in melanoma. *Nature genetics* **44:** 133-139.

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368:** 753-756.

Nohl H, Kozlov AV, Gille L, Staniek K (2003). Cell respiration and formation of reactive oxygen species: facts and artefacts. *Biochem Soc Trans* **31:** 1308-1311.

Nonaka D, Chiriboga L, Rubin BP (2008). Differential expression of S100 protein subtypes in malignant melanoma, and benign and malignant peripheral nerve sheath tumors. *J Cutan Pathol* **35:** 1014-1019.

Okamoto K, Toyokuni S, Uchida K, Ogawa O, Takenewa J, Kakehi Y *et al* (1994). Formation of 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in human renal-cell carcinoma. *Int J Cancer* **58:** 825-829.

Omholt K, Krockel D, Ringborg U, Hansson J (2006). Mutations of PIK3CA are rare in cutaneous melanoma. *Melanoma Res* **16:** 197-200.

Pashaei S, Li L, Zhang H, Spencer HJ, Schichman SA, Fan CY *et al* (2008). Concordant loss of heterozygosity of DNA repair gene, hOGG1, in melanoma in situ and atypical melanocytic hyperplasia. *J Cutan Pathol* **35:** 525-531.

Patnana M, Bronstein Y, Szklaruk J, Bedi DG, Hwu WJ, Gershenwald JE *et al* (2011). Multimethod imaging, staging, and spectrum of manifestations of metastatic melanoma. *Clin Radiol* **66:** 224-236.

Pollock PM, Pearson JV, Hayward NK (1996). Compilation of somatic mutations of the CDKN2 gene in human cancers: non-random distribution of base substitutions. *Genes Chromosomes Cancer* **15:** 77-88.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM *et al* (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* **8:** 9-22.

Raper HS (1927). The Tyrosinase-tyrosine Reaction: Production from Tyrosine of 5: 6-Dihydroxyindole and 5: 6-Dihydroxyindole-2-carboxylic Acid-the Precursors of Melanin. *Biochem J* **21:** 89-96.

Reid TM, Loeb LA (1993). Tandem double CC-->TT mutations are produced by reactive oxygen species. *Proceedings of the National Academy of Sciences of the United States of America* **90:** 3904-3907.

Retel J, Hoebee B, Braun JE, Lutgerink JT, van den Akker E, Wanamarta AH *et al* (1993). Mutational specificity of oxidative DNA damage. *Mutat Res* **299:** 165-182.

Ruas M, Peters G (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* **1378:** F115-F177.

Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ (2004). Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. *Int J Dermatol* **43:** 326-335.

Scandalios JG (2002). The rise of ROS. *Trends Biochem Sci* **27:** 483-486.

Schwarz KB, Kew M, Klein A, Abrams RA, Sitzmann J, Jones L *et al* (2001). Increased hepatic oxidative DNA damage in patients with hepatocellular carcinoma. *Dig Dis Sci* **46:** 2173-2178.

Sedgwick SG, Smerdon SJ (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* **24:** 311-316.

Sharpless NE, DePinho RA (1999). The INK4A/ARF locus and its two gene products. *Current opinion in genetics & development* **9:** 22-30.

Sheehan JM, Cragg N, Chadwick CA, Potten CS, Young AR (2002). Repeated ultraviolet exposure affords the same protection against DNA photodamage and erythema in human skin types II and IV but is associated with faster DNA repair in skin type IV. *J Invest Dermatol* **118:** 825-829.

Sherr CJ, Roberts JM (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13:** 1501-1512.

Sherr CJ (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer research* **60:** 3689-3695.

Shibutani S, Takeshita M, Grollman AP (1991). Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* **349:** 431-434.

Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J *et al* (1994). Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. *Cancer research* **54:** 3171-3172.

Slupska MM, Baikalov C, Luther WM, Chiang JH, Wei YF, Miller JH (1996). Cloning and sequencing a human homolog (hMYH) of the Escherichia coli mutY gene whose function is required for the repair of oxidative DNA damage. *J Bacteriol* **178:** 3885-3892.

Soufir N, Avril MF, Chompret A, Demenais F, Bombled J, Spatz A *et al* (1998). Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. *Human molecular genetics* **7:** 209-216.

Takeuchi T, Morimoto K (1993). Increased formation of 8-hydroxydeoxyguanosine, an oxidative DNA damage, in lymphoblasts from Fanconi's anemia patients due to possible catalase deficiency. *Carcinogenesis* **14:** 1115-1120.

Tawbi HA, Buch SC (2010). Chemotherapy resistance abrogation in metastatic melanoma. *Clin Adv Hematol Oncol* **8:** 259-266.

Tevelev A, Byeon IJ, Selby T, Ericson K, Kim HJ, Kraynov V *et al* (1996). Tumor suppressor p16INK4A: structural characterization of wild-type and mutant proteins by NMR and circular dichroism. *Biochemistry* **35:** 9475-9487.

Theriault LL, Hurley LS (1970). Ultrastructure of developing melanosomes in C57 black and pallid mice. *Dev Biol* **23:** 261-275.

Toyokuni S, Okamoto K, Yodoi J, Hiai H (1995). Persistent oxidative stress in cancer. *FEBS Lett* **358:** 1-3.

Tsao H, Zhang X, Fowlkes K, Haluska FG (2000). Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. *Cancer research* **60:** 1800-1804.

Tsao H, Atkins MB, Sober AJ (2004). Management of cutaneous melanoma. *N Engl J Med* **351:** 998-1012.

Vulimiri SV, Wu X, Baer-Dubowska W, de Andrade M, Detry M, Spitz MR *et al* (2000). Analysis of aromatic DNA adducts and 7,8-dihydro-8-oxo- 2'-deoxyguanosine in lymphocyte DNA from a case-control study of lung cancer involving minority populations. *Mol Carcinog* **27:** 34-46.

Vurusaner B, Poli G, Basaga H (2012). Tumor suppressor genes and ROS: complex networks of interactions. *Free Radic Biol Med* **52:** 7-18.

Walker GJ, Flores JF, Glendening JM, Lin AH, Markl ID, Fountain JW (1998). Virtually 100% of melanoma cell lines harbor alterations at the DNA level within CDKN2A, CDKN2B, or one of their downstream targets. *Genes Chromosomes Cancer* **22:** 157-163.

Wang D, Kreutzer DA, Essigmann JM (1998). Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutat Res* **400:** 99-115.

Waris G, Alam K (1998). Attenuated antigenicity of ribonucleoproteins modified by reactive oxygen species. *Biochem Mol Biol Int* **45:** 33-45.

Waris G, Turkson J, Hassanein T, Siddiqui A (2005). Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. *J Virol* **79:** 1569-1580.

Waris G, Ahsan H (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog* **5:** 14-21.

Weinstock MA (1993). Epidemiology of melanoma. *Cancer Treat Res* **65:** 29-56.

Weisiger RA, Fridovich I (1973). Superoxide dismutase. Organelle specificity. *The Journal of biological chemistry* **248:** 3582-3592.

Zindy F, Quelle DE, Roussel MF, Sherr CJ (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15:** 203-211.

Zindy F, Williams RT, Baudino TA, Rehg JE, Skapek SX, Cleveland JL *et al* (2003). Arf tumor suppressor promoter monitors latent oncogenic signals in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 15930-15935.

Zyrek-Betts J, Micale M, Lineen A, Chaudhuri PK, Keil S, Xue J *et al* (2008). Malignant blue nevus with lymph node metastases. *J Cutan Pathol* **35:** 651-657.

CHAPTER 2

THE $p16^{INK4A}$ TUMOR SUPRESSOR REGULATES CELLULAR OXIDATIVE STRESS

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ORIGINAL ARTICLE

The p16^{INK4A} tumor suppressor regulates cellular oxidative stress

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Mutations or deletions in the cyclin-dependent kinase inhibitor *p16^{INK4A}* are associated with multiple cancer types, but are more commonly found in melanoma tumors and associated with familial melanoma predisposition. Although p16 is thought to function as a tumor suppressor by negatively regulating the cell cycle, it remains unclear why the genetic compromise of p16 predisposes to melanoma over other cancers. Here we describe a novel role for p16 in regulating oxidative stress in several cell types, including melanocytes. Expression of p16 was rapidly upregulated following ultraviolet-irradiation and in response to H_2O_2-induced oxidative stress in a p38 stress-activated protein kinase-dependent manner. Knockdown of p16 using small interfering RNA increased intracellular reactive oxygen species (ROS) and oxidative (8-oxoguanine) DNA damage, which was further enhanced by H_2O_2 treatment. Elevated ROS levels were also observed in p16-depleted human keratinocytes and in whole skin and dermal fibroblasts from *Cdkn2a*-deficient mice. Aberrant ROS and p38 signaling in *Cdkn2a*-deficient fibroblasts was normalized by expression of exogenous p16. The effect of p16 depletion on ROS was not recapitulated by the knockdown of retinoblastoma protein (Rb) and did not require Rb. Finally, p16-mediated suppression of ROS could not be attributed to the potential effects of p16 on cell cycle phase. These findings suggest a potential alternate Rb-independent tumor-suppressor function of p16 as an endogenous regulator of carcinogenic intracellular oxidative stress. Compared with keratinocytes and fibroblasts, we also found increased susceptibility of melanocytes to oxidative stress in the context of p16 depletion, which may explain why the compromise of p16 predisposes to melanoma over other cancers.
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Introduction

Inactivation of the *p16^{INK4A}* (hereafter referred to as *p16*) gene commonly occurs in many tumors (Sharpless and DePinho, 1999), although germ-line mutations in *p16* are more commonly associated with hereditary melanoma predisposition (Goldstein *et al.*, 2006). Somatic mutations of *p16* (Flores *et al.*, 1996) or allelic deletions of the *p16*-containing CDKN2a locus at 9p21 (Curtin *et al.*, 2005) have also been described in a large percentage of sporadic melanomas. The p16 tumor suppressor protein normally inhibits the kinase activity of cyclin-dependent kinases 4 and 6, thereby inhibiting the hyperphosphorylation of retinoblastoma (Rb)-related pocket proteins required for cell cycle progression (Lukas *et al.*, 1995). Native p16 thus functionally serves to prevent inappropriate division of stressed or damaged cells by holding them in the late G1 S transition and may promote irreversible exit from the cell cycle into a senescent state (Alcorta *et al.*, 1996).

Acute or chronic exposure to ultraviolet (UV) radiation produces reactive oxygen species (ROS) in the skin (Herrling *et al.*, 2006), which may contribute to the development of skin cancers including melanoma. There is a good deal of correlative evidence to suggest that the link between UV radiation and melanoma may lie in the generation of oxidative damage (Meyskens *et al.*, 2001). Interestingly, melanocytes isolated from melanoma patients display increased sensitivity to peroxidizing agents that correlates with endogenous antioxidant imbalance (Grammatico *et al.*, 1998), and elevated ROS levels have been found in melanocytes from dysplastic nevi relative to normal skin of the same individuals (Pavel *et al.*, 2004). In addition, mutation or loss of the enzyme hOGG-1, which repairs mutagenic oxidative DNA lesions (namely 8-oxoguanine (8-OG)), has been associated with melanoma progression (Pashaei *et al.*, 2008; Zyrek-Betts *et al.*, 2008). Recently, we demonstrated a role for UV-induced oxidative stress and damage in an animal model of UV-induced melanoma (Cotter *et al.*, 2007).

Given the role of p16 as a melanoma tumor suppressor and recent implication of oxidative stress in UV-induced melanoma noted above, we investigated a potential link between p16 and regulation of intracellular ROS. We found that multiple cell types exhibit increased levels of intracellular ROS in the context of

deficiency or loss of p16, which can be restored upon re-expression of p16. Regulation of ROS by p16 occurred independently of Rb and potential effects on cell cycle. Thus, the tumor suppressor function of p16 may extend beyond cell cycle control and include a novel role in regulating oxidative stress in skin cells. Oxidative dysregulation in the context of loss of p16 function may lead to accumulation of mutations, which could predispose to tumor development. The increased susceptibility of melanocytes (compared with keratinocytes and fibroblasts) to oxidative stress in the context of p16 depletion may explain why compromise of p16 predisposes to melanoma over other cancers.

Results

Oxidative stress upregulates p16 in melanocytes, which are more susceptible to oxidative stress than other cell types

The p16 tumor suppressor is known to function by inducing cell cycle arrest or senescence when cells encounter potentially oncogenic DNA damage (Shapiro *et al*., 1998). In response to UV exposure, melanocytes acutely upregulate p16 at both protein and mRNA levels (Piepkorn, 2000). We confirmed that p16 protein levels are elevated in normal human melanocytes following UV exposure, detected as early as 1 h and peaking at 5 h (Figure 1a, top). Direct induction of oxidative stress had comparable effects on p16, as melanocytes treated with H_2O_2 demonstrated similar upregulation of p16 protein levels, which was blocked by pre-addition of the antioxidant *N*-acetylcysteine (NAC) (Figure 1a, bottom). The induction of ROS under these conditions, and the capacity of NAC to reduce ROS levels when added before H_2O_2 treatment, was confirmed by addition of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and subsequent fluorimetric analysis (Supplementary Figure S1a). In addition, p16 upregulation following H_2O_2 treatment was associated with dose-dependent increased positive staining for 8-OG, which was reduced by pre-addition of NAC (Supplementary Figure S1b). Addition of NAC to untreated cells reduced endogenous oxidative stress and p16 levels, and pre-addition of NAC significantly reduced UV-induced oxidative stress (Figure 1b, top) and attenuated UV-induced upregulation of p16 (Figure 1b, bottom).

Significant upregulation of p16 was also observed at the RNA level in H_2O_2-treated melanocytes, as well as in similarly treated human keratinocytes and fibroblasts (Figure 1c). This response of p16 to oxidative stress may be particularly important in melanocytes, however, which demonstrated significantly higher levels of basal and H_2O_2-induced oxidative stress (Figure 1d, left) and 8-OG (Figure 1d, right) than the other cell types.

ROS-mediated p16 upregulation occurs through the p38 stress-activated protein kinase (SAPK)

Previous studies in hematopoietic stem cells found that p38 SAPK phosphorylation is associated with upregulation of p16 (Ito *et al*., 2006). To investigate this pathway in melanocytes, cells were tested for upregulation of p16 by exogenous oxidative stress in the presence of a p38 phosphorylation inhibitor. As shown in Figure 2a, H_2O_2 treatment resulted in p38 phosphorylation and addition of the inhibitor attenuated H_2O_2-induced upregulation of p16. Addition of NAC inhibited both upregulation of p16 and phosphorylation of p38 in H_2O_2-treated cells (Figure 2a). Thus, ROS-induced p38 phosphorylation is required for the upregulation of p16, demonstrating a functional ROS-dependent p38 SAPK-p16 signaling pathway in melanocytes.

We further investigated this pathway by examining the effect of p38 phosphorylation on intracellular ROS levels. Interestingly, in the absence of an exogenous oxidative insult, inhibition of p38 phosphorylation significantly increased intracellular ROS to levels comparable to those observed in H_2O_2-treated cells (Figure 2b). This finding is consistent with a recent report in which inhibition or genetic deficiency of p38 resulted in increased intracellular ROS (Naidu *et al*., 2009), suggesting that activated p38 may also act to suppress ROS levels (Figure 2c). However, the induction of ROS upon inhibition of p38 phosphorylation also suggests the possible presence of a negative feedback loop in the ROS-dependent p38 p16 pathway in which p16 may act to suppress endogenous ROS (Figure 2c).

Intracellular ROS and oxidative DNA damage is increased in p16-deficient cells

We next asked whether p16 is required for normal regulation of intracellular ROS levels. Depletion of p16 in melanocytes by RNA interference (RNAi) (Figure 3a) was associated with an increase in intracellular ROS both in the presence and in the absence of exogenous oxidative (H_2O_2) stress (Figure 3b). ROS levels in p16-depleted cells were normalized by pre-addition of NAC (Figure 3b). We next assessed the extent of oxidative DNA damage (that is, 8-OG) in p16-depleted cells. As shown in Figure 3c, the fraction of untreated melanocytes positive for 8-OG was significantly higher in cells transfected with p16-specific compared with control small interfering RNA (siRNA). Although 8-OG positivity increased in both groups following treatment with H_2O_2, an increased fraction of 8-OG-positive cells was observed in the p16-depleted group (Figure 3c). Thus, p16 functions as a negative regulator of oxidative stress in melanocytes, lowering intracellular ROS and reducing oxidative DNA damage both in untreated cells and in conditions of exogenous oxidative stress.

To extend these findings to other cell types, melanocytes, fibroblasts, and keratinocytes were propagated from four individual donors and depleted of p16 using siRNA (Supplementary Figure S2). When intracellular ROS levels were compared in different cell types from the same donor, p16-depleted melanocytes exhibited significantly higher levels both basally and following H_2O_2 treatment (Figure 3d). Thus, p16 appears to regulate oxidative stress in multiple cell types and p16 depletion results in significantly greater levels of

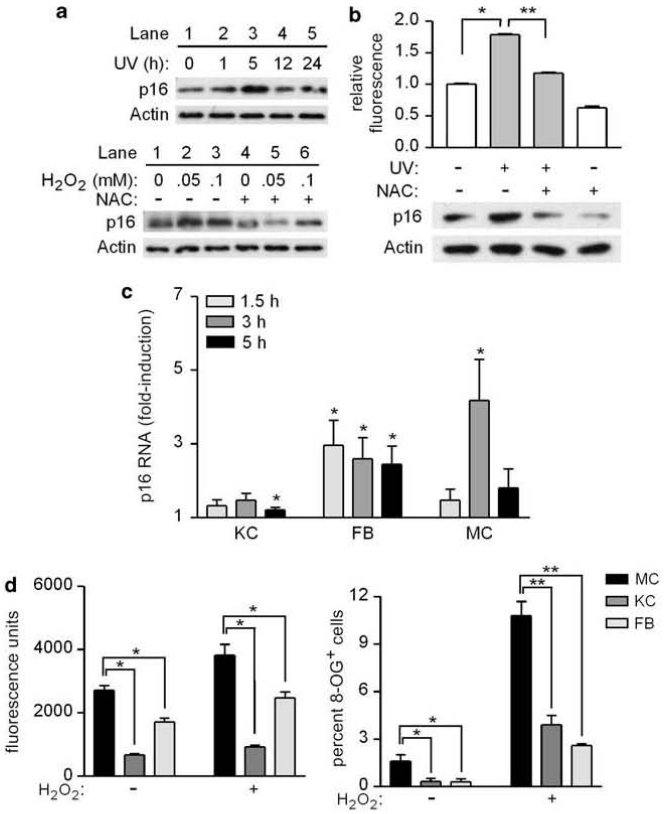

Figure 1 Exogenous oxidative stress acutely upregulates p16 in human skin cells, and melanocytes are more susceptible than other cell types to oxidative stress and damage. (**a**) Melanocytes were untreated (0 h) or UV-irradiated (480 J/m², lanes 2–5), and cell lysates were prepared over a 24-h period and blotted with antibodies against p16 and actin (upper panel). Melanocytes were treated with H_2O_2 at the concentrations indicated, in the absence (lanes 1–3) or presence (lanes 4–6) of 5 mM NAC, and 5 h later cell lysates were blotted for p16 and actin (lower panel). (**b**) Melanocytes were untreated (0 h) or UV-irradiated (480 J/m²) in the absence or presence of 5 mM NAC and 5 h later ROS levels were measured by DCFDA assay (upper panel, values normalized to mean of control conditions which were set at 1) and cell lysates were blotted for p16 and actin (lower panel). Error bars indicate s.e.m. from three independent experiments. $^*P<0.001$ (one-sample *t* test), $^{**}P<0.001$ (two-sample *t* test). (**c**) Keratinocytes (KC), fibroblasts (FB) and melanocytes (MC) isolated from each of six donors were untreated or treated with 0.05 mM H_2O_2 for 1.5, 3 or 5 h. RNA was isolated and expression of p16 and GAPDH was quantitated by qRT PCR, with p16 levels normalized to GAPDH at each time point (and then normalized to control conditions which were set at 1). Error bars indicate s.e.m. from six independent determinations. $^*P<0.05$ (one-sample *t* tests). (**d**) Melanocytes (MC), keratinocytes (KC), and fibroblasts (FB) isolated from each of seven donors were untreated or treated with 0.05 mM H_2O_2 for 5 h, and ROS levels were measured by DCFDA assay (left panel). Error bars indicate s.e.m. from seven independent determinations. $^*P<0.001$ (repeated-measures analysis of variance (ANOVA), *P*-values adjusted for multiple comparisons). Cells isolated from each of three donors were untreated or treated with 0.5 mM H_2O_2 for 48 h, then fixed and immobilized for 8-OG staining. Error bars indicate s.e.m. of percent 8-OG-positive cells assessed under each condition from three independent determinations. $^*P=0.06$, $^{**}P<0.001$ (adjusted for multiple comparisons).

intracellular ROS in melanocytes compared with other skin cell types.

The Cdkn2a locus regulates oxidative stress in vivo

The p16-encoding *Cdkn2a* locus is mutated in approximately half of the familial melanoma cases (Curtin *et al*., 2005). We next investigated *Cdkn2a*-dependent regulation of oxidative stress *in vivo* by measuring ROS levels in the skin of *Cdkn2a*-deficient mice. As shown in Figure 4a, we observed a significant increase in basal ROS in skin isolated from these mice compared with wild-type animals.

Oxidative dysregulation in Cdkn2a-deficient cells and restoration by exogenous p16

Two fibroblast lines were derived from both wild-type and *Cdkn2a*-null mice. Fibroblast lines from knockout

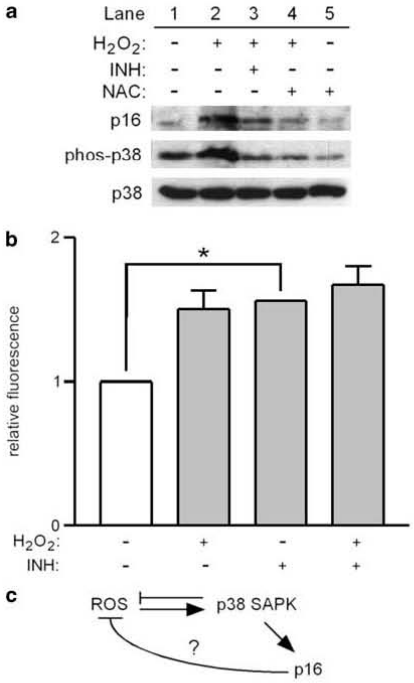

Figure 2 ROS upregulate p16 through the p38 SAPK in human melanocytes. (**a**) Melanocytes were cultured for 5 h alone (lane 1) or in the presence of 0.05 mM H_2O_2 (lanes 2–4), phospho-p38 inhibitor (INH), and with or without pre-addition of 5 mM NAC (lanes 4 and 5). Cell lysates were blotted for p16, phospho-p38 and p38, with p38 serving as a loading control. (**b**) Melanocytes were cultured for 5 h alone or in the presence of 0.05 mM H_2O_2 and/or phospho-p38 inhibitor (INH). ROS levels were measured by DCFDA assay, and values normalized to those of control conditions, which were set at 1. Error bars indicate s.e.m. from three independent experiments. $^*P<0.001$ (one-sample *t* test). (**c**) Schematic depicting the ROS-dependent p38-p16 signaling pathway, and the possibility that p16 suppresses endogenous ROS.

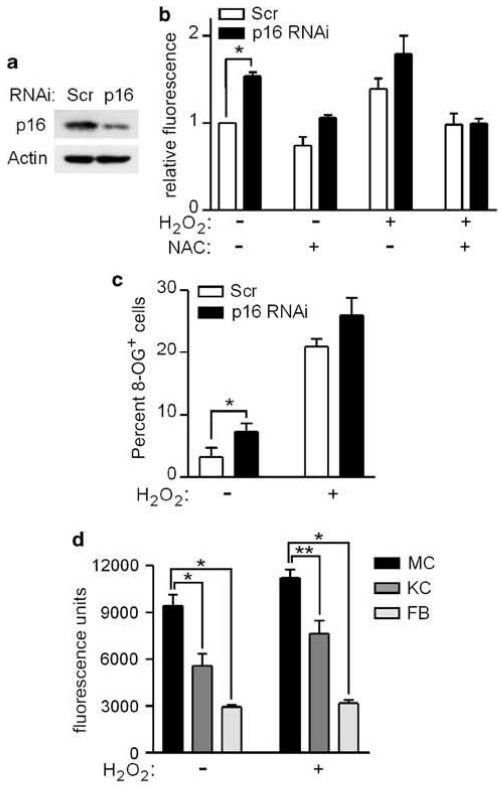

Figure 3 Intracellular ROS and oxidative DNA damage is increased in p16-depleted cells. (**a**) Melanocytes were transfected with control scrambled (Scr) or p16-specific siRNA, and 48 h later cell lysates were blotted for p16 and Actin. (**b**) Melanocytes were transfected with siRNA, and 48 h later either untreated or treated with 5 mM NAC and/or 0.05 mM H_2O_2. After 5 h, ROS levels were measured by DCFDA assay, and values normalized to those of control conditions, which were set at 1. Error bars indicate s.e.m. from three independent experiments. $^*P=0.005$ (one-sample *t* test). (**c**) Melanocytes were transfected with siRNA, and 48 h later either untreated or treated with 0.5 mM H_2O_2. After an additional 48 h, cells were fixed and immobilized for 8-OG staining. Error bars indicate s.e.m. of percent 8-OG-positive cells assessed under each condition in three independent experiments. $^*P=0.05$ (two-sample *t* test). (**d**) Melanocytes (MC), keratinocytes (KC) and fibroblasts (FB) isolated from four individual donors were transfected with p16-specific siRNA, and then 48 h later cultured in the absence or presence of 0.05 mM H_2O_2. After 5 h, intracellular ROS levels were measured by DCFDA assay. Error bars indicate s.e.m. from four independent experiments with different donor cells. $^*P<0.001$, $^{**}P=0.002$ (repeated-measures analysis of variance (ANOVA)).

mice demonstrated significantly elevated ROS levels compared with lines derived from wild-type mice (Figure 4b), recapitulating our findings in whole-mouse skin and p16-depleted human cells. Having demonstrated that p16 is required for normal regulation of oxidative stress, we next asked whether restoration of p16 expression in p16-deficient cells would be *sufficient* to normalize intracellular ROS levels. It is important to note that the *Cdkn2a*-null mice and derived fibroblasts used in these experiments are also deficient in $p14^{ARF}$, although the siRNA we employed (Figure 3) is specific for p16 (and not cross-reactive with ARF). Fibroblasts isolated from wild-type and *Cdkn2a*-null mice were infected with control lentivirus expressing green flourescent protein (GFP), and *Cdkn2a*-null fibroblasts were seperately infected with lentivirus expressing p16/GFP, which conferred a level of p16 expression comparable to that of wild-type cells (Figure 4c, right). We found that restoration of p16 expression in *Cdkn2a*-null fibroblasts was sufficient to neutralize elevated oxidative stress, as ROS levels significantly decreased (Figure 4c, left) and p38 phosphorylation was attenuated (Figure 4c, right). Thus, p16 is both necessary and sufficient for proper maintenance of endogenous cellular ROS levels.

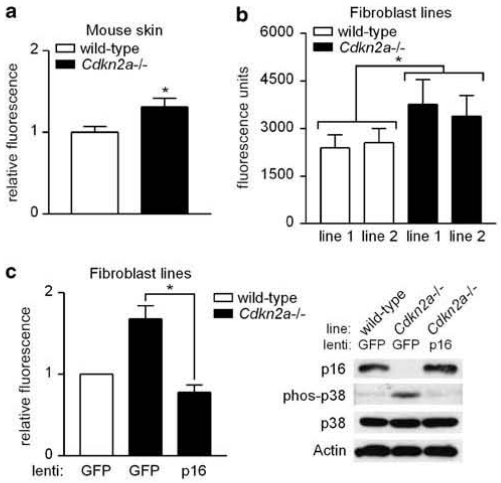

Figure 4 Suppression of ROS by *Cdkn2a in vivo*, and normalization of ROS in *Cdkn2a*-deficient cells upon restoration of p16 expression. (**a**) Dorsal skin was removed from wild-type and *Cdkn2a*-deficient mice, and ROS levels were measured in tissue lysates by DCFDA assay. Mean values normalized to those of wild-type mice, which were set at 1. Error bars indicate s.e.m. from independent measurements in wild-type ($n=10$) and *Cdkn2a*-deficient ($n=11$) mice. $^*P=0.03$ (two-sample t test). (**b**) Two lines of fibroblasts were derived from both wild-type and *Cdkn2a*-deficient mice, and ROS levels determined by DCFDA assay. Error bars indicate s.e.m. from five independent experiments. $^*P<0.001$ (repeated-measures analysis of variance (ANOVA)). (**c**) Wild-type (WT) and *Cdkn2a*-deficient fibroblasts were infected with either GFP control lentivirus or lentivirus expressing p16/GFP as indicated. Cell lysates were prepared 48 h later and subjected to DCFDA assay for ROS (left panel) and western blotting for p16, phospho-p38, p38 or actin (right panel). Error bars indicate s.e.m. from three independent experiments. $^*P=0.01$ (two-sample t test).

p16 regulates oxidative stress independently of the Rb pathway

Given the prominent upstream role of p16 in negative regulation of the Rb pathway (Lukas *et al*., 1995), we examined the phosphorylation status of Rb in H_2O_2-treated human melanocytes and its requirement for p16-mediated regulation of oxidative stress. Following exposure to H_2O_2, there was loss of Rb phosphorylation (as observed previously in similarly-treated endothelial cells (Cicchillitti *et al*., 2003)) coincident with upregulation of p16 at 5 h, with recovery of Rb phosphorylation and normalization of p16 expression by 24 h (Figure 5a, left). Cell cycle analysis revealed modest but statistically significant increase in the G1 fraction and decrease in the G2M fraction in H_2O_2-treated melanocytes at 5 h (Figure 5a, right). Next, p16 and Rb were either separately or simultaneously depleted in melanocytes by two-step RNAi (Figure 5b, lower left). Although p16 knockdown was associated with elevated ROS, there was no effect of Rb knockdown alone and, furthermore, combined Rb and p16 knockdown increased oxidative stress to a level comparable to that seen with p16 knockdown alone (Figure 5b, upper left). Thus, the dysregulation of ROS by depletion of p16 cannot be recapitulated by depletion of Rb, and Rb is not required for p16 regulation of ROS levels.

Oxidative dysregulation in p16-depleted cells was not due to effects on cell cycle

Finally, we considered the possibility that the observed suppressive effect of p16 on ROS levels could be related to its role in cell cycle regulation. It is established that p16 is a negative regulator of the cell cycle and cells with p16 mutations exhibit faster proliferation rates and decreased fraction of cells in the G1 phase compared with cells with wild-type p16 (Serrano *et al*., 1996). Consistent with these findings, we observed an increased G2M fraction in fibroblasts isolated from *Cdkn2a*-null mice compared with cells from wild-type mice, as well as increased BrdU staining in the skin epidermis of *Cdkn2a*-null compared with wild-type mice (not shown). It is also known that increased proliferation is associated with increased mitochondrial respiration and increased ROS leakage from the mitochondrial chain into the cytoplasm (Chung *et al*., 2009). Thus, dysregulation of intracellular ROS in p16-deficient cells could be secondary to increased proliferation resulting from loss of p16-mediated control of the cell cycle.

Indeed, we observed that p16 depletion was associated with a modest but significant shift in cell cycle phase distribution reflected by decreased (81–65%) G1 and increased (15–26%) G2M fractions (Figure 5b, right). However, a significant but less pronounced effect was also seen on G1 and G2M fractions with Rb knockdown (Figure 5b, right), which was not associated with any increase in ROS levels (Figure 5b, upper left). Thus, small increases in the cycling fraction are not necessarily associated with increased ROS levels. We noted that the degree of p16 knockdown using two-step RNAi (Figure 5b, lower left) was more complete than in our earlier experiments that depleted p16 by single-step RNAi in melanocytes (Figure 3a, Supplementary Figure 2a), in which a lower concentration of transfection reagent was used. We repeated the single-step p16 RNAi as in earlier experiments using cells isolated from four different donors, this time assessing whether alterations in oxidative stress and damage were associated with changes in cell cycle phase. Under such conditions of partial depletion of p16 (Supplementary Figure S3a), cell cycle distribution was not significantly altered compared with control RNAi-transfected cells (Supplementary Figure S3b). In these cells matched for cell cycle phase, we confirmed increased ROS levels (Supplementary Figure S3c) and increased proportion of 8-OG-positive cells (Supplementary Figure S3d) in p16-RNAi-transfected compared with control RNAi-transfected cells. Taken together, these data suggest that dysregulated oxidative stress and resulting oxidative damage observed in p16-depleted cells occurs independently of potential effects of p16 on cell-cycle control.

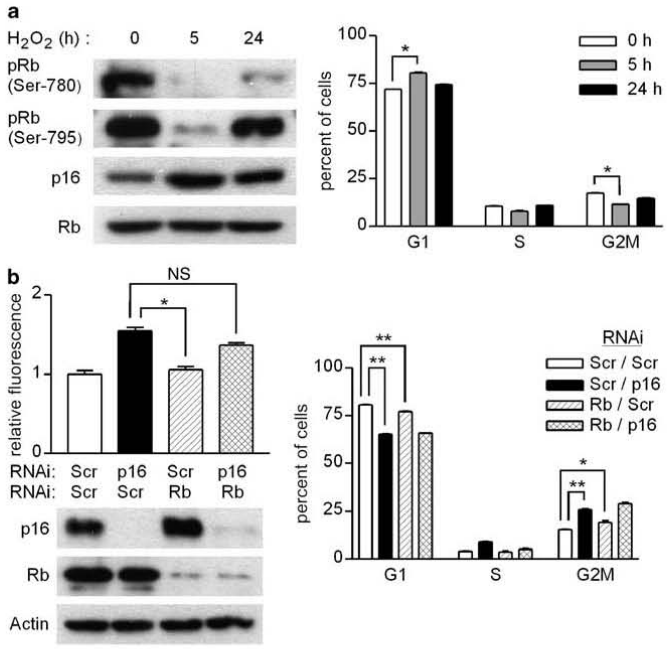

Figure 5 p16 regulation of ROS occurs independently of Rb. (**a**) Melanocytes were untreated (0 h), or treated with 50 μM H_2O_2 for 5 or 24 h. Cells were lysed for western blotting (left panel), or analyzed by flow cytometry to determine cell cycle phase (right panel). Error bars represent s.e.m. of triplicate determinations, $^*P<0.001$ (two-sample *t* test). Representative of two experiments performed. (**b**) Melanocytes were transfected with scrambled (Scr) or indicated specific siRNA, and then 48 h later ROS levels were measured by DCFDA assay, cell lysates were prepared for western blotting, and cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM of duplicate or triplicate determinations. $^*P=0.01$, $^{**}P<0.001$; NS, not significant. Representative of two experiments performed.

Discussion

It is widely accepted that p16 acts as a tumor suppressor by inhibiting Rb phosphorylation and inducing cell cycle arrest in the G1 phase in response to potentially genotoxic stimuli (Alcorta *et al.*, 1996). This p16-mediated cell cycle arrest allows time for repair of DNA damage before replication, thereby reducing the chance of propagating mutations (Figure 6). However, it is unknown whether other mechanisms exist by which p16 may protect against tumor formation. Here we show that, in addition to UV irradiation, exogenous oxidative stress rapidly upregulates expression of p16. We present evidence that p16 deficiency leads to dysregulation of intracellular ROS in multiple cell types and accumulation of oxidative DNA damage, and that p16 is both necessary and sufficient for normal regulation of p38 SAPK signaling and intracellular oxidative status. Our findings further suggest that compromise of p16 may allow cells to progress to S phase bearing oxidative DNA lesions that may result in carcinogenic mutations (Figure 6). Our results indicate that p16-mediated regulation of intracellular oxidative stress

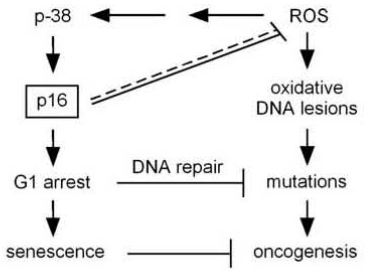

Figure 6 Potential tumor-suppressive functions of p16. In the canonical pathway, p16 mediates cell cycle arrest as part of the DNA damage response pathway, allowing time for DNA repair enzymes to correct potentially oncogenic mutations or avoiding transformation by inducing senescence. Loss of p16 leads to increased ROS levels (direct or indirect effect, indicated by dashed/solid line) and oxidative DNA damage, which, in p16-deficient cells with impaired capacity for cell cycle arrest and senescence, may result in increased accumulation of mutations promoting oncogenesis. Melanocytes are particularly susceptible to oxidative stress (Figure 1d), perhaps explaining why loss of p16 predisposes to melanoma rather than other cancers.

appears to be independent of the Rb pathway and not secondary to potential cell cycle effects, thus confirming a novel function for p16.

It is unknown why compromise of p16 is more commonly associated with melanoma over other cancers. Although we demonstrate here that p16 is necessary for proper oxidative regulation in multiple cell types, our data also indicate that melanocytes maintain higher levels of intracellular ROS and incur greater oxidative DNA damage in response to exogenous oxidative stress than other cell types (Figure 1d). This finding also holds true in the context of p16 depletion, wherein different skin cell types were matched by donor and analyzed for intracellular ROS (Figure 3d). This inherent predisposition to oxidative damage may underlie an increased susceptibility of melanocytes over other cell types to transformation in the setting of p16 deficiency. The basis for higher basal levels of oxidative stress in melanocytes may relate to their synthesis of melanin pigment, which is distributed to adjacent keratinocytes for the purpose of absorbing photons and scavenging free radicals (Riley, 1997). Although UV exposure stimulates melanocytes to proliferate and produce melanin that can absorb UV-generated ROS (Gilchrest *et al*., 1996), higher UV doses can oxidize melanin and increase ROS production (Wood *et al*., 2006), which may further increase oxidative stress in melanocytes (Urabe *et al*., 1994). Moreover, under conditions of increased oxidative stress, there is less efficient repair of 8-OG lesions (Eiberger *et al*., 2008). Consistent with this notion, a recent study has shown that melanocytes are deficient (compared with fibroblasts) in repair of oxidative DNA damage (Wang *et al*., 2010).

It has been suggested that loss of p16 in melanocytes may lead to failure of senescence, which underlies malignant transformation of melanocytic nevi (Figure 6) (Mooi and Peeper, 2006). Oncogene activation in melanocytes also generates oxidative stress, and increased ROS and p16 expression has been implicated in oncogene-induced melanocyte senescence (Leikam *et al*., 2008). Consistent with this notion, it has been shown that several melanoma-associated p16 mutants lack the capacity to induce senescence (Haferkamp *et al*., 2008), although p16 is not consistently expressed in melanocytes of senescent nevi *in vivo* (Gray-Schopfer *et al*., 2006) and more recent studies indicate that oncogene-induced senescence does not require p16 or $p14^{ARF}$ (Dhomen *et al*., 2009; Haferkamp *et al*., 2009).

Our data showing negative control of ROS by p16 may seem at odds with these (Leikam *et al*., 2008) and other reports in the literature, suggesting that the relationship between p16 levels and oxidative stress may be highly dependent on the circumstances and cell system utilized. For example, it was reported that over-expression of p16 increases cellular ROS levels in a human diploid fibroblast line (TIG-3) and p16 knock-down decreases ROS in a conditionally immortalized human fibroblast line (SVts8) (Takahashi *et al*., 2006). Another study using EJ human carcinoma cells found that although tet-regulated over-expression of p21 elevated cellular ROS, induction of p16 over-expression had no discernable effect on ROS (Macip *et al*., 2002). By contrast, our studies utilized siRNAi to knock down p16 in primary normal human cells expressing wild-type p16, or lentivirus to express p16 in freshly isolated mouse fibroblasts that were genetically deficient in p16. These various findings suggest that regulation of ROS by p16 in melanocytes and melanoma cells may be context-specific and could reflect differences between cell types, or the immortalized or senescent state of the cells. Nevertheless, it seems plausible from our findings that p16 may act to suppress tumorigenesis through two inter-related pathways (Figure 6): (1) mediating cell cycle arrest to facilitate repair of DNA damage, and (2) controlling accumulation of ROS that may cause oxidative DNA damage. Moreover, melanocytes may be more perturbed by oxidative dysregulation induced by compromise of p16 than other cell types, leading to increased susceptibility to melanoma over other cancers.

The precise mechanism by which p16 deficiency increases intracellular ROS in our system remains to be elucidated. Several recent reports also describe possible novel tumor-suppressive roles of p16 that are independent of its role in cell cycle control. For example, several p16 mutants responsible for inherited melanoma susceptibility in humans retain robust cyclin-dependent kinase 4-binding capacity (Becker *et al*., 2001). It has been reported that p16 interacts with brahma-related gene 1, a chromatin remodeling factor (Bochar *et al*., 2000) whose expression is frequently lost in primary and metastatic melanomas (Becker *et al*., 2009). Given the myriad of possible transcriptional targets of the BRG1 p16 complex, it is possible that p16 may regulate cellular oxidative stress through this newly discovered interaction. It has also been reported that p16 can bind to c-Jun N-terminal kinase (JNK) 3, thereby blocking JNK-mediated phosphorylation of c-Jun following UV exposure and activation of the Ras-JNK-Jun-AP-1 signaling cascade (Choi *et al*., 2005). Therefore it is possible that loss of p16 removes an important inhibitor of Ras-JNK-Jun-AP-1 signaling (and cellular transformation), resulting in an upregulation of genes responsible for increased intracellular ROS. Interestingly, the residue Arg24 of p16 is thought to have a crucial role in the stabilization of the p16 JNK3 complex and an Arg24Pro mutation in p16 co-segregates in nine melanoma-prone families (Becker *et al*., 2001).

Taken together, our work presented here suggests that p16 may exert tumor-suppressive effects that extend beyond its known function as a cell cycle regulator. Further study of the mechanistic basis for oxidative regulation by p16 may shed further light on why loss of p16 commonly occurs in tumors and why inherited p16 mutations predispose to melanoma susceptibility.

Materials and methods

Skin cells

Normal human melanocytes and keratinocytes were prepared from neonatal foreskins as described previously (Bowen *et al*., 2003). Human fibroblasts were also prepared from foreskins

following overnight treatment with dispase and removal of the epidermis (Bowen *et al*., 2003). Briefly, tissue fragments were incubated sequentially with collagenase (260 U/ml, Sigma, St Louis, MO, USA) for 1.5 h and then with trypsin/EDTA (0.05%, Invitrogen, Carlsbad, CA, USA) for 15 min at 37 °C. Cultures were expanded in high-glucose Dulbecco's modied Eagle's medium supplemented with 10% fetal bovine serum (complete medium). Mouse fibroblasts were isolated from neonatal wild-type (C57/BL6, National Cancer Institute, Rockville, MD, USA) and background-matched *Cdkn2a* homozygous-null mice (B6.129-*Cdkn2a*^{tm1Rdp}, MMHCC, National Cancer Institute) in a sterile manner as previously described (Bockholt and Burridge, 1995). Briefly, the skin was dissected away and the ribcage/torso region was removed and then mechanically disrupted using a razor blade in complete medium. Cultures were expanded in complete medium. All cell cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. Mouse fibroblasts were stored at −80 °C, thawed, maintained in exponential growth phase and used for experiments within 2–3 weeks.

Generation and measurement of oxidative stress

Cells were UV-irradiated as previously described (Cotter *et al*., 2007), or H_2O_2 (Sigma) was added to cultures. Endogenous ROS of protein equivalents were quantified by DCFDA assay as previously described (Cotter *et al*., 2007). Although DCFDA is somewhat non-specific, fluorescence levels likely reflect H_2O_2 levels (Cathcart *et al*., 1983). For measurements in whole skin, dorsal skin was excised from newborn pups and incubated in high-glucose Dulbecco's modied Eagle's medium supplemented with 10% fetal bovine serum and 20 μM DCFDA for 30 min at 37 °C. Skins were then rinsed in phosphate-buffered saline, manually homogenized in a disposable eppendorf tube with plastic pestle (Fisher Scientific, Pittsburgh, PA, USA) containing 150 μl lysis buffer (2% SDS, 50 mM Tris and 10% glycerol), and then 30 μg lysate was subjected to fluorimetric analysis. NAC solution (American Regent, Shirley, NY, USA) diluted from newly opened vials was added at a final concentration of 5 mM to cells 30 min before other treatments. The phospho-p38 specific inhibitor (SB203580, EMD Chemicals, Gibbstown, NJ, USA) was added directly to cultures at a final concentration of 20 μM.

Western blotting

Specific proteins were detected in cell lysates as previously described (Raj *et al*., 2008). Primary antibodies were used against p16 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:10 000, A-3853, Sigma,), p38 SAPK (1:1000, ab31828, Abcam, Cambridge, MA, USA), phospho-p38 (1:1000, ab32557, Abcam), HO-1 (1:1000, OSA 110, Stressgen, Ann Arbor, MI, USA), Rb (1:1000, sc-50, Santa Cruz Biotechnology), and Rb phosphorylated at residues 780 and 795 (1:1000, 9307 and 9301, respectively, Cell Signaling Technology, Danvers, MA, USA).

Oxidative damage in melanocytes

The presence of 8-OG was detected in cells immobilized in agarose by immunohistochemistry as previously described (Cotter *et al*., 2007). Approximately 300 cells were counted per experimental condition for each experiment.

RNAi

Cells were transfected at 70% confluency in six-well plates with oligonucleotides targeting *p16* (sc-36143 targeting exon 1β and non-overlapping with p14^{ARF}, Santa Cruz Biotechnology), *Rb* (Raj *et al*., 2008) or a control non-specific siRNA (Raj *et al*., 2008). For *p16* and the control non-specific siRNA, 6 μl RNAi duplexes (10 μM stocks) were mixed with 100 μl transfection medium (sc-36868, Santa Cruz Biotechnology). For Rb knockdown, two duplexes were used in combination at a final concentration of 50 nM each in 100 μl transfection medium. These mixtures were added to a 6-μl transfection reagent (sc-29528, Santa Cruz Biotechnology) and 100 μl transfection medium was added to each well and after 30 min added dropwise to wells containing 800 μl transfection media. For (two-step) knockdown of p16 and Rb, twice the amount of transfection reagent was used. After 6 h, 1 ml of normal media for the given cell type with 20% fetal bovine serum was added to the cells. After an additional 24 h, normal media was exchanged. Conditions were optimized for p16 and Rb knockdown at 48 h.

qRT–PCR

Cellular RNA (0.5 μg) prepared using the RNeasy kit (Qiagen, Valencia, CA, USA) was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen), and equal volumes of cDNA (1 μl of each 20 μl reaction) were subjected to PCR using primers specific for *p16* (5′-CCCAACGCACCGAATA GTTAC-3′ and 5′-ACCACCAGCGTGTCCAGGAA-3′) or *GAPDH* (5′-CCCTCAACGACCACTTTGTC-3′ and 5′-GGGTC TACATGGCAACTGTG-3′) for up to 40 cycles (95 °C for 10 s, 59 °C for 10 s and 72 °C for 20 s). The SyBR Advantage qPCR Premix Kit (Clontech, Mountain View, CA, USA) was used according to the manufacturer's instructions, and samples were run using a CFD-3240 Chromo4 detector (MJ Research, Waltham, MA, USA) equipped with Opticon Monitor software (Promega Corporation, Madison, WI, USA) for data acquisition, monitoring and analysis. Expression levels for each gene were normalized to GAPDH. GAPDH expression was not affected by H_2O_2 treatment or p16 knockdown.

p16-expressing lentivirus

The *p16* gene was PCR-amplified from human melanocyte cDNA using primers (5′-GGACTGCAGCATGGAGCCGGC GG-3′ and 5′-TTTCTCGAGCCTCTCTGGTTCTTTCA-3′) and PfuUltra II Fusion HS polymerase (Stratagene, La Jolla, CA, USA). Underlined nucleotides indicate *Pst*I and *Xho*I sites introduced by PCR for further subcloning. The PCR product was cloned into pSC-B-Amp/Kan (Stratagene), confirmed by sequencing, then subcloned into the *Sbf*I/*Xho*I sites of the modified pEI2 lentiviral expression vector (Welm *et al*., 2008) obtained from Bryan Welm (Huntsman Cancer Institute). The lentiviral construct was validated for p16 expression by transient transfection into HeLa cells followed by western blotting. For viral production, HEK 293T/17 cells (ATCC, Manassas, VA, USA) grown in Dulbecco's modied Eagle's medium with 10% fetal bovine serum were co-transfected with 5 μg lentiviral vector and helper plasmids (Lenti-X HT packaging mix, Clontech) and 30 μg of polyethylenimine (pH 7.0, Sigma) in 1 ml of OptiMEM (Invitrogen) as described (Welm *et al*., 2008). Viral particles were collected 48 and 72 h after transfection, purified by centrifugation (× 1500 g) and filtration (0.45 μm), followed by ultracentrifugation (× 100 000 g) to achieve 100-fold concentration. Viral titers were determined by limiting dilution and visualization of GFP-positive cells. For cellular infection, 8 μg/ml polybrene (Sigma) was added as previously described (Raj *et al*., 2008).

Cell cycle analysis

Human melanocytes at 60–70% confluency were cultured in six-well plates and subjected to RNAi. After 48 h, cells harvested by trypsinization were washed with cold phos-

phate-buffered saline, resuspended in 500 µl of 70% ethanol and then stored overnight at 4 °C. After washing in phosphate-buffered saline, cells were resuspended in phosphate-buffered saline containing 50 µg/ml propidium iodide (Sigma) and then 10 000 cells were analyzed on a FACSort using ModFit LT version 3.1 software (Verity Software House, Topsham, ME, USA) as described previously (Raj *et al*., 2008).

Statistics

Statistical analysis was performed using Prism 3.0 (GraphPad Software, La Jolla, CA, USA) and R 2.80 (R Foundation for Statistical Computing, Vienna, Austria) and supervised by a biostatistician (KMB). Data from experimental groups were subjected to standard one- and two-sample *t* tests. For experiments using cells from multiple donors, a repeated-measures analysis of variance analysis was performed with *P*-values adjusted for multiple comparisons using Tukey's honest significant difference. For experiments in which individual cells were counted (that is, determination of 8-OG), data were analyzed using logistic regression. The pairing of donors was taken into account as a factor in the analysis and a Bonferonni correction was applied for two comparisons. *P*-values <0.05 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc Natl Acad Sci USA* **93**: 13742–13747.

Becker TM, Haferkamp S, Dijkstra MK, Scurr LL, Frausto M, Diefenbach E *et al*. (2009). The chromatin remodelling factor BRG1 is a novel binding partner of the tumor suppressor p16INK4a. *Mol Cancer* **8**: 4.

Becker TM, Rizos H, Kefford RF, Mann GJ. (2001). Functional impairment of melanoma-associated p16(INK4a) mutants in melanoma cells despite retention of cyclin-dependent kinase 4 binding. *Clin Cancer Res* **7**: 3282–3288.

Bochar DA, Wang L, Beniya H, Kinev A, Xue Y, Lane WS *et al*. (2000). BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell* **102**: 257–265.

Bockholt SM, Burridge K. (1995). An examination of focal adhesion formation and tyrosine phosphorylation in fibroblasts isolated from src-, fyn-, and yes- mice. *Cell Adhes Commun* **3**: 91–100.

Bowen AR, Hanks AN, Allen SM, Alexander A, Diedrich MJ, Grossman D. (2003). Apoptosis regulators and responses in human melanocytic and keratinocytic cells. *J Invest Dermatol* **120**: 48–55.

Cathcart R, Schwiers E, Ames BN. (1983). Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem* **134**: 111–116.

Choi BY, Choi HS, Ko K, Cho YY, Zhu F, Kang BS *et al*. (2005). The tumor suppressor p16(INK4a) prevents cell transformation through inhibition of c-Jun phosphorylation and AP-1 activity. *Nat Struct Mol Biol* **12**: 699–707.

Chung JS, Lee SB, Park SH, Kang ST, Na AR, Chang TS *et al*. (2009). Mitochondrial reactive oxygen species originating from Romo1 exert an important role in normal cell cycle progression by regulating p27(Kip1) expression. *Free Radic Res* **43**: 729–737.

Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, Martelli F. (2003). Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *J Biol Chem* **278**: 19509–19517.

Cotter MA, Thomas J, Cassidy P, Robinette K, Jenkins N, Florell SR *et al*. (2007). N-acetylcysteine protects melanocytes against oxidative stress/damage and delays onset of ultraviolet-induced melanoma in mice. *Clin Cancer Res* **13**: 5952–5958.

Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H *et al*. (2005). Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353**: 2135–2147.

Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V *et al*. (2009). Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer Cell* **15**: 294–303.

Eiberger W, Volkmer B, Amouroux R, Dherin C, Radicella JP, Epe B. (2008). Oxidative stress impairs the repair of oxidative DNA base modifications in human skin fibroblasts and melanoma cells. *DNA Repair (Amst)* **7**: 912–921.

Flores JF, Walker GJ, Glendening JM, Haluska FG, Castresana JS, Rubio MP *et al*. (1996). Loss of the p16INK4a and p15INK4b genes, as well as neighboring 9p21 markers, in sporadic melanoma. *Cancer Res* **56**: 5023–5032.

Gilchrest BA, Park HY, Eller MS, Yaar M. (1996). Mechanisms of ultraviolet light-induced pigmentation. *Photochem Photobiol* **63**: 1–10.

Goldstein AM, Chan M, Harland M, Gillanders EM, Hayward NK, Avril MF *et al*. (2006). High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res* **66**: 9818–9828.

Grammatico P, Maresca V, Roccella F, Roccella M, Biondo L, Catricala C *et al*. (1998). Increased sensitivity to peroxidizing agents is correlated with an imbalance of antioxidants in normal melanocytes from melanoma patients. *Exp Dermatol* **7**: 205–212.

Gray-Schopfer VC, Cheong SC, Chong H, Chow J, Moss T, Abdel-Malek ZA *et al*. (2006). Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br J Cancer* **95**: 496–505.

Haferkamp S, Becker TM, Scurr LL, Kefford RF, Rizos H. (2008). p16INK4a-induced senescence is disabled by melanoma-associated mutations. *Aging Cell* **7**: 733–745.

Haferkamp S, Scurr LL, Becker TM, Frausto M, Kefford RF, Rizos H. (2009). Oncogene-induced senescence does not require the p16(INK4a) or p14ARF melanoma tumor suppressors. *J Invest Dermatol* **129**: 1983–1991.

Herrling T, Jung K, Fuchs J. (2006). Measurements of UV-generated free radicals/reactive oxygen species (ROS) in skin. *Spectrochim Acta A Mol Biomol Spectrosc* **63**: 840–845.

Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K *et al.* (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* **12**: 446–451.

Leikam C, Hufnagel A, Schartl M, Meierjohann S. (2008). Oncogene activation in melanocytes links reactive oxygen to multinucleated phenotype and senescence. *Oncogene* **27**: 7070–7082.

Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M *et al.* (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**: 503–506.

Macip S, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW *et al.* (2002). Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *EMBO J* **21**: 2180–2188.

Meyskens Jr FL, Farmer P, Fruehauf JP. (2001). Redox regulation in human melanocytes and melanoma. *Pigment Cell Res* **14**: 148–154.

Mooi WJ, Peeper DS. (2006). Oncogene-induced cell senescence—halting on the road to cancer. *N Engl J Med* **355**: 1037–1046.

Naidu S, Vijayan V, Santoso S, Kietzmann T, Immenschuh S. (2009). Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2. *J Immunol* **182**: 7048–7057.

Pashaei S, Li L, Zhang H, Spencer HJ, Schichman SA, Fan CY *et al.* (2008). Concordant loss of heterozygosity of DNA repair gene, hOGG1, in melanoma *in situ* and atypical melanocytic hyperplasia. *J Cutan Pathol* **35**: 525–531.

Pavel S, van Nieuwpoort F, van der Meulen H, Out C, Pizinger K, Cetkovska P *et al.* (2004). Disturbed melanin synthesis and chronic oxidative stress in dysplastic naevi. *Eur J Cancer* **40**: 1423–1430.

Piepkorn M. (2000). The expression of p16(INK4a), the product of a tumor suppressor gene for melanoma, is upregulated in human melanocytes by UVB irradiation. *J Am Acad Dermatol* **42**: 741–745.

Raj D, Liu T, Samadashwily G, Li F, Grossman D. (2008). Survivin repression by p53, Rb and E2F2 in normal human melanocytes. *Carcinogenesis* **29**: 194–201.

Riley PA. (1997). Melanin. *Int J Biochem Cell Biol* **29**: 1235–1239.

Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**: 27–37.

Shapiro GI, Edwards CD, Ewen ME, Rollins BJ. (1998). p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. *Mol Cell Biol* **18**: 378–387.

Sharpless NE, DePinho RA. (1999). The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev* **9**: 22–30.

Takahashi A, Ohtani N, Yamakoshi K, Iida S, Tahara H, Nakayama K *et al.* (2006). Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat Cell Biol* **8**: 1291–1297.

Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, Prota G *et al.* (1994). The inherent cytotoxicity of melanin precursors: a revision. *Biochim Biophys Acta* **1221**: 272–278.

Wang H-T, Choi B, Tang MS. (2010). Melanocytes are deficient in repair of oxidative DNA damage and UV-induced photoproducts. *Proc Natl Acad Sci USA* **107**: 2180–2185.

Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z. (2008). Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. *Cell Stem Cell* **2**: 90–102.

Wood SR, Berwick M, Ley RD, Walter RB, Setlow RB, Timmins GS. (2006). UV causation of melanoma in Xiphophorus is dominated by melanin photosensitized oxidant production. *Proc Natl Acad Sci USA* **103**: 4111–4115.

Zyrek-Betts J, Micale M, Lineen A, Chaudhuri PK, Keil S, Xue J *et al.* (2008). Malignant blue nevus with lymph node metastases. *J Cutan Pathol* **35**: 651–657.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Information

$p16^{INK4A}$ -dependent upregulation of antioxidant enzymes is secondary to modulation of ROS

We considered several mechanisms by which $p16^{INK4A}$ loss may elevate cellular oxidative stress. First, we asked whether increased ROS in $p16^{INK4A}$ -depleted cells resulted directly from dysregulation of intracellular antioxidant enzymes. We initially examined expression of several antioxidant enzymes in H_2O_2-treated melanocytes, and found that mRNA levels of the stress protein heme oxygenase-1 (HO-1) were significantly increased 3 h after treatment (Supplementary Figure S2.5). On the other hand, expression of additional antioxidant enzymes including superoxide dismutase 1, superoxide dismutase 2, thioredoxin, and peroxiredoxin 1 was not significantly increased in H_2O_2-treated cells (although there was an increased trend for thioredoxin and peroxiredoxin 1) (Supplementary Figure S2.5). HO-1 has been shown to be a critical oxidant stress- inducible antioxidant defense mechanism in fibroblasts (Vile et al. 1994), and also appears to be the predominant antioxidant enzyme modulated by oxidative stress in melanocytes, consistent with a previous report (Marrot et al. 2008).

Similarly, in the context of increased intracellular oxidative stress induced by $p16^{INK4A}$ knockdown, expression of HO-1 was significantly increased while that of thioredoxin and peroxiredoxin 1 was not (Supplementary Figure S2.6a). The effect of $p16^{INK4A}$ depletion on HO-1 expression, however, was secondary to increased ROS as addition of NAC effectively prevented this increase in HO-1 expression (Supplementary Figure S2.6a). These results were recapitulated at the protein level, as $p16^{INK4A}$

knockdown resulted in increased HO-1 protein levels which were attenuated by addition of NAC (Supplementary Figure S2.6b). Thus HO-1 is the major antioxidant enzyme upregulated in melanocytes by oxidative stress, and its upregulation in $p16^{INK4A}$ -depleted cells appears to be secondary to increased ROS following $p16^{INK4A}$ knockdown since the antioxidant NAC blocks increased HO-1 expression.

Oxidative dysregulation in p16-deficient cells is independent of cell cycle regulation

In our published study, we considered the possibility that the observed suppressive effect of $p16^{INK4A}$ on ROS levels was related to its role in cell cycle regulation. $p16^{INK4A}$ is an established negative regulator of the cell cycle, and it has been reported that cells with $p16^{INK4A}$ mutations exhibit faster proliferation rates and decreased fraction of cells in the G1 phase compared to cells with wild-type $p16^{INK4A}$ (Serrano et al. 1996). It is also known that increased proliferation is associated with increased mitochondrial respiration and in turn increased ROS leakage from the mitochondrial chain into the cytoplasm (Halliwell and Gutteridge 1990). Thus dysregulation of intracellular ROS in $p16^{INK4A}$ -deficient cells could be secondary to increased proliferation resulting from loss of $p16^{INK4A}$ regulatory control of the cell cycle. Indeed, skin from $p16^{INK4A}$ -null mice showed a significant increase in proliferating (BrdU-positive) cells in the epidermis compared to that from wild-type mice (Supplementary Figure S2.7). Similarly, fibroblast lines from $p16^{INK4A}$ -null mice displayed a much larger population of proliferating cells than lines from wild-type mice, as reflected by a greater fraction of cells in the G2M phase with a minority of cells in the G1 phase (Supplementary Figure S2.8a). We again found elevated ROS levels in $p16^{INK4A}$ -deficient fibroblasts compared to wild-type cells

(Supplementary Figure S2.8b). Increased ROS levels in $p16^{INK4A}$ -deficient cells correlated with an increased fraction of cells staining for 8-OG (Supplementary Figure S2.9).

To determine whether increased ROS associated with $p16^{INK4A}$ loss was due to increased proliferation, fibroblast lines from both $p16^{INK4A}$ -deficient and wild-type mice were serum-starved to synchronize both cell types with respect to cell cycle phase. Following serum starvation, the majority of $p16^{INK4A}$ -deficient cells were in the G1 phase (Supplementary Figure S2.8a), resembling the cell cycle profile of wild-type cells. When cell lysates obtained from serum-starved cells were analyzed for intracellular ROS, we again found increased ROS levels in $p16^{INK4A}$ -deficient compared to wild-type cells despite their similar cell cycle profiles (Supplementary Figure S2.8b). Likewise, despite comparable cell cycle profiles, we also found an increased proportion of 8-OG positive cells in $p16^{INK4A}$ -deficient compared to wild-type cell lines (Supplementary Figure S2.9). Taken together, these data suggest that the dysregulated oxidative stress and resulting oxidative damage observed in $p16^{INK4A}$ -deficient cells is independent of $p16^{INK4A}$ -regulated cell-cycle control.

Supplementary Methods

qRT-PCR

RNA was harvested from cells using the RNeasy kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. Approximately 0.5 μg total RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen), and equal volumes of cDNA (1 μl of each 20 μl reaction) were subjected to PCR using primers specific for *p16* (5'-CCCAACGCACCGAATAGTTAC-3' and 5' ACCACCAGCGTGTCCAGGAA-

3'), *HMOX1* (5'-CTGTGTCCCTCTCTCTGGAAA-3' and 5' TCCAGGCTCTGCTGCAGGAA-3'), *SOD1* (5'-TCACTTTAATCCTCTATCCAGAAA-3' and 5'-CACCACAAGCCAAACGACTTC-3'), *SOD2* (5'-GGAGTTGCTGGAAGCCATCAA-3' and 5'-TCTCCCAGTTGATTACATTCCAA-3'), *TRXRD1* (5' GTGATGGAACAACTGTCAAATCA-3' and 5'-ATAGCCTCCAAGGGAGCCAAA-3'), *PRDX1* (5'- TTTGGTATCAGACCCGAAGC-3' and 5'-TCCCCATGTTTGTCAGTGAA-3'), or *GAPDH* (5'-CCCTCAACGACCACTTTGTC 3' and 5'-GGGTCTACATGGCAACTGTG-3') for up to 40 cycles (95 °C for 10 s, 59 °C for 10 s and 72 °C for 20 s). The SyBR Advantage qPCR Premix Kit (Clontech, Mountain View, CA) was used according to the manufacturer's instructions, and samples were run using a CFD-3240 Chromo4 detector (MJ Research, Waltham, MA) equipped with Opticon Monitor software (Promega Corporation, Madison, WI) for data acquisition, monitoring, and analysis.

Cell cycle analysis

Mouse fibroblasts at 40-50% confluency were cultured in 6-well plates with high glucose DMEM either unsupplemented (serum-starved) or supplemented with 10% FBS. After 72 h, cells harvested by trypsinization were washed with cold PBS, resuspended in 500 μl of 70% ethanol, and then stored overnight at 4 °C. After washing in PBS, cells were resuspended in PBS containing 50 μg/ml propidium iodide (Sigma) and then analyzed on a FACSort using ModFit LT version 3.1 software (Verity Software House, Topsham, ME) as described previously (Raj et al 2008).

Statistics

Data from experimental groups were subjected to standard one- and two-sample t tests. P values $\leq .05$ were considered statistically significant.

Proliferation in vivo

Neonatal mice were injected *i.p.* with 5-bromo-2' deoxyuridine (BrdU, 50 mg/kg, Sigma), then 2 h later dorsal skin was excised and proliferating cells were detected by immunohistochemistry as described previously (Zhang et al. 2005).

Supplementary References

Halliwell B, Gutteridge JM (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* **186:** 1-85.

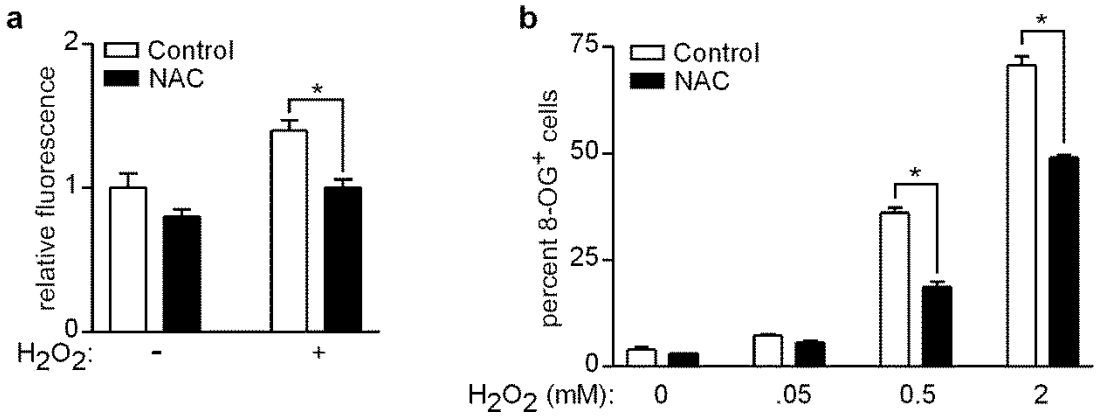
Marrot L, Jones C, Perez P, Meunier JR (2008). The significance of Nrf2 pathway in (photo)-oxidative stress response in melanocytes and keratinocytes of the human epidermis. *Pigment Cell Melanoma Res* **21:** 79-88.

Raj D, Liu T, Samadashwily G, Li F, Grossman D (2008). Survivin repression by p53, Rb and E2F2 in normal human melanocytes. *Carcinogenesis* **29:** 194-201.

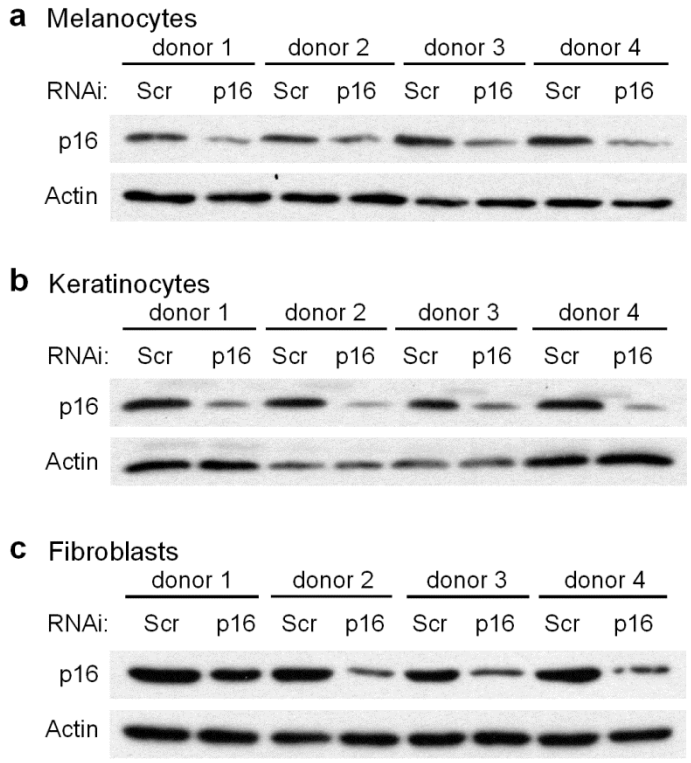
Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85:** 27-37.

Vile GF, Basu-Modak S, Waltner C, Tyrrell RM (1994). Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 2607-2610.

Zhang W, Hanks AN, Boucher K, Florell SR, Allen SM, Alexander A *et al* (2005). UVB-induced apoptosis drives clonal expansion during skin tumor development. *Carcinogenesis* **26:** 249-257.

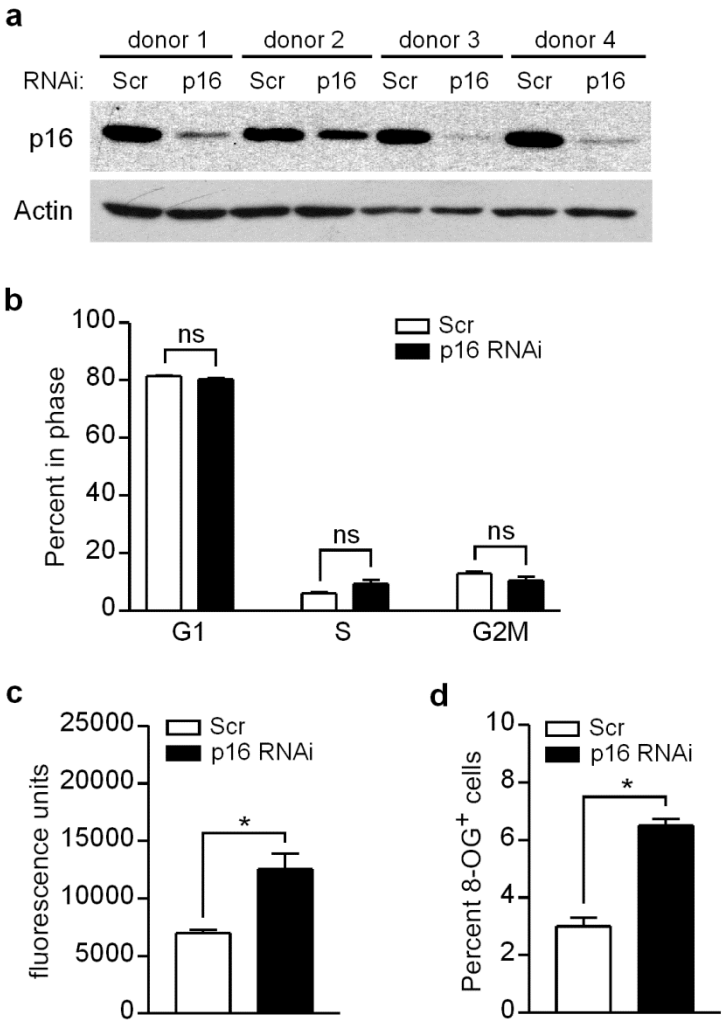


Supplementary Figure S2.1 Generation of intracellular ROS and oxidative DNA damage in melanocytes treated with H_2O_2. (**a**) Melanocytes were either untreated or treated with 5 mM NAC and/or 0.05 mM H_2O_2. After 5 h, ROS levels were measured by DCFDA assay, and values normalized to control conditions which were set at 1. Error bars indicate SEM from three independent experiments. *P=.01 (two-sample t test). (**b**) Cells were either untreated or treated with 5 mM NAC and/or 0-2 mM H_2O_2. After 48 h, cells were fixed and immobilized for 8-OG staining. Error bars indicate SEM of percent 8-OG positive cells assessed under each condition in three independent experiments. *P=.01, **P<.001 (two-sample t tests).



Supplementary Figure S2.2 $p16^{INK4A}$ depletion in multiple human cell types. (**a**) Melanocytes, (**b**) keratinocytes, and (**c**) fibroblasts independently isolated from four seperate donors were transfected either with control scrambled (Scr) or p16-specific siRNA, and 48 h later cell lysates were blotted for p16 or Actin.

Supplementary Figure S2.3 Oxidative dysregulation in $p16^{INK4A}$ -depleted melanocytes not due to alterations in cell-cycle phase. (**a**) Human melanocytes independently isolated from four separate donors were transfected with either control scrambled (Scr) or $p16^{INK4A}$ -specific siRNA, and 48 h later cell lysates were blotted for p16 or Actin. (**b**) Cell cycle analysis was performed on siRNA-transfected cells in (a), with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from four separate donors. ns, not significant (paired two-sample t tests). (**c**) ROS levels were determined by DCFDA assay in siRNA-transfected cells in (a). Error bars indicate SEM from four separate donors. *P<.001 (repeated measures analysis of variance, ANOVA). (**d**) 8-OG staining was performed in siRNA-transfected cells in (a) after an additional 48 h. Error bars indicate SEM from four separate donors. *P=.008 (repeated measures analysis of variance, ANOVA).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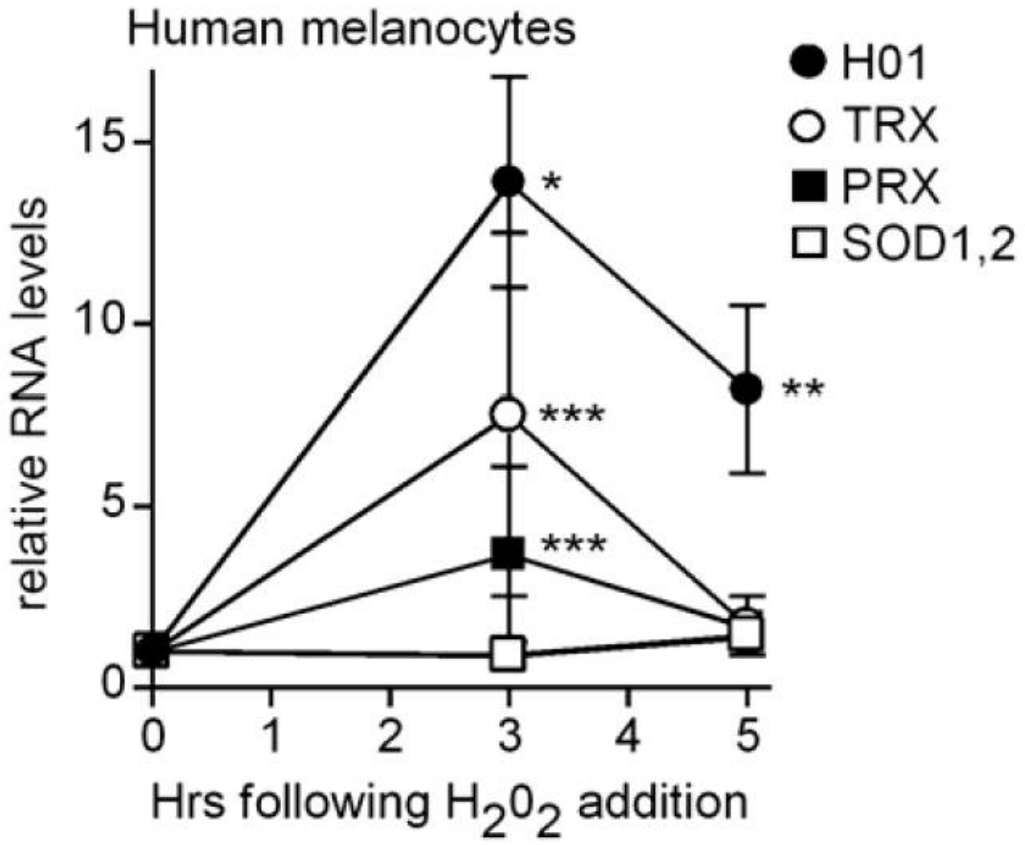
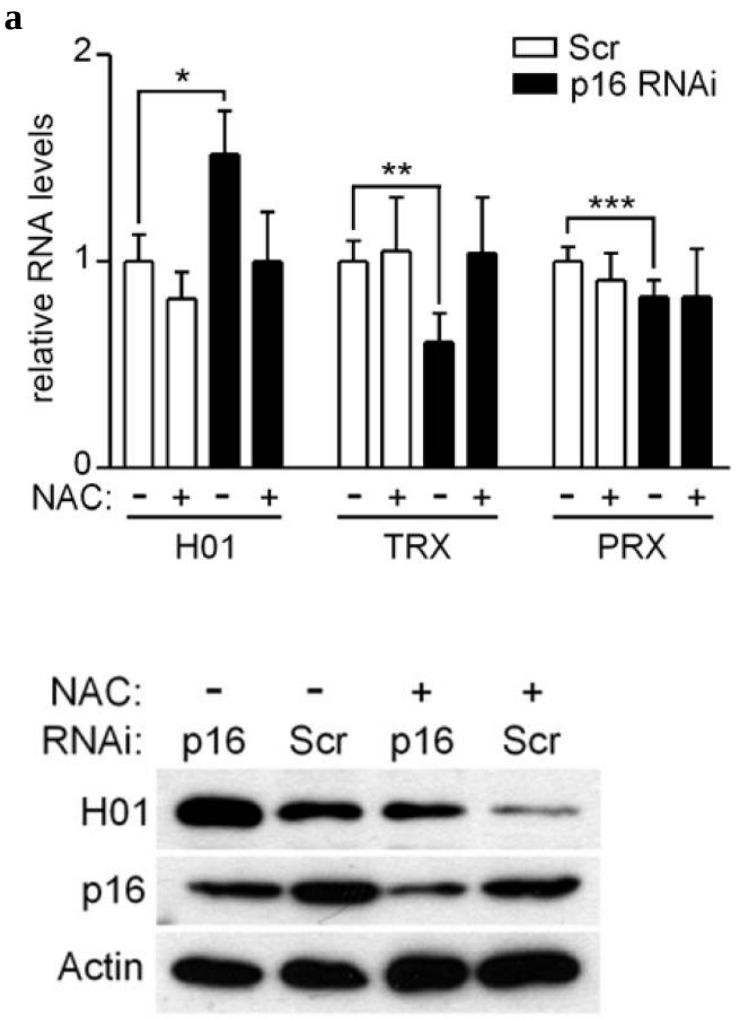
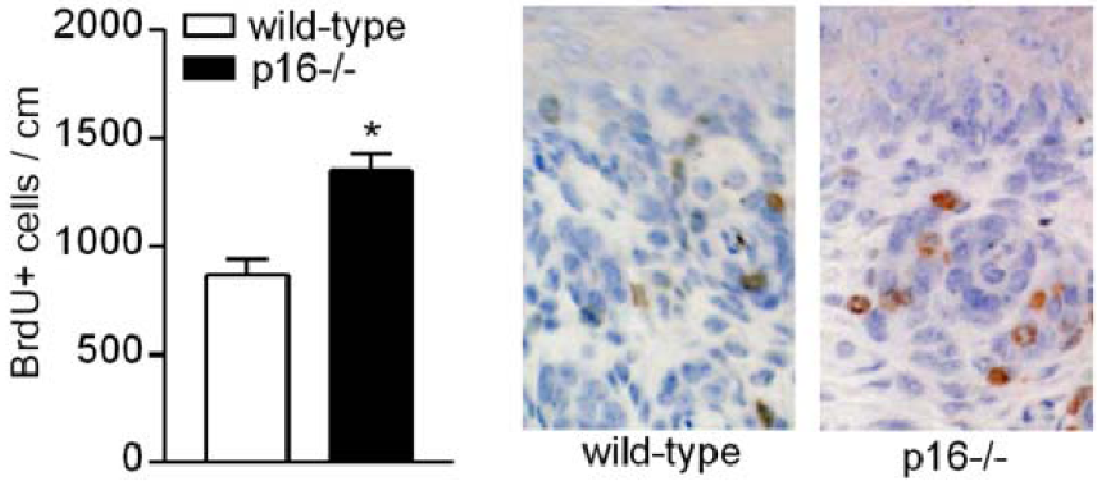


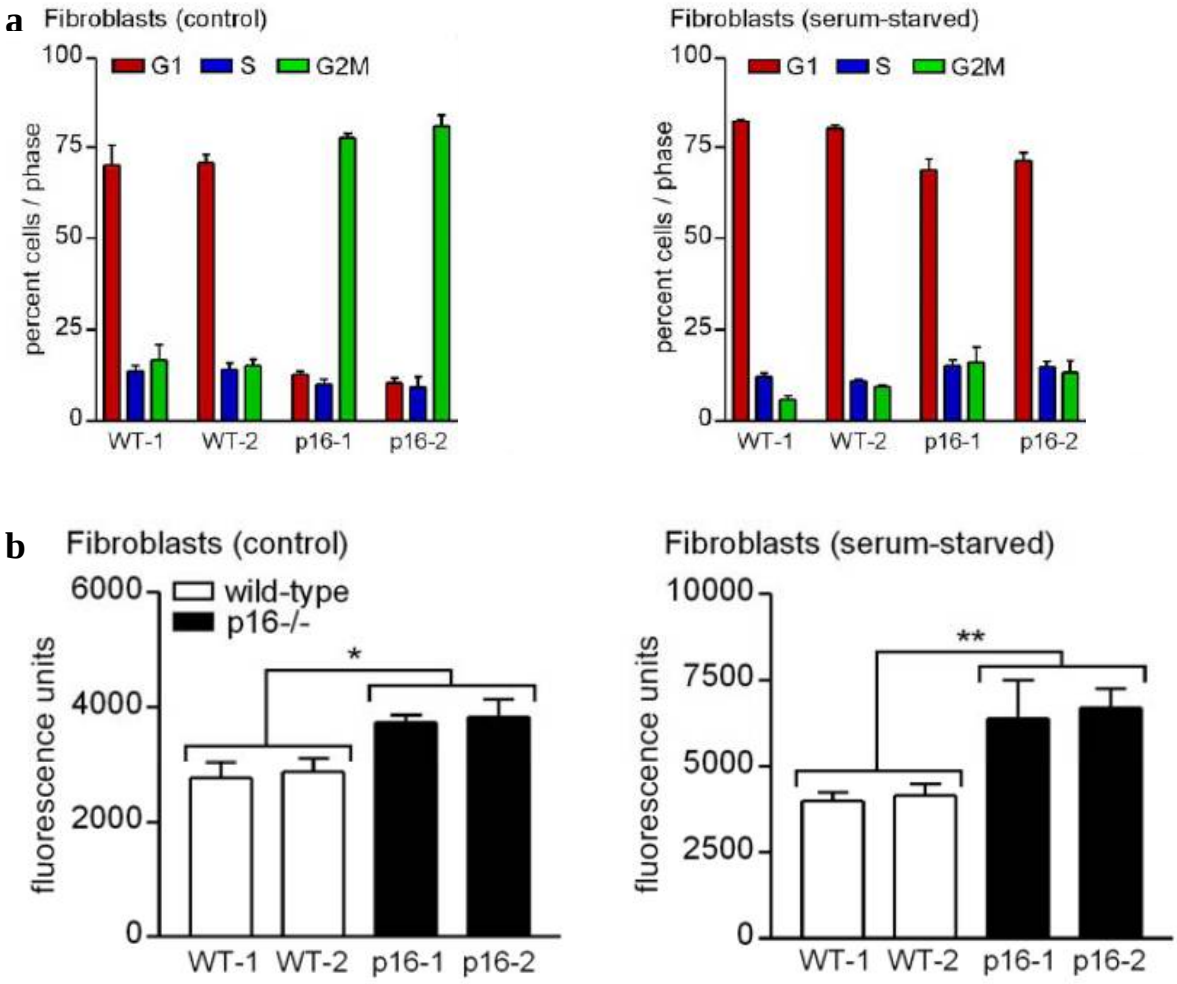
Supplementary Figure S2.4 Upregulation of the predominant antioxidant enzyme HO-1 occurs in response to H_2O_2. Melanocytes were treated with 0.05 mM 20 H_2O_2, then RNA was isolated 3 and 5 h later for qRT-PCR analysis of expression of HO-1, superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), thioredoxin (TRX), and peroxiredoxin 1 (PRX) as indicated. Error bars indicate SEM from three independent experiments. *P=.05, **P=.09, ***P>.30 (one-sample t tests).



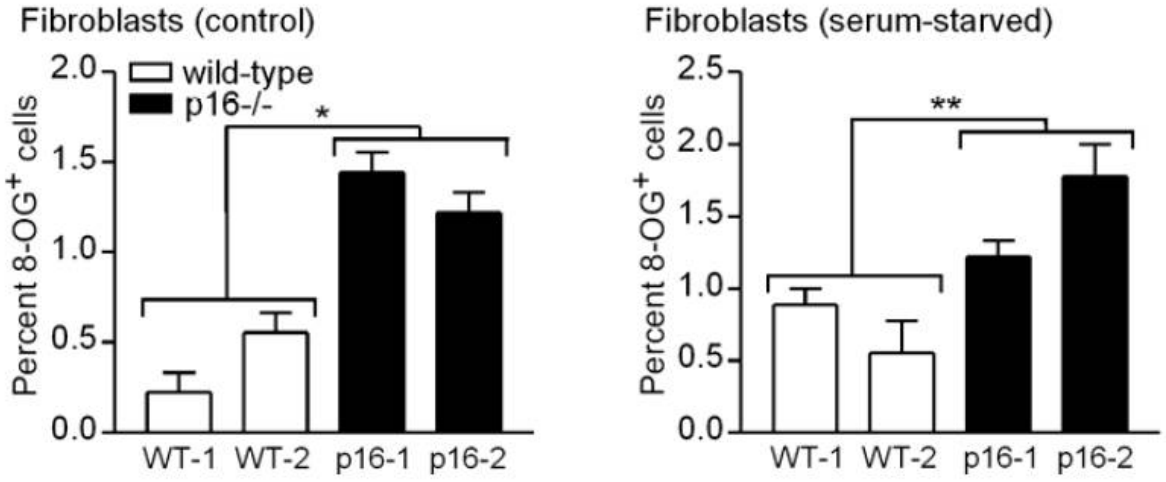
Supplementary Figure S2.5 Upregulation of the predominant antioxidant enzyme HO-1 occurs in response to p16 knockdown, and in $p16^{INK4A}$ -deficient cells is blocked by NAC. (**a**) Melanocytes were transfected with siRNA against control scrambled sequence (Scr) or p16 in the absence or presence of NAC, then 48 h later RNA was isolated and qRT-PCR was performed for HO-1, thioredoxin (TRX), and peroxiredoxin 1 (PRX) as indicated. Expression levels for each gene were normalized to GAPDH, and under control conditions value was set at 1. Error bars indicate SEM from three independent experiments. *P=.05, **P=.09, ***P=.19 (two-sample t tests). (**b**) Melanocytes were transfected with siRNA against control scrambled sequence (Scr) or $p16^{INK4A}$ in the absence or presence of NAC, then 48 h later cell lysates were blotted for HO-1, $p16^{INK4A}$, and Actin.



Supplementary Figure S2.6 $p16^{INK4A}$ -deficient cells show increased proliferation *in vivo*. Skin was obtained from wild-type and p16-null mice following injection of BrdU, and proliferating epidermal cells were quantitated in sections stained for BrdU. Error bars indicate SEM of measurements from three mice of each genotype. *P<.01 (two-sample t test).



Supplementary Figure S2.7 Oxidative dysregulation in $p16^{INK4A}$ -deficient cells is independent of $p16^{INK4A}$ -regulated cell-cycle control. *B.* Two fibroblast lines derived from both wild-type and p16-null mice were cultured in the presence (control, left panel) or absence (serum-starved, right panel) of FBS for 72 h. Cell cycle analysis was then performed, and percentages of cells in each phase are indicated. Error bars indicate SEM from five independent experiments. (**a**) Lysates from fibroblast lines under control (left panel) or serum-starved (right panel) conditions were subjected to DCFDA assay for ROS. Error bars indicate SEM from three independent experiments. *P<.001, **P<.001 (repeated measures analysis of variance, ANOVA). (**b**) Fibroblast lines under control (left panel) or serum-starved (right panel) conditions were stained for 8-OG. Error bars indicate SEM from three independent experiments. *P=.001, **P=.027 (Chi-square tests).



Supplementary Figure S2.8 Oxidative damage in $p16^{INK4A}$ -deficient cells is independent of $p16^{INK4A}$ -regulated cell-cycle control. Fibroblast lines under control (left panel) or serum-starved (right panel) conditions were stained for 8-OG. Error bars indicate SEM from three independent experiments. *P=.001, **P=.027 (Chi-square tests).

CHAPTER 3

FAMILIAL MELANOMA-ASSOCIATED MUTATIONS IN P16 UNCOUPLE ITS TUMOR SUPPRESSOR FUNCTIONS

Abstract

Familial melanoma is associated with point mutations in the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4A}$. We recently reported that $p16^{INK4A}$ regulates intracellular oxidative stress in a cell cycle-independent manner. Here, we constructed 12 different familial melanoma-associated point mutants spanning the $p16^{INK4A}$ coding region and analyzed their capacity to regulate cell-cycle phase and suppress reactive oxygen species (ROS). Compared to wild-type $p16^{INK4A}$ which fully restored both functions in $p16^{INK4A}$-deficient fibroblasts, various $p16^{INK4A}$ mutants differed in their capacity to normalize ROS and cell cycle profiles. While some mutations did not impair either function, others impaired both. Interestingly, several impaired cell-cycle (R24Q, R99P, V126D) or oxidative function (A36P, A57V, P114S) selectively, indicating that these two functions of $p16^{INK4A}$ can be uncoupled. Similar activities were confirmed with selected mutants in human melanoma cells. Many mutations impairing both cell-cycle and oxidative functions, or only cell cycle function, localize to the third ankyrin repeat of the $p16^{INK4A}$ molecule. Alternatively, most mutations impairing oxidative but not cell-cycle function, or those not impairing either function, lie outside this region. These results demonstrate that particular familial melanoma-associated mutations in $p16^{INK4A}$ can selectively compromise these two independent tumor-suppressor functions, which may be mediated by distinct regions of the protein.

Introduction

The CDK4/6 inhibitor $p16^{INK4A}$ is encoded by the chromosomal locus *CDKN2A* and altered in the majority of human tumors (Sharpless and DePinho 1999). Germ-line mutations in $p16^{INK4A}$ have been associated more commonly with a subset of cancers,

namely pancreatic carcinoma and melanoma, and are inherited in approximately 40% of melanoma-prone families (Goldstein et al. 2007). In the presence of potentially oncogenic stress such as DNA damage, the canonical tumor-suppressor function of $p16^{INK4A}$ involves binding either to cyclin-dependent kinases 4 and/or 6 (CDK4/6) or preassembled CDK4/6-cyclin D complexes (Hirai et al. 1995, Serrano et al. 1993), inhibiting hyperphosphorylation of Retinoblastoma-associated pocket proteins and delaying cell cycle progression from the G1 to S phase (Alcorta et al. 1996, Lukas et al. 1995). In this setting, $p16^{INK4A}$ may induce cellular senescence or allow time for DNA repair prior to cell division (Shapiro et al. 1998). Interestingly, several studies have demonstrated that many familial melanoma-associated $p16^{INK4A}$ mutants retain CDK4-binding capacity *in vitro* (Becker et al. 2001, Hashemi et al. 2000, Kannengiesser et al. 2009, McKenzie et al. 2010), suggesting that $p16^{INK4A}$ may mediate an additional important function(s) independent of cell-cycle regulation.

Since penetrance of melanoma in *$p16^{INK4A}$* mutant kindreds is highly associated with chronic exposure to ultraviolet radiation (Bishop et al. 2002), which produces reactive oxygen species (ROS) in the skin (Herrling et al. 2006), we recently investigated a possible role for $p16^{INK4A}$ in regulating intracellular oxidative stress. We found increased oxidative stress in multiple skin cell types when depleted of $p16^{INK4A}$ that was independent of cell-cycle regulation (Jenkins et al. 2011). Melanocytes demonstrated increased susceptibility to oxidative stress in the context of $p16^{INK4A}$ depletion compared to keratinocytes and fibroblasts (Jenkins et al. 2011). Melanocytes thus appear to be more dependent on $p16^{INK4A}$ for normal oxidative regulation than other cell types, which may

in part explain why inherited mutations in *p16^{INK4A}* predispose to melanoma over other cancers.

Given this newly identified role of p16^{INK4A} in regulating intracellular oxidative stress, we investigated whether different familial melanoma-associated p16^{INK4A} mutations can differentially modulate its cell cycle and oxidative regulatory functions. A panel of p16^{INK4A} mutants was constructed and compared to wild-type p16^{INK4A} in functional assays using p16$^{-/-}$Arf$^{+/+}$ cells. Interestingly, several mutations selectively compromised control of cell-cycle or oxidative stress, effectively uncoupling these two functions. Taken together, these data show that these two potential tumor-suppressor functions of p16^{INK4A} can be independently disrupted by distinct familial melanoma-associated mutations, and different regions of the protein may be important for these separate functions.

Results

Wild-type p16 suppresses ROS and cell cycle progression, and induces senescence in p16$^{-/-}$ Arf$^{+/+}$ cells

Our previous experiments (Jenkins et al. 2011) demonstrating sufficiency of p16^{INK4A} in mediating control of intracellular oxidative stress were performed in fibroblasts deficient in *CDKN2A*, which encodes both the p16^{INK4A} and Alternative reading frame (Arf, p19) proteins (Sharpless and DePinho 1999). We began by confirming these results using cells that were selectively deficient in p16^{INK4A} (i.e., wild-type for Arf). Fibroblasts from wild-type mice were infected with control lentivirus expressing green fluorescent protein (GFP), while p16$^{-/-}$Arf$^{+/+}$ fibroblasts were separately infected with either lentivirus expressing p16^{INK4A} and GFP or GFP alone. We had

previously optimized conditions for viral transduction to achieve 80-90% infection rates (as measured by GFP visualization using fluorescence microscopy) and expression of exogenous $p16^{INK4A}$ (by Western blotting) roughly equivalent to $p16^{INK4A}$ levels in wild-type fibroblasts (72 h after lentiviral infection). Infection of $p16^{INK4A}$ -deficient cells with $p16^{INK4A}$ lentivirus resulted in $p16^{INK4A}$ levels comparable to that observed in wild-type cells (Figure 3.1a, bottom), and was associated with normalization of ROS while ROS levels were significantly higher in $p16^{INK4A}$ -deficient cells infected with GFP lentivirus (Figure 3.1a, top). These control (GFP) $p16^{-/-}Arf^{+/+}$ cells also exhibited a dysregulated cell cycle profile evidenced by marked decrease in the proportion of cells in G1 phase and increase in the proportion in G2/M phase (Figure 3.1b). Introduction of $p16^{INK4A}$ expression in $p16^{-/-}Arf^{+/+}$ cells normalized the cell cycle distribution, increasing the fraction of cells in G1 phase and decreasing the fraction in G2/M phase (Figure 3.1b). These results provide evidence that expression of $p16^{INK4A}$ is both necessary and sufficient in $p16^{-/-}Arf^{+/+}$ cells to mediate oxidative and cell-cycle regulation.

Excessive ROS may lead to cellular senescence in some circumstances (Macleod 2008). Previous reports indicated in some experimental systems that $p16^{INK4A}$ expression was associated with both senescence and increased ROS (Takahashi et al. 2006), while in others increased $p16^{INK4A}$ expression was not associated with increased ROS (Macip et al. 2002). Thus, we examined whether reduced ROS associated with introduction of $p16^{INK4A}$ into $p16^{-/-}Arf^{+/+}$ cells was associated with cellular senescence. The $p16^{-/-}Arf^{+/+}$ fibroblasts were separately infected with either lentivirus expressing $p16^{INK4A}$ /GFP or GFP alone, and then assessed for β-galactosidase (β-gal) activity at pH 6.0 over a 7-day period. β-gal staining is an accepted marker of senescence in cultured cells (Dimri et al.

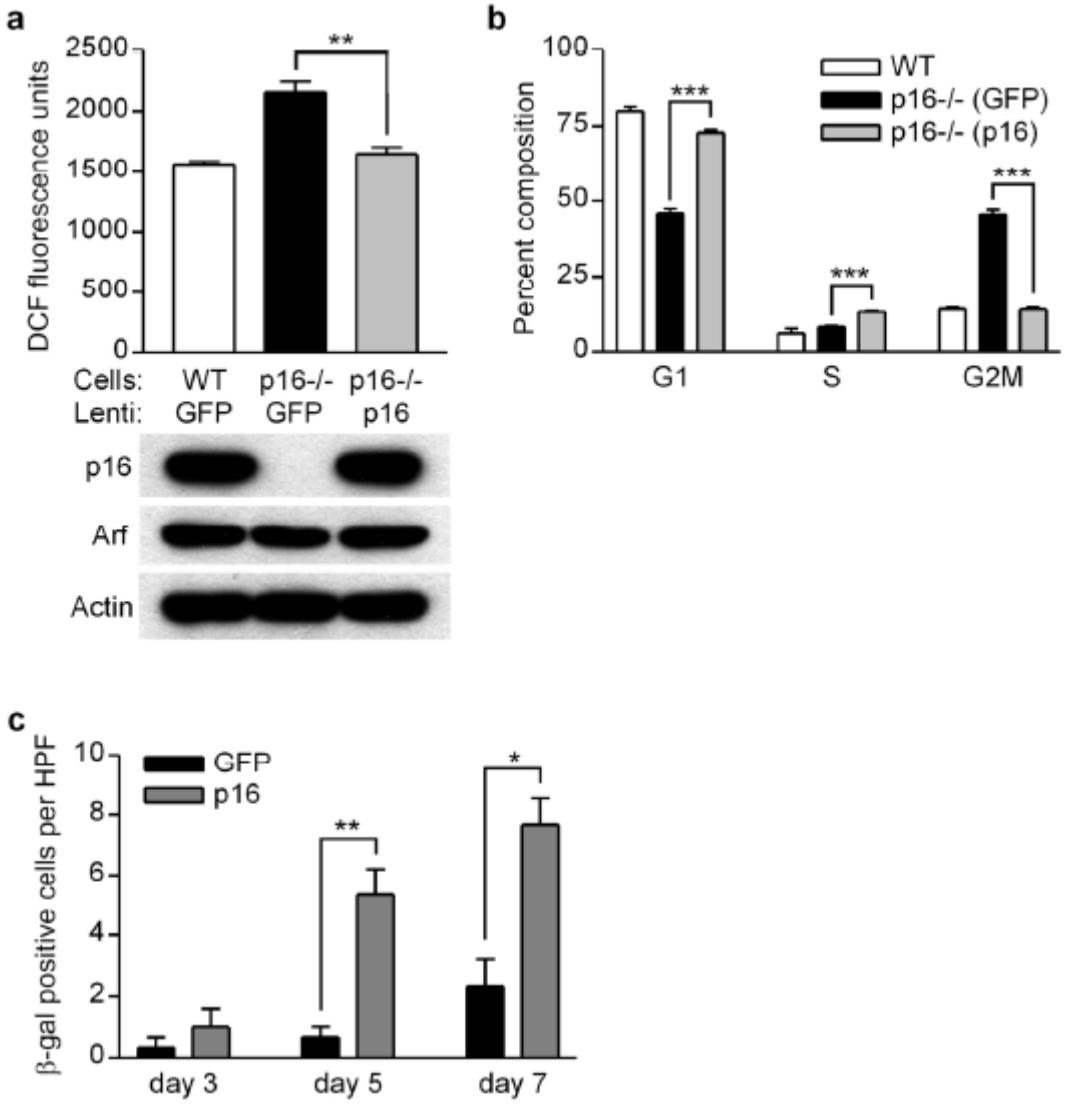


Figure 3.1. $p16^{INK4A}$ expression normalizes ROS and cell-cycle profile in $p16^{-/-}Arf^{+/+}$ cells. (**a**) Wild-type (WT) and *p16*^{INK4A}-deficient fibroblasts were infected with either GFP (control) lentivirus or lentivirus expressing wild-type p16 as indicated. After 72 h, cell lysates were subjected to DCFDA assay for intracellular ROS (upper panel) and western blotting for p16, Arf, or actin (lower panel). Error bars indicate SEM from triplicate determinations. (**b**) After 72 h, cell cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from triplicate determinations. (**c**) *p16*^{INK4A}-deficient fibroblasts were infected with either GFP (control) lentivirus or lentivirus expressing wild-type p16, then after the indicated time staining for β-gal was performed at pH 6.0. Average values were determined from three fields. Error bars indicate SEM from triplicate determinations.

1995). We found that while no senescent cells were evident in cultures of $p16^{-/-}Arf^{+/+}$ fibroblasts infected with control GFP lentivirus, cells infected with $p16^{INK4A}$ lentivirus were increasingly positive for senescence-associated β-gal staining over 7 days (Figure 3.1c, Figure 3.2). Thus although the relationship between $p16^{INK4A}$ expression and ROS appears subject to experimental context (Vurusaner et al. 2012), in our system restoring $p16^{INK4A}$ expression correlates with reduced ROS and increased G1 arrest and senescence.

Functional activities of familial melanoma-associated $p16^{INK4A}$ mutants

To investigate the potential functional consequences of particular mutations in $p16^{INK4A}$ that have been identified in human melanoma kindreds (Becker et al. 2001, Hashemi et al. 2000, Kannengiesser et al. 2009, McKenzie et al. 2010), we prepared lentiviral constructs encoding 12 point mutants that span the length of the $p16^{INK4A}$ coding region (Table 3.1). While nine of the mutations would be predicted to affect only the $p16^{INK4A}$ and not Arf coding sequences (R24P, R24Q, G35A, G35V, A36P, A57V, L97R, R99P, V126D), the remaining three mutations would be predicted to affect both $p16^{INK4A}$ and Arf (P81T, R87W, P114S). Each mutant was separately expressed in $p16^{-/-}Arf^{+/+}$ fibroblasts, and levels of ROS and cell cycle distribution were determined and compared to that found in cells expressing either GFP or wild-type $p16^{INK4A}$. We found that several $p16^{INK4A}$ mutants exhibited an impaired capacity to regulate both oxidative stress and the cell cycle. For example, ROS levels and cell cycle distribution remained dysregulated in cells expressing the P81T mutant compared to wild-type $p16^{INK4A}$ (Figure 3.3a). A similar phenotype was observed with the L97R (Figure 3.3b), and R87W (Figure 3.3c) mutants.

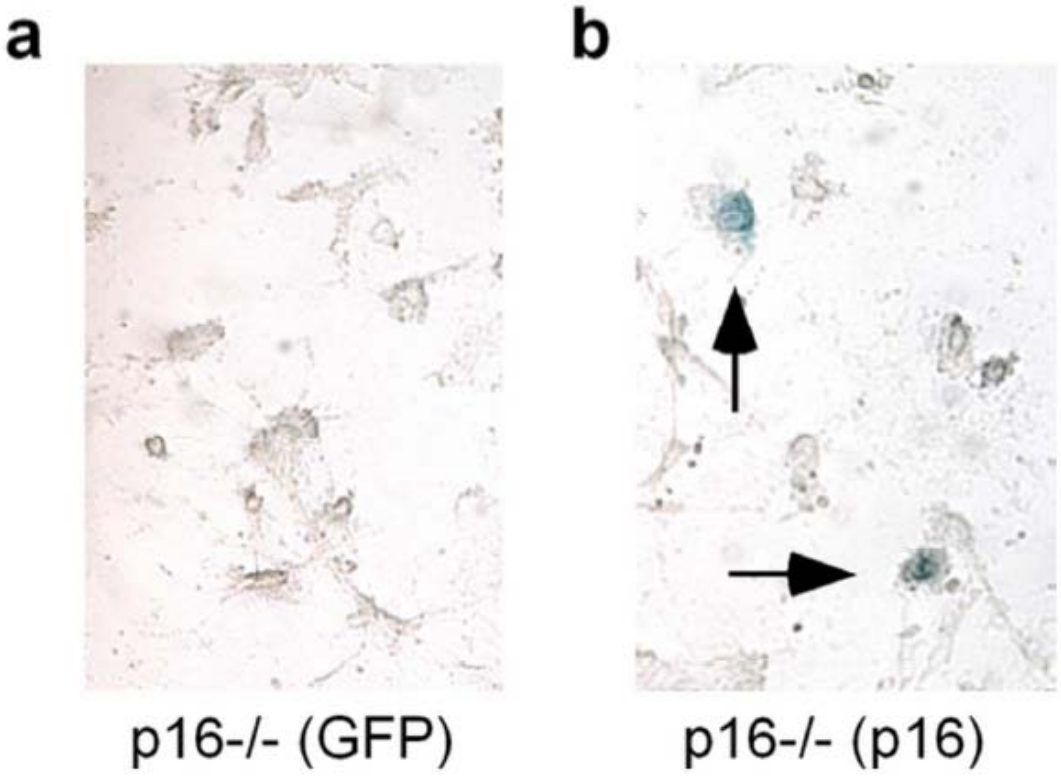


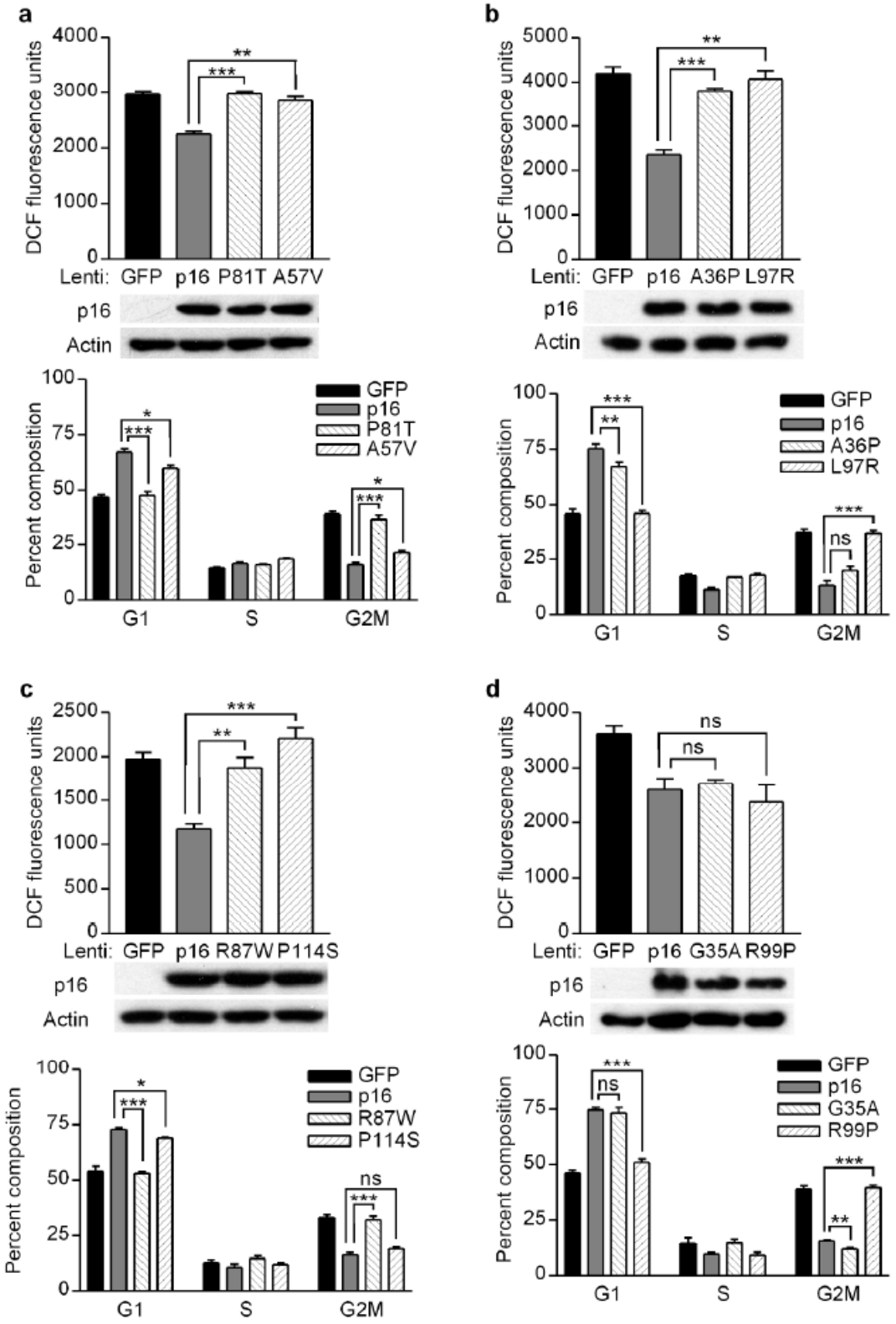
Figure 3.2 $p16^{INK4A}$ expression induces senescence. *$p16^{INK4A}$*-deficient fibroblasts were infected with either (**a**) GFP (control) lentivirus or (**b**) lentivirus expressing $p16^{INK4A}$, then after 7d staining for β-gal was performed at pH 6.0. Representative staining (arrows indicate positive cells) is shown.

Table 3.1 List of primers used to construct either wild-type $p16^{INK4A}$ or the 12 familial melanoma-associated point mutations of interest. Underlined nucleotides in the wild-typer primer sequences indicate PstI and XhoI sites introduced by PCR for further subcloning. Lower case bold letters in other primer sequences represent bases used to induce relevant point mutations into final $p16^{INK4A}$ mutant constructs.

Amino Acid Change	Base Change	Primer 1	Primer 2
WT	WT	GGACTGCAGCATGGAGCCGGCGG	TTTCTCGAGCCTCTCTGGTTCTTTCA
R24P	G71C	C**c**GGTAGAGGAGGTGCGGGCGCTGCTGG	CCAGCAGCGCCCGCACCTCCTCTACC**g**G
R24Q	G71A	C**a**GGTAGAGGAGGTGCGGGCGCTGCTGG	CCAGCAGCGCCCGCACCTCCTCTACC**t**G
G35A	G104C	GCGGGCGCTGCTGGAGGCGG**c**GGCGCTGCCC	GGGCAGCGCC**g**CCGCCTCCAGCAGCGCCCGC
G35V	G104T	GCGGGCGCTGCTGGAGGCGG**t**GGCGCTGCCC	GGGCAGCGCCaCCGCCTCCAGCAGCGCCCGC
A36P	G106C	GGAGGCGGGG**c**CGCTGCCCA	TGGGCAGCG**g**CCCCGCCTCC
A57V	C170T	CATGATGATGGGCAGCG**t**CCGAGTGGCGG	CCGCCACTCGG**a**CGCTGCCCATCATCATG
P81T	C241A	CACCCGA**a**CCGTGCACGACGCTGCCCGGG	CCCGGGCAGCGTCGTGCACGG**t**TCGGGTG
R87W	C259T	GCACGACGCTGCCtGGGAGGGCTTCCTGG	CCAGGAAGCCCTCCC**a**GGCAGCGTCGTGC
L97R	T290G	GC**g**GCACCGGGCCGGGGCGCGGCTGG	CCAGCCGCGCCCCGGCCCGGTGC**c**GC
R99P	G296C	GCACC**c**GGCCGGGGCGCGGCTGG	CCAGCCGCGCCCCGGCC**g**GGTGC
P114S	G339C	CT**c**CCCGTGGACCTGGCTGAGG	CCTCAGCCAGGTCCACGGG**g**AG
V126D	T377A	GAGGAGCTGGGCCATCGCGATG**a**CGCACGGT	ACCGTGCGTCA**t**CGCGATGGCCCAGCTCCTC

All sequences are 5' to 3'. Lower case bold letters in sequences indicate point mutations introduced.
WT, wild-type $p16^{INK4A}$

Figure 3.3 Functional activities of familial melanoma-associated $p16^{INK4A}$ mutants. *p16*^{INK4A}-deficient fibroblasts were infected with the indicated lentiviral constructs expressing GFP, wild-type $p16^{INK4A}$, or (**a**) mutants P81T or A57V, (**b**) mutants A36P or L97R, (**c**) mutants R87W or P114S, or (**d**) mutants G35A or R99P. Cell lysates were prepared for detection of ROS and p16 protein levels (upper panels in each). Cell cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated (lower panels in each). Error bars indicate SEM from triplicate determinations. ns, not significant.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3000
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***
Lenti: GFP p16 P81T A57V
p16
Actin
Percent composition
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Lenti: GFP p16 A36P L97R
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Thus three of the 12 mutants could be categorized as “double loss of function” (Figure 3.4). Interestingly, several $p16^{INK4A}$ mutants largely restored regulation of both oxidative stress and cell cycle distribution. For example, expression of the G35A mutant resulted in ROS levels and cell cycle distribution comparable to that of cells expressing wild-type $p16^{INK4A}$ (Figure 3.3d). A similar phenotype was observed for the R24P (Figure 3.5) and G35V (Figure 3.6) mutants. The identification of these three mutants (none of which affect Arf) that largely retain both oxidative and cell cycle regulatory functions (Figure 3.4) suggests that some mutations in $p16^{INK4A}$ may affect melanoma predisposition by disrupting other (tumor suppressor-related yet undefined) functional activities.

Uncoupling of cell cycle and oxidative regulatory functions

For the remaining six $p16^{INK4A}$ mutants, we found that the oxidative or cell cycle regulatory activity was selectively compromised. For example, the A57V mutant normalized cell-cycle distribution comparable to wild-type $p16^{INK4A}$, but did not correct elevated ROS levels (Figure 3.2a). Similarly, the A36P (Figure 3.3b) and P114S mutants (Figure 3.3c) demonstrated selective loss of oxidative compared to cell cycle regulation. The inverse result was observed with the R99P mutant, which effectively suppressed ROS levels but did not restore cell-cycle distribution (Figure 3.3d, Figure 3.7). Similarly, selective loss of cell cycle compared to oxidative regulatory function was observed in the V126D (Figure 3.5) and R24Q (Figure 3.7) mutants. Thus the identification of these six mutants in which the oxidative and cell cycle regulatory functions are relatively uncoupled (Figure 3.4) supports our previous contention that $p16^{INK4A}$ regulates oxidative stress in a cell cycle-independent manner (Jenkins et al. 2011).

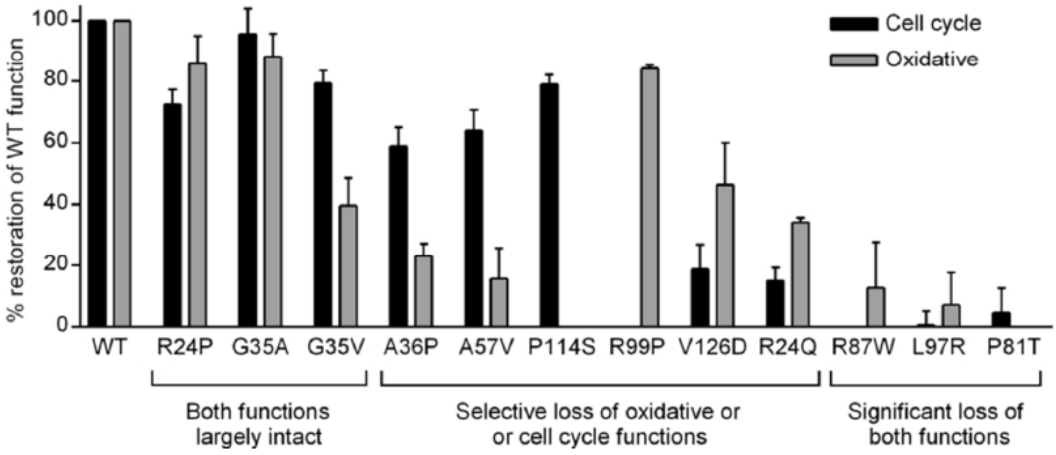


Figure 3.4 Summary of functional analyses of familial melanoma-associated $p16^{INK4A}$ mutants. Percent restoration (relative to wild-type $p16^{INK4A}$, set at 100%) of cell cycle or oxidative regulatory function is shown after each construct was expressed in $p16^{INK4A}$ -deficient fibroblasts. Error bars indicate SEM of triplicate determinations.

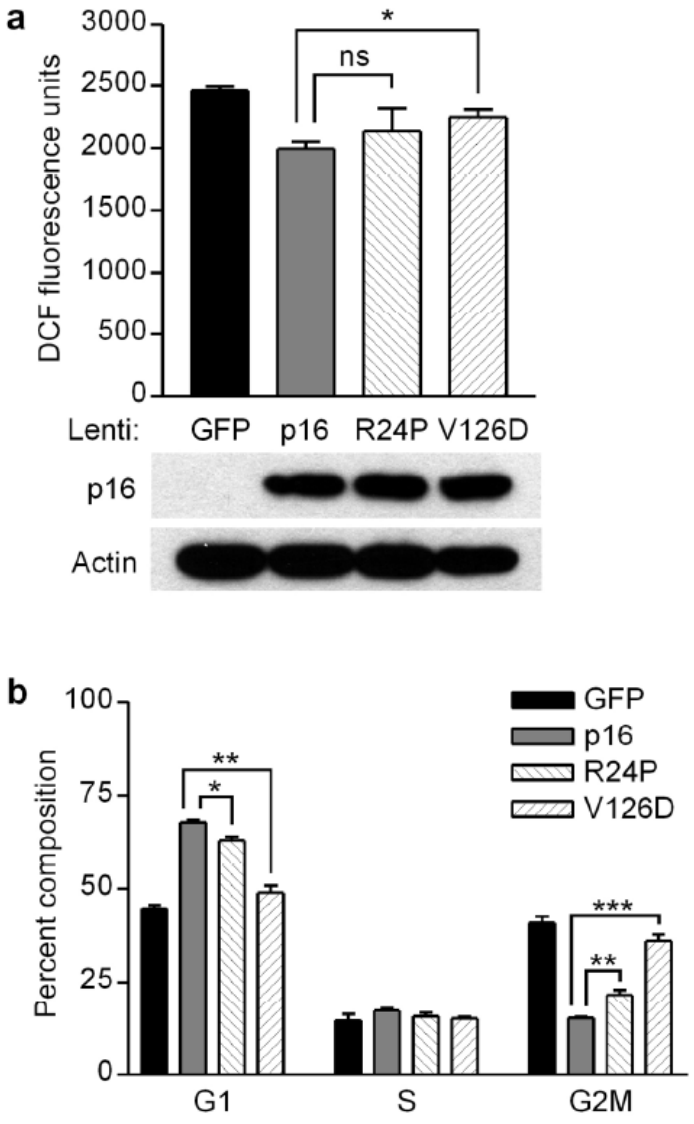


Figure 3.5 Functional activities of familial melanoma-associated p16^{INK4A} mutants R24P and V126D. (**a**) *p16^{INK4A}* -deficient fibroblasts were infected with the indicated lentiviral constructs, and cell lysates were used for detection of ROS and p16^{INK4A} protein levels. Error bars indicate SEM from triplicate determinations. ns, not significant. (**b**) Cell cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from triplicate determinations.

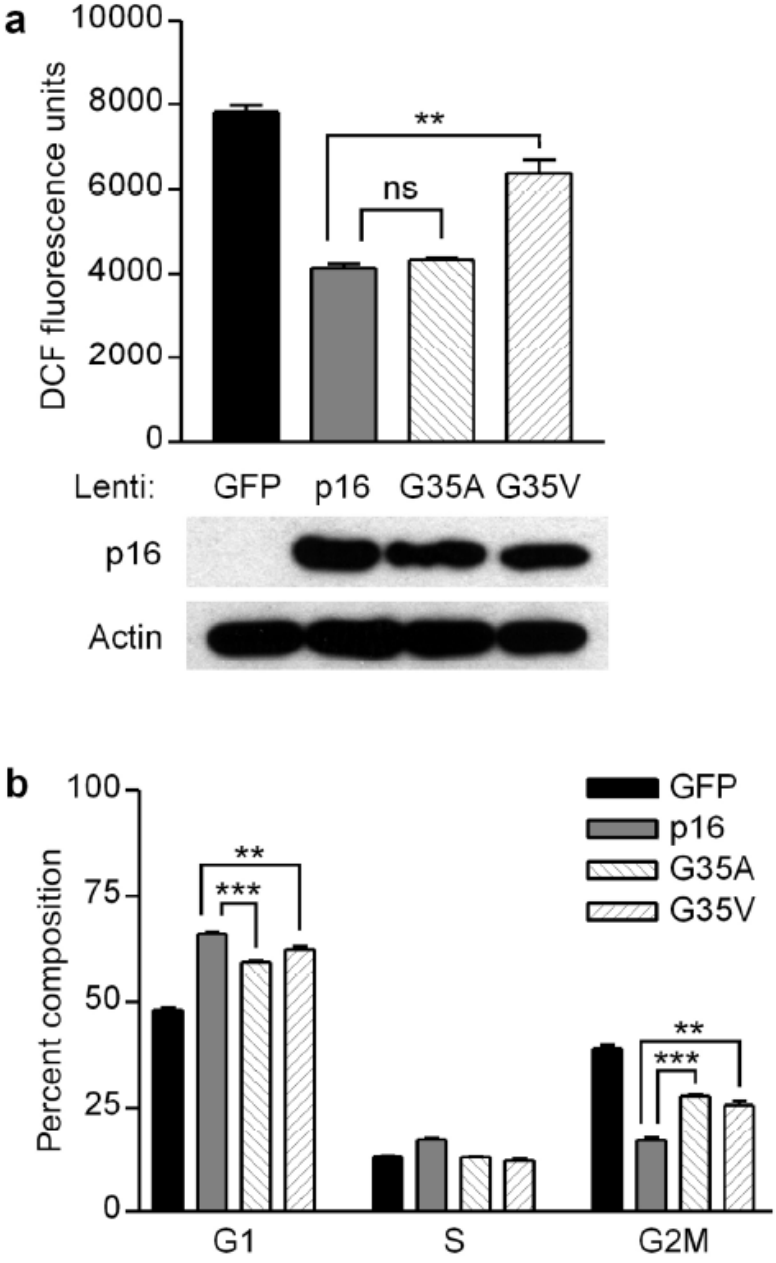


Figure 3.6 Functional activities of familial melanoma-associated p16 mutants G35A and G35V. (**a**) *p16*^{INK4A} -deficient fibroblasts were infected with the indicated lentiviral constructs, and cell lysates were used for detection of ROS and p16^{INK4A} protein levels. Error bars indicate SEM from triplicate determinations. ns, not significant. (**b**) Cell cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from triplicate determinations

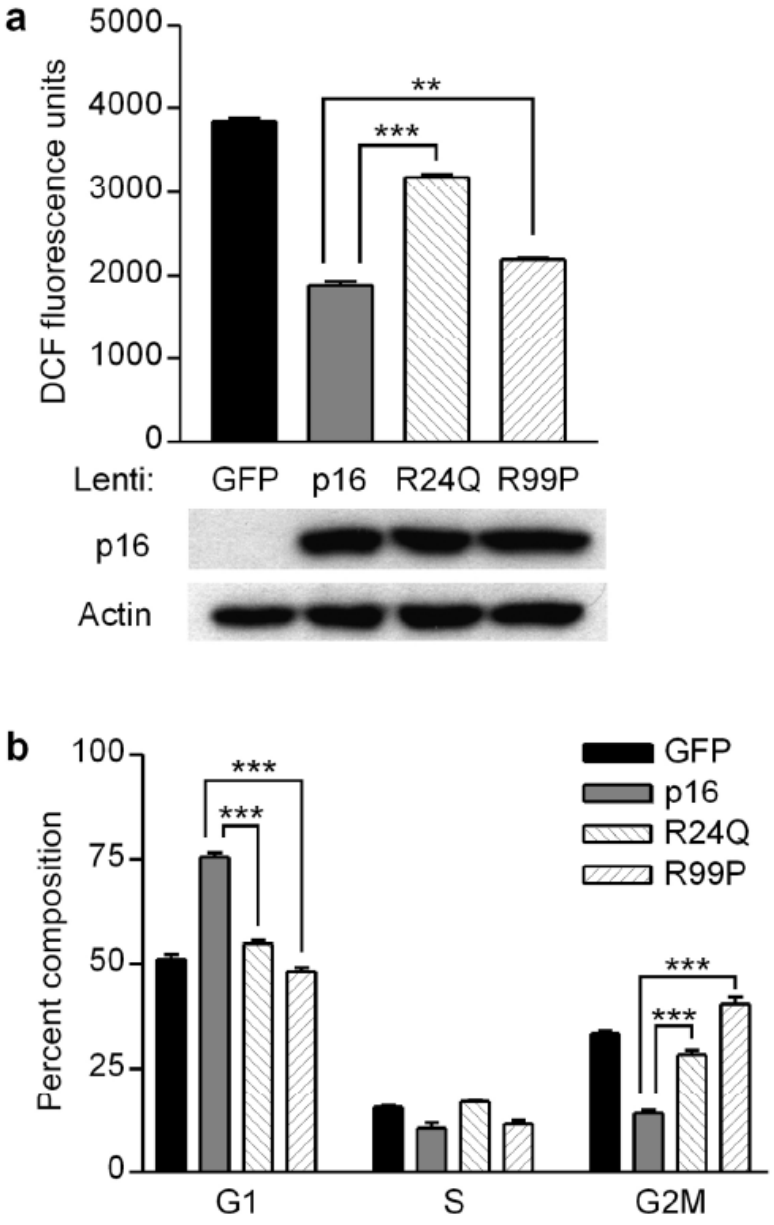


Figure 3.7 Functional activities of familial melanoma-associated $p16^{INK4A}$ mutants R24Q and R99P. (**a**) *$p16^{INK4A}$* -deficient fibroblasts were infected with the indicated lentiviral constructs, and cell lysates were used for detection of ROS and $p16^{INK4A}$ protein levels. Error bars indicate SEM from triplicate determinations. (**b**) Cell cycle analysis was performed with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from triplicate determinations.

p16^{INK4A} mutants with altered functional activities retain appropriate subcellular localization

It is generally thought that p16^{INK4A} localizes to the nucleus to exert its CDK-inhibitory function (Bartkova et al., 1996; Lukas et al., 1995), although there are reports of p16^{INK4A} revealing both nuclear and (sparse) cytoplasmic localization (Geradts et al., 2000; McKenzie et al., 2010). Others have found that exogenous over-expression of p16^{INK4A} can lead to protein aggregation and unfolding in the cytoplasm, resulting in loss of function (Tevelev et al., 1996). It has also been suggested that cytoplasmic localization of p16^{INK4A} may represent a specific mechanism of its inactivation in tumors (Evangelou et al., 2004). Given these considerations, it was important to demonstrate that alterations in functional activities seen here with some p16^{INK4A} mutants were not due to mislocalization of the protein. Therefore, we assessed subcellular localization of each p16^{INK4A} mutant in p16$^{-/-}$Arf$^{+/+}$ fibroblasts by immunofluorescence. First, we confirmed that wild-type p16^{INK4A} was strongly nuclear, colocalizing with DAPI-staining nuclei, and no cytosolic expression was detected (Figure 3.8). Analysis of the 12 p16^{INK4A} point mutants consistently showed similar nuclear localization (Figure 3.8), providing strong evidence that their various altered functional activities could not be attributed to mislocalization of p16^{INK4A}.

Analysis of p16^{INK4A} -regulatory functions in human melanoma cells

Next, we examined a subset of these mutants in human melanoma cells – perhaps a more relevant model for analyzing p16 mutations associated with familial melanoma. WM793 cells that do not express p16^{INK4A} were transduced with lentivirus expressing

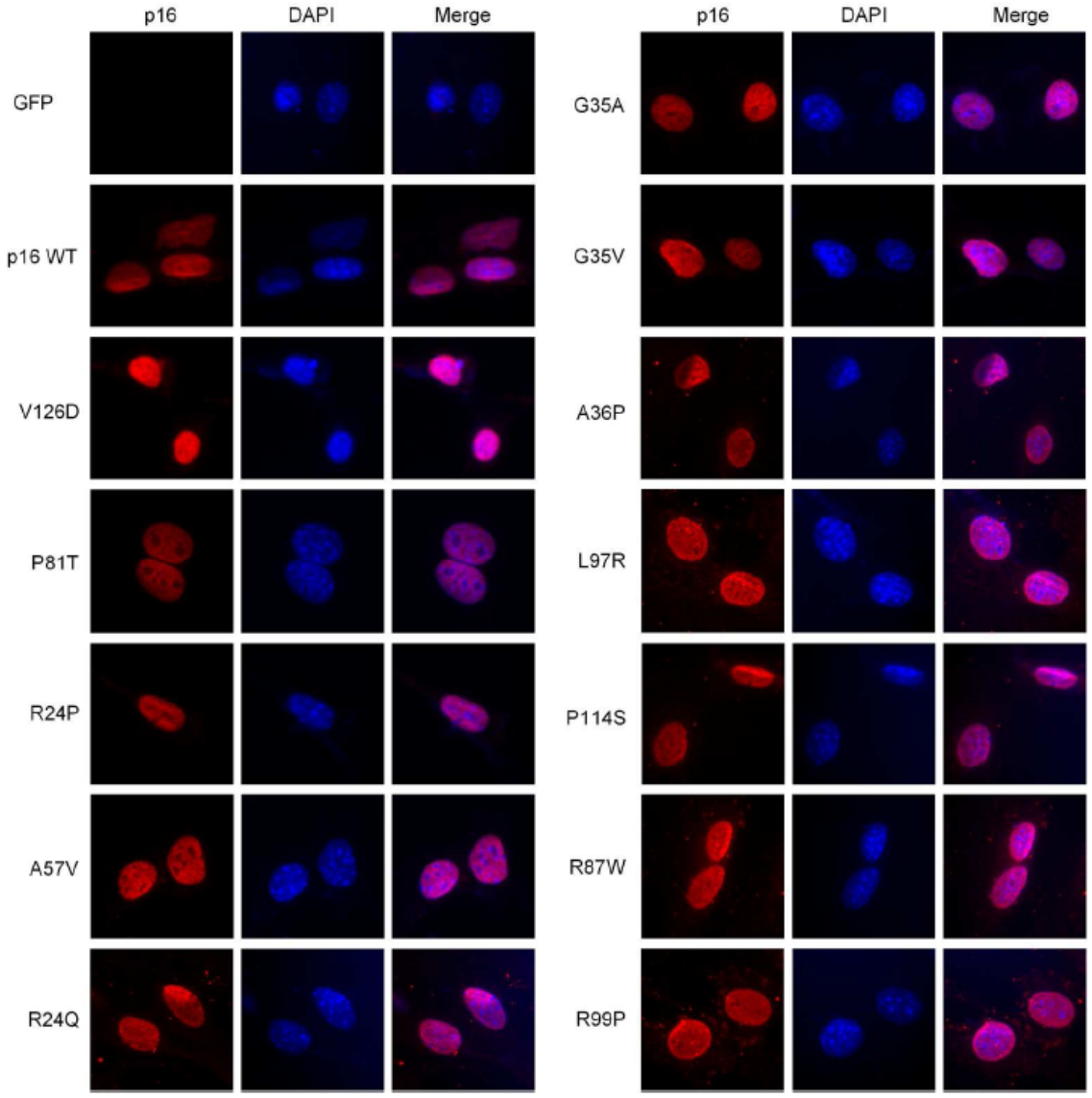


Figure 3.8 Nuclear localization of $p16^{INK4A}$ mutants. $p16^{-/-}Arf^{+/+}$ fibroblasts were infected with the indicated lentiviral constructs. Cells were fixed, permeabilized and stained for $p16^{INK4A}$ expression (red) and the DNA marker 4',6-diamidino-2-phenylindole (DAPI, blue).

either GFP, wild-type $p16^{INK4A}$, or a selected $p16^{INK4A}$ mutant. As above, we optimized expression of individual mutants to be comparable to expression levels of wild-type $p16^{INK4A}$ by Western blotting (Figure 3.9a, b). As we observed in $p16^{-/-}Arf^{+/+}$ mouse fibroblasts (Figure 3.1), expression of wild-type $p16^{INK4A}$ (compared to GFP control) was associated both with suppression of ROS levels (Figure 3.9c, d) and shift of the cell cycle distribution (Figure 3.9e,f). Mirroring the phenotypes seen above (Figure 3.2c, d), the R99P mutant retained oxidative but not cell cycle function while the P114S mutant exhibited the reciprocal phenotype (Figure 3.9c, e) in WM793 cells. Compared to wild-type $p16^{INK4A}$, the R24Q mutant was unable to restore significant oxidative (Figure 3.9d) or cell cycle function (Figure 3.9f) consistent with our earlier findings (Figure 3.7). Finally, as seen above (Figure 3.6), the G35V mutant retained cell cycle function comparable to wild type $p16^{INK4A}$ (Figure 3.9f), but exhibited limited capacity for reducing ROS (Figure 3.9d). Importantly, the differential capacity of three p16 mutants (R99P, P114S, G35V) to regulate oxidative versus cell cycle regulatory functions was recapitulated in human melanoma cells.

Structure-function relationships among $p16^{INK4A}$ mutants

In order to gain insight into the different functional activities associated with particular $p16^{INK4A}$ mutants, we examined their relative localization based on published structures of the molecule (Byeon et al., 1998; Russo et al., 1998). $p16^{INK4A}$ consists mainly of four ankyrin repeats, a conserved motif of approximately 30 amino acids, which is involved in various protein-protein interactions (Li et al., 2006). These repeats create a pair of antiparallel helices forming a stem and a β-hairpin forming the base of an L-shaped structure (Gorina and Pavletich 1996; Russo et al., 1998). While some studies

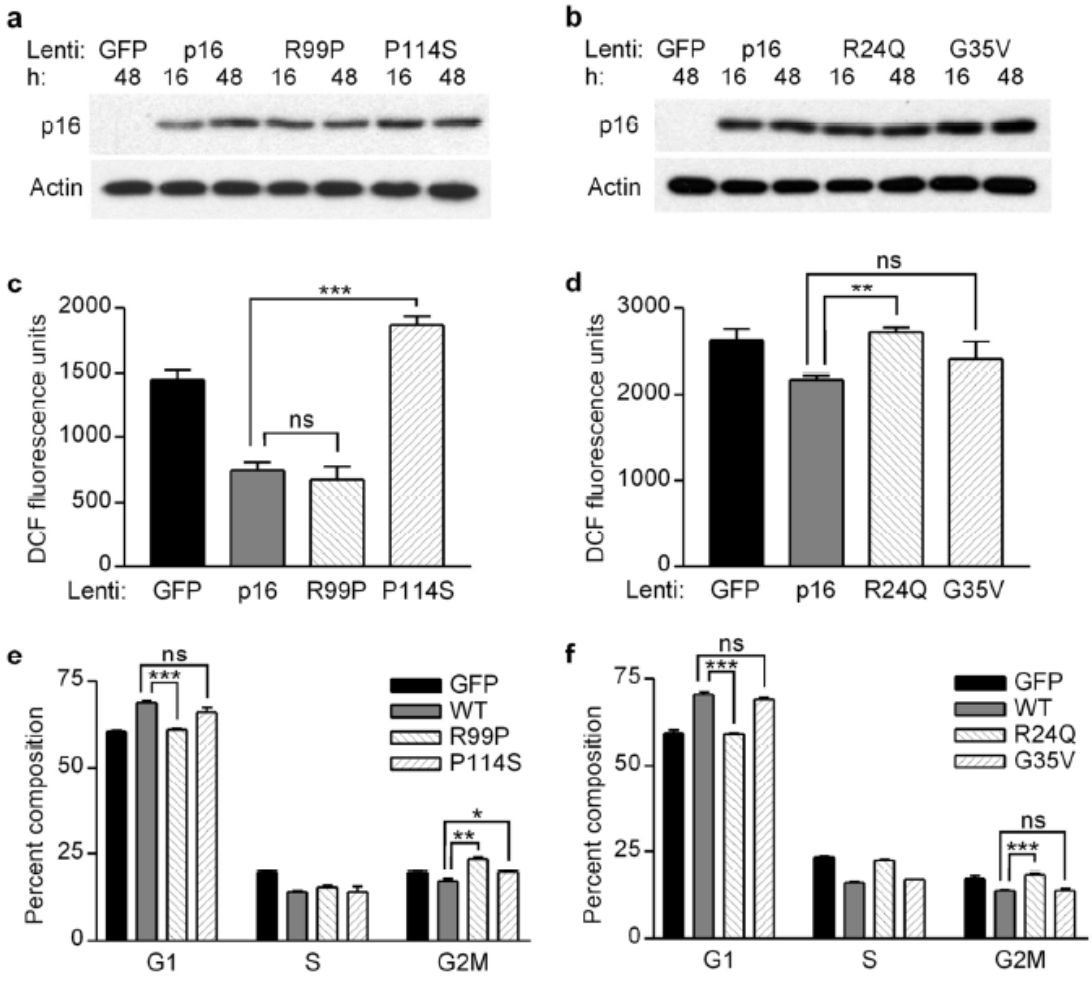


Figure 3.9 Uncoupling of oxidative and cell cycle regulatory functions by p16^{INK4A} mutants in WM793 human melanoma cells. (**a**, **b**) WM793 cells were infected with the indicated lentiviral constructs, and cell lysates were collected either 16 h or 48 h post-infection for western blotting. (**c, d**) ROS levels were determined in cell lysates 16 h postinfection with the indicated lentivirus. Error bars indicate SEM from triplicate determinations. ns, not significant. (**e**, **f**) Cell cycle analysis was performed 48 h post-infection with percentages of cells in each phase (G1, S, G2M) indicated. Error bars indicate SEM from triplicate determinations. ns, not significant.

have implicated all four ankyrin repeats as important for CDK4/6-binding and cell cycle inhibition, others indicate that the third ankyrin repeat (residues 81-113) as well as the β-hairpin loop within the second ankyrin repeat (residues 52-54) are the most critical regions for mediating these functions (Byeon et al., 1998, Mahajan et al., 2007, Russo et al., 1998). Consistent with this notion, several residues that we found to be important for both cell-cycle and oxidative regulation (P81, R87, L97), or only cell-cycle regulation (R99), reside in the third ankyrin repeat (Figure 3.10). By contrast, most residues important for oxidative but not cell-cycle regulation (A36, A57, P114), or those not important for either function (G35, R24), are not found within the third ankyrin repeat or the β-hairpin loop of the second ankyrin repeat (Figure 3.10).

Discussion

We recently described a novel role for $p16^{INK4A}$ in suppressing intracellular oxidative stress, functioning independently of cell cycle and its control of the Rb pathway (Jenkins et al., 2011). These two regulatory functions are likely to be complementary in preventing potentially oncogenic oxidative DNA lesions by decreasing their formation (reduction of ROS) and propagation (induction of cell cycle arrest to allow DNA repair). In this study we examined separately the cell cycle and oxidative stress regulatory capacities of a panel of familial melanoma-associated $p16^{INK4A}$ point mutations spanning the *$p16^{INK4A}$* coding region. These mutants varied in their abilities to restore these two regulatory functions when expressed in $p16^{INK4A}$ –deficient fibroblasts, and could be grouped into distinct categories: those that restored both functions (R24P, G35A, G35V), selectively restored cell-cycle (A36P, A57V, P114S) or oxidative (R24Q, R99P, V126D)

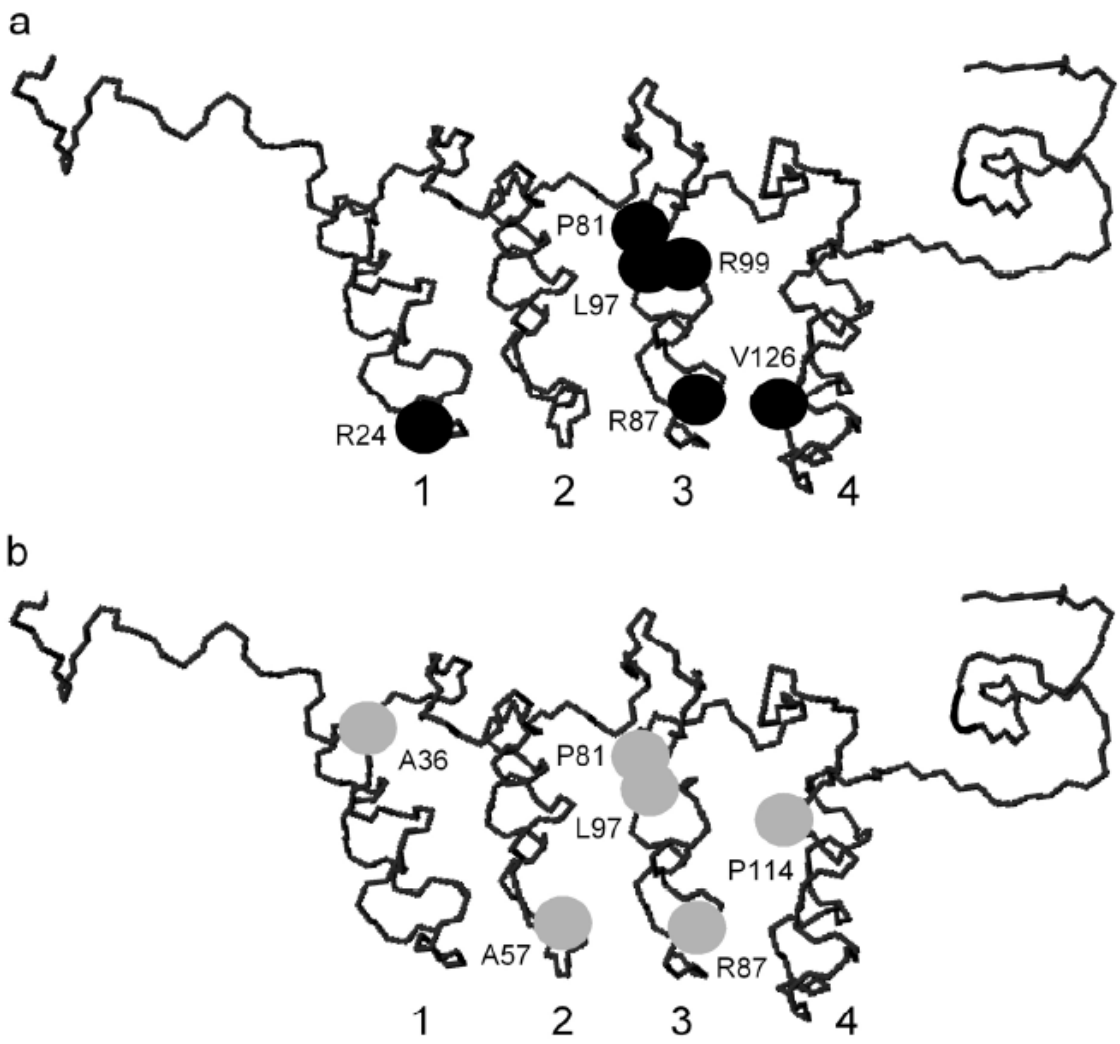


Figure 3.10 Localization of critical residues on $p16^{INK4A}$. Residues important for regulation of (**a**) cell-cycle (black circles) and (**b**) oxidative stress (gray circles) are highlighted on the $p16^{INK4A}$ backbone structure which exhibits 4 ankyrin repeats (numbered 1-4). Note that G35 is not depicted since neither G35A nor G35V compromised either function.

regulation, or failed to restore either function (R87W, L97R, P81T). These findings are further evidence that $p16^{INK4A}$ is sufficient to regulate intracellular oxidative stress independently of its canonical role in cell-cycle regulation, and that these roles can be individually compromised by particular familial melanoma-associated mutations.

Historically, the cell cycle regulatory function of some of these and other $p16^{INK4A}$ mutants found in patients was assessed by measuring CDK4-binding – a reasonable surrogate since $p16^{INK4A}$ binding to CDK4/6 is the critical step leading to reduction in Rb phosphorylation and inhibition of the G1/S transition of the cell cycle (Alcorta et al., 1996, Lukas et al., 1995). The two primary assays employed were based on yeast two-hybrid (Yang et al., 1995) and immunoprecipitation (Becker et al., 2001; Hashemi et al., 2000; Kannengiesser et al., 2009) approaches. These assays, however, have been problematic for two reasons. First, several mutants were found to retain the capacity to bind CDK4, yet were greatly reduced in their capacity to regulate the cell cycle (Becker et al., 2001, Koh et al., 1995). These discrepancies could reflect the additional known capacity of $p16^{INK4A}$ to bind CDK6 and intact CDK4/6-cyclinD complexes in addition to CDK4 (Hirai et al., 1995, Serrano et al., 1993), neither of which was measured in these studies. Differences in functional assays may also relate to the potential ability of p16 to bind and inhibit CDK7, a kinase subunit of the TFIIH transcription factor (Serizawa 1998). Given the requirement of TFIIH-mediated phosphorylation of RNA polymerase II for transcription, $p16^{INK4A}$ may induce cell cycle arrest independently of CDK4/6 by binding to CDK7 (Nishiwaki et al., 2000). In addition to lack of correlation between CDK4- binding and cell-cycle inhibitory functions found in some cases, other studies have reported differences in CDK4-binding activity for the

same p16 mutant. For example, the reported CDK4-binding activity of the relatively common G101W mutant ranged from 5 to 73% of wildtype, based on yeast two-hybrid (Reymond and Brent 1995, Yang et al., 1995) and immunoprecipitation assays (Becker et al., 2001, Parry and Peters 1996, Ranade et al., 1995, Walker et al., 1995). A mammalian two-hybrid assay has also been used to measure interactions between $p16^{INK4A}$ mutants and CDK4 in human osteosarcoma (Saos-2) cells (McKenzie et al., 2010). While this experimental system has the advantage of retaining a more appropriate intracellular environment allowing for posttranslational modifications, there could be important differences between these tumor cells and melanocytes or melanoma cells. Rather than developing our own assay based on CDK4-, CDK6- or CDK4/6-cyclin D binding, we wanted to avoid these pitfalls and directly measure cell-cycle regulatory activity; thus we determined cell-cycle distribution by flow cytometry (which was highly reproducible) as a readout of the cell-cycle regulatory function of these $p16^{INK4A}$ mutants.

Several previous studies have characterized the cell cycle regulatory capacity of different familial melanoma-associated $p16^{INK4A}$ point mutants. Overall, a wide range of phenotypes were reported among different mutants, as well as conflicting results concerning the same mutants. The basis for some of the discrepancies may lie in the different assays and cell types used for assessing cell cycle function, which included ability to induce phase arrest (Becker et al., 2001, Becker et al., 2005, Koh et al., 1995, McKenzie et al., 2010, Miller et al., 2011), limit cell numbers in culture (Jones et al., 2007, Kannengiesser et al., 2009), reduce proliferation by Ki67/BrdU staining (Jones et al., 2007, McKenzie et al., 2010), and reduce colony formation (Becker et al., 2005,

Jones et al., 2007) in fibroblasts, osteosarcoma, and melanoma cells. For the mutants studied here, however, our results largely agreed with what has been reported in the literature. For example, our observations that cell cycle function was retained (R24P, G35A, G35V) or only partially diminished (A36P, A57V, P114S) in these particular mutants is consistent with prior reports (Jones et al., 2007, Kannengiesser et al., 2009, McKenzie et al., 2010). Similarly, our findings that cell cycle function was largely diminished (V126D) or completely absent (R99P, R87W, L97R) in other mutants is consistent with previous studies (Becker et al., 2001, Kannengiesser et al., 2009; McKenzie et al., 2010, Miller et al., 2011). On the other hand, the lack of cell cycle regulatory function that we observed for mutants R24Q and P81T was not consistent with earlier studies in which the R24Q (Kannengiesser et al., 2009) and P81T (McKenzie et al., 2010) mutants were found to be comparable to wild-type $p16^{INK4A}$. For the R24Q mutant, we confirmed lack of cell cycle function in WM793 human melanoma cells (Figure 3.9f). As suggested above, one explanation for these discrepancies in addition to the different assays is that different cell types were employed. The capacity of some mutants to regulate cell cycle may be unmasked in particular cellular contexts depending on the different interactions of $p16^{INK4A}$ (i.e. with various CDKs) that could be affected. In addition, some cell lines may be less susceptible to regulation by exogenous $p16^{INK4A}$ due to the presence of background mutations, or loss of the entire *CDKN2A* locus with corresponding lack of dependency on $p16^{INK4A}$ or ARF. By testing the regulatory functions of these different mutants in primary fibroblasts, we gain insight into their behavior as potential tumor suppressors in an otherwise wild-type genetic background.

It has been reported that the third ankyrin repeat of $p16^{INK4A}$ (residues 81-113) and a β-hairpin loop in the second ankyrin repeat (residues 52-54) are the most important regions for CDK4- binding, as a 20-residue synthetic peptide (consisting of amino acids 84-103) was able to bind CDK4 (Fahraeus et al., 1996). Subsequent studies reported that numerous residues along the entire $p16^{INK4A}$ molecule are important in some capacity for CDK4-binding, but still identified residues very near or in the third ankyrin repeat as being the most significant (Byeon et al., 1998; Li et al., 1999; Mahajan et al., 2007). Our results further confirm the importance of this region in $p16^{INK4A}$ for cell cycle regulation, as the R99P mutant which demonstrates the most dramatic loss of cell cycle function while retaining oxidative function (Figure 3.4) is located in the third ankyrin repeat (Figure 3.10). This region forms both an extensive hydrogen-bond network at the interface of CDK4/6 (involving residues 74, 84, and 87 of p16) and a mostly hydrophobic structural core that interacts with the other internal helices that may help stabilize the protein (Russo et al., 1998). Perhaps several mutations in this region upset either the hydrogen bond network of the binding interface or these internal stabilizing helices, as most mutants that fail to restore both cell cycle and oxidative regulatory function (P81T, R87W, L97R) are located here (Figure 3.10). Consistent with this notion, the mutants we found that fail to impair either function (R24P, G35A, G35V), or that selectively impaired oxidative regulation (A36P, A57V, P114S), are located outside of this region and the β-hairpin loop in the second ankyrin repeat known to form hydrogen-bond backbone contacts with CDK4/6 (Russo et al., 1998). These residues may be involved in direct or indirect interactions with yet uncharacterized binding partners of $p16^{INK4A}$, or

mutations of these residues could alter the secondary structure of the $p16^{INK4A}$ molecule that precludes interactions required for oxidative regulatory function. The effects of particular mutations studies here (if any) on $p16^{INK4A}$ structure are difficult to predict without analysis of crystal structures of the mutant $p16^{INK4A}$ molecules.

The identification of several familial melanoma-associated mutants that largely retain both oxidative and cell cycle regulatory function (Figure 3.4) suggests that some mutations in $p16^{INK4A}$ may affect melanoma predisposition by disrupting some other yet-to-be defined tumor suppressor function. There is precedent for other well-studied tumor suppressor genes that appears to regulate ROS independently of their canonical functions (Vurusaner et al., 2012). For example, p53 which is involved in multiple cellular processes, including apoptosis, cell cycle arrest and senescence (Lane 1992), is a regulator of ROS. Several p53-target genes include redox-active proteins and ROS-generating enzymes (Macip et al., 2003, Polyak et al., 1997). In addition, many post-translational modifications of p53 generate ROS leading to activation of p38 mitogen-activated protein kinase (Bragado et al., 2007). Another example is the CDK inhibitor p21 that promotes cell cycle arrest and DNA repair (Li et al., 1994) that was reported to regulate oxidative stress through the Nrf2 pathway. p21 increases stability of Nrf2 by competing for Keap1 binding, which protects Nrf2 from ubiquination and subsequent degradation, allowing increased Nrf2-mediated transactivation of several antioxidant enzymes through binding to antioxidant response elements in their promoters (Chen et al., 2009). Finally, the breast cancer susceptibility genes BRCA1 and BRCA2 that are implicated in regulating cell cycle progression and maintaining genomic integrity (Rosen et al., 2003) also appear to be involved in regulating oxidative stress. BRCA1 upregulates

multiple antioxidant genes, including glutathione S-transferases and oxidoreductases (Bae et al., 2004). In addition, both BRCA1 and BRCA2 are required for the repair of the oxidative DNA lesion 8-oxoguanine (Le Page et al., 2000). The elucidation of non-canonical roles of $p16^{INK4A}$ as well as these other tumor suppressors in the regulation of cellular oxidative stress may signal the development of a new paradigm in which tumor-suppressor proteins employ multiple mechanisms that may be disabled in cancer, or in patients with cancer predisposition syndromes.

Materials and Methods

Cell culture

Murine fibroblasts were isolated from newborn wild-type (FVB) and background-matched $p16^{-/-}$ $Arf^{+/+}$ (#01XE4, FVB.129-*Cdkn2a*$^{tm2.1Rdp}$) homozygous mice (Kamijo *et al.,* 1997), both obtained from the National Cancer Institute (Rockville, MD, USA), as we have previously described (Jenkins et al., 2011). These procedures were approved by the University of Utah IACUC. Early passage cells (approximately two weeks after isolation) were aliquoted and stored at -80 °C. For each set of experiments, fresh cells were thawed and used over a 2-3 week period. WM793 melanoma cells were originally obtained from Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA).

Western blotting

Specific proteins were detected in cell lysates by Western blotting as previously described (Jenkins et al., 2011). Primary antibodies were used against $p16^{INK4A}$ (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:10 000, A-3853, Sigma-Aldrich, St. Louis, MO, USA), and ARF (1:1000, sc-22784, Santa Cruz Biotechnology).

Measurement of oxidative stress

Endogenous ROS of protein equivalents (30 µg) were quantified using 2,7-dichlorodihydrofluorescein diacetate (DCFDA, Life Technologies, Grand Island, NY, USA) as previously described (Jenkins et al., 2011). All experiments were performed in triplicate.

$p16^{INK4A}$ -expressing lentiviruses

The lentivirus expressing human wild-type $p16^{INK4A}$ is previously described (Jenkins et al., 2011). The $p16^{INK4A}$ point mutant constructs were generated by PCR-based segment overlap as described previously (Raj et al., 2008), using human $p16^{INK4A}$ cDNA as a template and primers designed to create specific point mutations. Briefly, an initial PCR reaction was used to separately create the 5' and 3' fragments for each mutant. The 5' fragment was constructed using wild-type $p16^{INK4A}$ sequence as "primer 1" and mutant sequence as "primer 2", and the 3' fragment was constructed using wild-type p16 sequence as "primer 2" and mutant sequence as "primer 1" (see Supplemental text, Table S1). A second PCR reaction was then used to anneal these individual segments, using equimolar amounts of the 5' and 3' fragments as template and primers corresponding to wild-type $p16^{INK4A}$. The final PCR product was cloned into a modified pHIV-Zsgreen (Addgene #18121) lentiviral expression vector (Welm et al., 2008) and confirmed by DNA sequencing. Each lentiviral construct was validated for $p16^{INK4A}$ expression by transient transfection into HeLa cells followed by western blotting. Viruses were produced in HEK 293T/17 cells (ATCC, Manassas, VA, USA) co-transfected with 5 µg lentiviral vector and 1.7 µg of each helper plasmid (pRSV-REV, pMDLg/pRRE and pVSVG, generously provided by Brian Welm, Huntsman Cancer Institute) and 30 µg of

polyethylenimine (pH 7.0, Sigma) in 1 mL of OptiMEM (Life Technologies). Viral particles were collected, purified, concentrated, titered, and stored as described previously (Jenkins et al., 2011). For cellular infection, 8 µg per mL polybrene (Sigma) was added. Assays for oxidative stress and cell cycle distribution in WM793 cells were performed 16 h and 48 h after infection, respectively, and after 72 h in fibroblasts. Experiments involving each mutant were performed at least twice.

Cell cycle analysis

Cells were harvested by trypsinization, washed, fixed, stained with 50 mg per mL propidium iodide (Sigma), and analyzed as described previously (Jenkins et al., 2011). All experiments were performed in triplicate.

Senescence-associated β-gal staining

Staining was performed as described previously (Cotter et al., 2007). Briefly, cells were fixed in 1% paraformaldehyde and then stained overnight at 37°C in a solution (pH 6.0) containing potassium ferrocyanide, potassium ferricyanide, and 5-bromo-4-chloro-3-indolyl-b-Dgalactoside (X-gal). All experiments were performed in triplicate.

Immunofluorescence

Cultured fibroblasts were seeded on coverslips in 12-well plates at 30-40% confluency, tranduced by lentivirus, and then fixed 72 h post-infection with PBS containing 4 % paraformaldehyde for 15 min. Cells were permeabilized with 0.2 %Triton X-100 in PBS, then immunostained for 60 min with anti- $p16^{INK4A}$ (1:1000, sc-1661, Santa Cruz Biotechnology), followed by a 60 min exposure to Alexa Fluor 594-conjugated secondary IgG (1:200, A-11062, Life Technologies). Images were captured

on a Zeiss Axioskop2 automated microscope, using an Axio Cam MRm camera and AxioVision 4.8.1 software (Carl Zeiss Microscopy, Thornwood, NY, USA), and then processed with ImageJ software (http://rsbweb.nih.gov/ij/download.html).

Structural analysis

Structural modeling of $p16^{INK4A}$ was performed using SwissPdb Viewer (http://www.expasy.org/spdbv) as described elsewhere (Guex and Peitsch 1997), based on the $p16^{INK4A}$ published structure 1a5e (Byeon et al., 1998).

Statistics

Analyses were performed with Prism 3.0 software (GraphPad). Data derived from multiple determinations were subjected to two-sided *t* tests. P values < 0.05 were considered statistically significant. Statistical significance is denoted within each figure by asterisks with *, **, and *** indicating P values of <0.05, <0.01, and <0.001, respectively.

References

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **93:** 13742-7.

Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ *et al.* (2004). BRCA1 induces antioxidant gene expression and resistance to oxidative stress. *Cancer research* **64:** 7893-909.

Bartkova J, Lukas J, Guldberg P, Alsner J, Kirkin AF, Zeuthen J *et al.* (1996). The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis. *Cancer research* **56:** 5475-83.

Becker TM, Rizos H, Kefford RF, Mann GJ (2001). Functional impairment of melanoma-associated p16(INK4a) mutants in melanoma cells despite retention of cyclin-dependent kinase 4 binding. *Clinical cancer research : an official journal of the American Association for Cancer Research* **7:** 3282-8.

Becker TM, Ayub AL, Kefford RF, Mann GJ, Rizos H (2005). The melanoma-associated 24 base pair duplication in p16INK4a is functionally impaired. *Int J Cancer* **117:** 569-73.

Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B *et al.* (2002). Geographical variation in the penetrance of CDKN2A mutations for melanoma. *J Natl Cancer Inst* **94:** 894-903

Bragado P, Armesilla A, Silva A, Porras A (2007). Apoptosis by cisplatin requires p53 mediated p38-alpha MAPK activation through ROS generation. *Apoptosis* **12:** 1733-42.

Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ *et al.* (1998). Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. *Molecular cell* **1:** 421-31.

Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D *et al.* (2009). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Molecular cell* **34:** 663-73.

Cotter MA, Florell SR, Leachman SA, Grossman D (2007). Absence of senescence-associated beta-galactosidase activity in human melanocytic nevi in vivo. *J Invest Dermatol* **127:** 2469-71.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 9363-7.

Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, Kalogeropoulos N *et al.* (2004). Electron microscopy evidence that cytoplasmic localization of the p16(INK4A) "nuclear" cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in nonsmall cell lung carcinomas. *Biotech Histochem* **79:** 5-10.

Fahraeus R, Paramio JM, Ball KL, Lain S, Lane DP (1996). Inhibition of pRb phosphorylation and cell-cycle progression by a 20-residue peptide derived from p16CDKN2/INK4A. *Curr Biol* **6:** 84-91.

Geradts J, Hruban RH, Schutte M, Kern SE, Maynard R (2000). Immunohistochemical p16INK4a analysis of archival tumors with deletion, hypermethylation, or mutation of the CDKN2/MTS1 gene. A comparison of four commercial antibodies. *Appl Immunohistochem Mol Morphol* **8:** 71-9.

Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT *et al.* (2007). Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet* **44:** 99-106.

Gorina S, Pavletich NP (1996). Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science* **274:** 1001-5.

Guex N, Peitsch MC (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18:** 2714-23.

Hashemi J, Platz A, Ueno T, Stierner U, Ringborg U, Hansson J (2000). CDKN2A germ-line mutations in individuals with multiple cutaneous melanomas. *Cancer research* **60:** 6864-7.

Herrling T, Jung K, Fuchs J (2006). Measurements of UV-generated free radicals/reactive oxygen species (ROS) in skin. *Spectrochim Acta A Mol Biomol Spectrosc* **63:** 840-5.

Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Molecular and cellular biology* **15:** 2672-81.

Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG *et al.* (2011). The p16(INK4A) tumor suppressor regulates cellular oxidative stress. *Oncogene* **30:** 265-74.

Jones R, Ruas M, Gregory F, Moulin S, Delia D, Manoukian S *et al.* (2007). A CDKN2A mutation in familial melanoma that abrogates binding of p16INK4a to CDK4 but not CDK6. *Cancer research* **67:** 9134-41.

Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA *et al.* (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91:** 649-59.

Kannengiesser C, Brookes S, del Arroyo AG, Pham D, Bombled J, Barrois M *et al.* (2009). Functional, structural, and genetic evaluation of 20 CDKN2A germ line mutations identified in melanoma-prone families or patients. *Hum Mutat* **30:** 564-74.

Koh J, Enders GH, Dynlacht BD, Harlow E (1995). Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375:** 506-10.

Lane DP (1992). Cancer. p53, guardian of the genome. *Nature* **358:** 15-6.

Le Page F, Randrianarison V, Marot D, Cabannes J, Perricaudet M, Feunteun J *et al.* (2000). BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8- oxoguanine lesion in human cells. *Cancer research* **60:** 5548-52.

Li J, Byeon IJ, Ericson K, Poi MJ, O'Maille P, Selby T *et al.* (1999). Tumor suppressor INK4: determination of the solution structure of p18INK4C and demonstration of the functional significance of loops in p18INK4C and p16INK4A. *Biochemistry* **38:** 2930-40.

Li J, Mahajan A, Tsai MD (2006). Ankyrin repeat: a unique motif mediating protein-protein interactions. *Biochemistry* **45:** 15168-78.

Li R, Waga S, Hannon GJ, Beach D, Stillman B (1994). Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* **371:** 534-7.

Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M *et al.* (1995). Retinoblastoma protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375:** 503-6.

Macip S, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW *et al.* (2002). Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *The EMBO journal* **21:** 2180-8.

Macip S, Igarashi M, Berggren P, Yu J, Lee SW, Aaronson SA (2003). Influence of induced reactive oxygen species in p53-mediated cell fate decisions. *Molecular and cellular biology* **23:** 8576-85.

Macleod KF (2008). The role of the RB tumour suppressor pathway in oxidative stress responses in the haematopoietic system. *Nat Rev Cancer* **8:** 769-81.

Mahajan A, Guo Y, Yuan C, Weghorst CM, Tsai MD, Li J (2007). Dissection of protein-protein interaction and CDK4 inhibition in the oncogenic versus tumor suppressing functions of gankyrin and P16. *J Mol Biol* **373:** 990-1005.

McKenzie HA, Fung C, Becker TM, Irvine M, Mann GJ, Kefford RF *et al.* (2010). Predicting functional significance of cancer-associated p16(INK4a) mutations in CDKN2A. *Hum Mutat* **31:** 692-701.

Miller PJ, Duraisamy S, Newell JA, Chan PA, Tie MM, Rogers AE *et al.* (2011). Classifying variants of CDKN2A using computational and laboratory studies. *Hum Mutat* **32:** 900-11.

Nishiwaki E, Turner SL, Harju S, Miyazaki S, Kashiwagi M, Koh J *et al.* (2000). Regulation of CDK7-carboxyl-terminal domain kinase activity by the tumor suppressor p16(INK4A) contributes to cell cycle regulation. *Molecular and cellular biology* **20:** 7726-34.

Parry D, Peters G (1996). Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. *Molecular and cellular biology* **16:** 3844-52.

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B (1997). A model for p53-induced apoptosis. *Nature* **389:** 300-5.

Raj D, Liu T, Samadashwily G, Li F, Grossman D (2008). Survivin repression by p53, Rb and E2F2 in normal human melanocytes. *Carcinogenesis* **29:** 194-201.

Ranade K, Hussussian CJ, Sikorski RS, Varmus HE, Goldstein AM, Tucker MA *et al.* (1995). Mutations associated with familial melanoma impair p16INK4 function. *Nature genetics* **10:** 114-6.

Reymond A, Brent R (1995). p16 proteins from melanoma-prone families are deficient in binding to Cdk4. *Oncogene* **11:** 1173-8.

Rosen EM, Fan S, Pestell RG, Goldberg ID (2003). BRCA1 gene in breast cancer. *J Cell Physiol* **196:** 19-41.

Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP (1998). Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. *Nature* **395:** 237-43.

Serizawa H (1998). Cyclin-dependent kinase inhibitor p16INK4A inhibits phosphorylation of RNA polymerase II by general transcription factor TFIIH. *The Journal of biological chemistry* **273:** 5427-30.

Serrano M, Hannon GJ, Beach D (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366:** 704-7.

Shapiro GI, Edwards CD, Ewen ME, Rollins BJ (1998). p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. *Molecular and cellular biology* **18:** 378-87.

Sharpless NE, DePinho RA (1999). The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev* **9:** 22-30.

Takahashi A, Ohtani N, Yamakoshi K, Iida S, Tahara H, Nakayama K *et al.* (2006). Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat Cell Biol* **8:** 1291-7.

Tevelev A, Byeon IJ, Selby T, Ericson K, Kim HJ, Kraynov V *et al.* (1996). Tumor suppressor p16INK4A: structural characterization of wild-type and mutant proteins by NMR and circular dichroism. *Biochemistry* **35:** 9475-87.

Vurusaner B, Poli G, Basaga H (2012). Tumor suppressor genes and ROS: complex networks of interactions. *Free Radic Biol Med* **52:** 7-18

Walker GJ, Hussussian CJ, Flores JF, Glendening JM, Haluska FG, Dracopoli NC *et al.* (1995). Mutations of the CDKN2/p16INK4 gene in Australian melanoma kindreds. *Human mol genetics* **4:** 1845-52.

Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z (2008). Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. *Cell Stem Cell* **2:** 90-102.

Yang R, Gombart AF, Serrano M, Koeffler HP (1995). Mutational effects on the p16INK4a tumor suppressor protein. *Cancer research* **55:** 2503-6.

CHAPTER 4

ROLE OF MELANIN IN MELANOCYTE DYSREGULATION OF REACTIVE OXYGEN SPECIES

Introduction

Inactivation or loss of $p16^{INK4A}$ is a common event in many tumors types (Sharpless and DePinho, 1999), although germ-line mutations in *p16*^{INK4A} are disproportionately associated with melanoma predisposition (Goldstein et al. 2006). The $p16^{INK4A}$ protein inhibits the kinase activity of cyclin-dependent kinases 4 and 6, preventing the hyperphosphorylation of retinoblastoma-related pocket proteins that are required to release E2F transcription factors necessary for cell cycle progression (Lukas et al. 1995). Thus the canonical tumor suppressor function of $p16^{INK4A}$ is to prevent division of stressed or damaged cells by holding them in the late G1–S transition to allow adequate time for DNA repair, or by promoting their irreversible exit from the cell cycle into a senescent state (Alcorta et al. 1996). We recently reported a potential novel tumor suppressor function for $p16^{INK4A}$ relating to its capacity to regulate oxidative stress. Depletion of $p16^{INK4A}$ by RNAi in human cells led to increased levels of intracellular reactive oxygen species (ROS) and the elevated levels of oxidative DNA lesion 8-oxoguanine. These effects on ROS and 8-OG were independent of the cycle cycle effects of $p16^{INK4A}$ (Jenkins et al. 2011). We observed that oxidative dysregulation in $p16^{INK4A}$ -depleted cells was most profound in melanocytes, compared to keratinocytes or fibroblasts. Moreover, in the absence of $p16^{INK4A}$ depletion or exogenous oxidative insult, melanocytes exhibited significantly higher basal levels of ROS than these other epidermal cell types. Given the role of oxidative stress in melanoma development (Meyskens et al. 2001; Cotter et al. 2007; Joosse et al. 2010) we speculated that this increased susceptibility of melanocytes to oxidative stress (and greater reliance on $p16^{INK4A}$ for suppression of ROS) may explain why genetic compromise of $p16^{INK4A}$ is

more commonly associated with predisposition to melanoma rather than other cancers. It is not known why melanocytes maintain higher levels of ROS than other cell types, but we hypothesized a role for melanin since its presence is a distinguishing feature of melanocytes and melanin synthesis is known to generate ROS (Urabe et al. 1994).

Results

A previous study found a correlation between levels of melanin and ROS, showing that both were elevated in melanocytes from dysplastic nevi compared to those from normal skin of the same individual (Pavel et al. 2004). Melanogenesis is pro-oxidative, commencing with the oxidation of L-tyrosine to dopaquinone (Figure 4.1), an enzymatic process that can be inhibited by N-phenylthiourea (PTU) (Ito and Watamatsu, 2008). To evaluate the role of melanin in melanocyte oxidative dysregulation, we derived melanocytes and fibroblasts from three separate individuals and cells were cultured in the absence or presence of PTU for 14 days. This was sufficient to deplete most of the melanin in melanocytes (Figure 4.2a, left). Intracellular ROS levels were then quantitated by fluorometric analysis following treatment with the cell-permeable fluorophore DCFDA (Jenkins et al. 2011). As previously reported (Jenkins et al. 2011), melanocytes exhibited significantly higher ROS levels compared to donor-matched fibroblasts (Figure 4.2a, right). By contrast, treatment with PTU resulted in a reduction of basal intracellular ROS levels in melanocytes comparable to that of fibroblasts (Figure 4.2a, right). PTU-treated fibroblasts, on the other hand, showed no significant difference in intracellular ROS from their untreated counterparts.

Next we evaluated the pro-oxidative role of melanin in the context of p16 depletion. Donor-matched fibroblasts and melanocytes were transfected with either

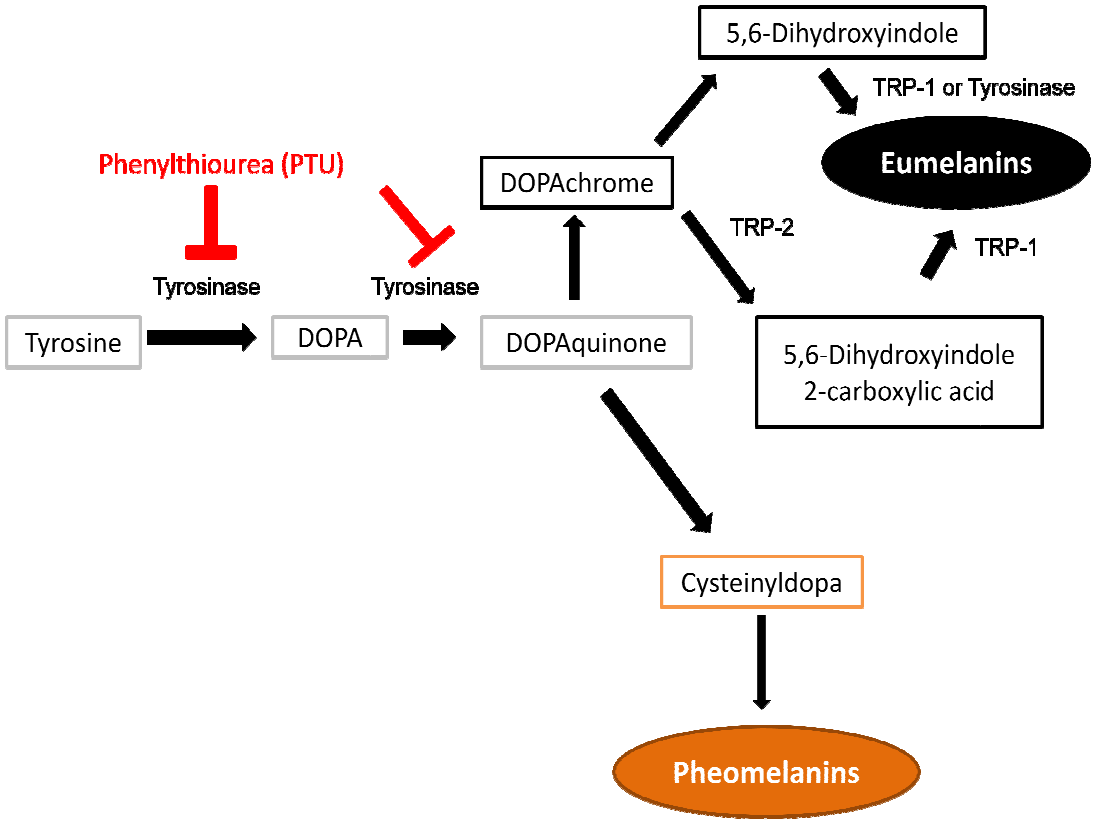
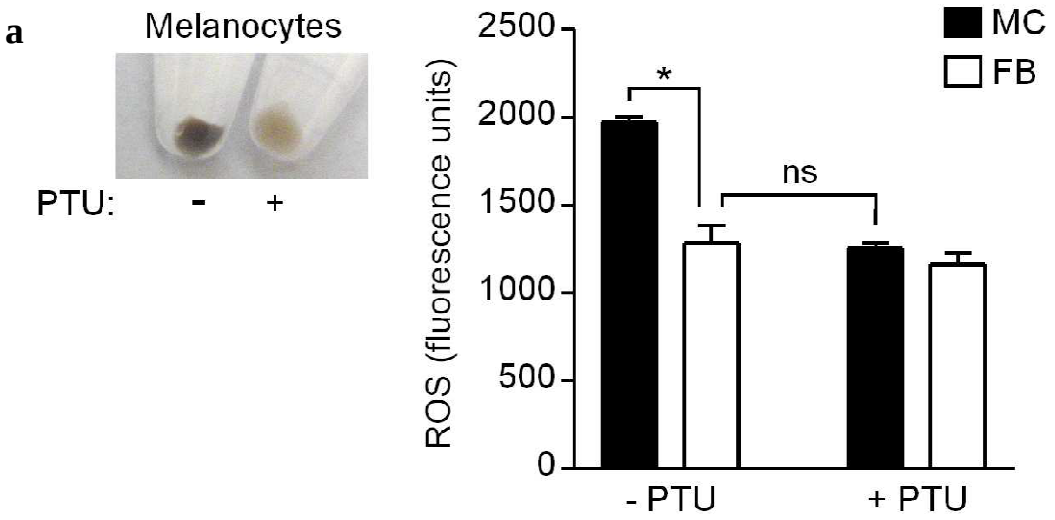


Figure 4.1 Intracellular production of melanins. The first two steps of both the reddish-brown pheomelanin and the dark brown/black eumelanin involves the hydroxylation of tyrosine to DOPA and the oxidation from DOPA to Dopaquinone. These reactions are both catalysed by tyrosinase, and enzyme that can be inhibited by phenylthiourea (PTU).

Figure 4.2 Inhibition of melanin synthesis reduces intracellular ROS in melanocytes. (**a**) Human melanocytes were either untreated (-) or treated (+) with 200 μM PTU (Sigma) for 14 days (left panel). Endogenous ROS were detected by addition of 20 μM DCFDA (Invitrogen) and measured as previously described (Jenkins et al., 2011). Error bars represent S.E.M. of triplicate determinations, and results are representative of two experiments performed. *P=.003 (two-sided t test). ns, not significant. (**b**) PTU treatment of melanocytes transfected with either a control scrambled (Scr) siRNA sequence, or siRNA specific for $p16^{INK4A}$, decreases melanin content (upper panel). Error bars represent S.E.M. of ROS determinations made from three separate donors (middle panel). *P= .04, **P=.03 (paired two-sided t test). ns, not significant. Representative Western blot showing $p16^{INK4A}$ levels in siRNAi-transfected cells (lower panel).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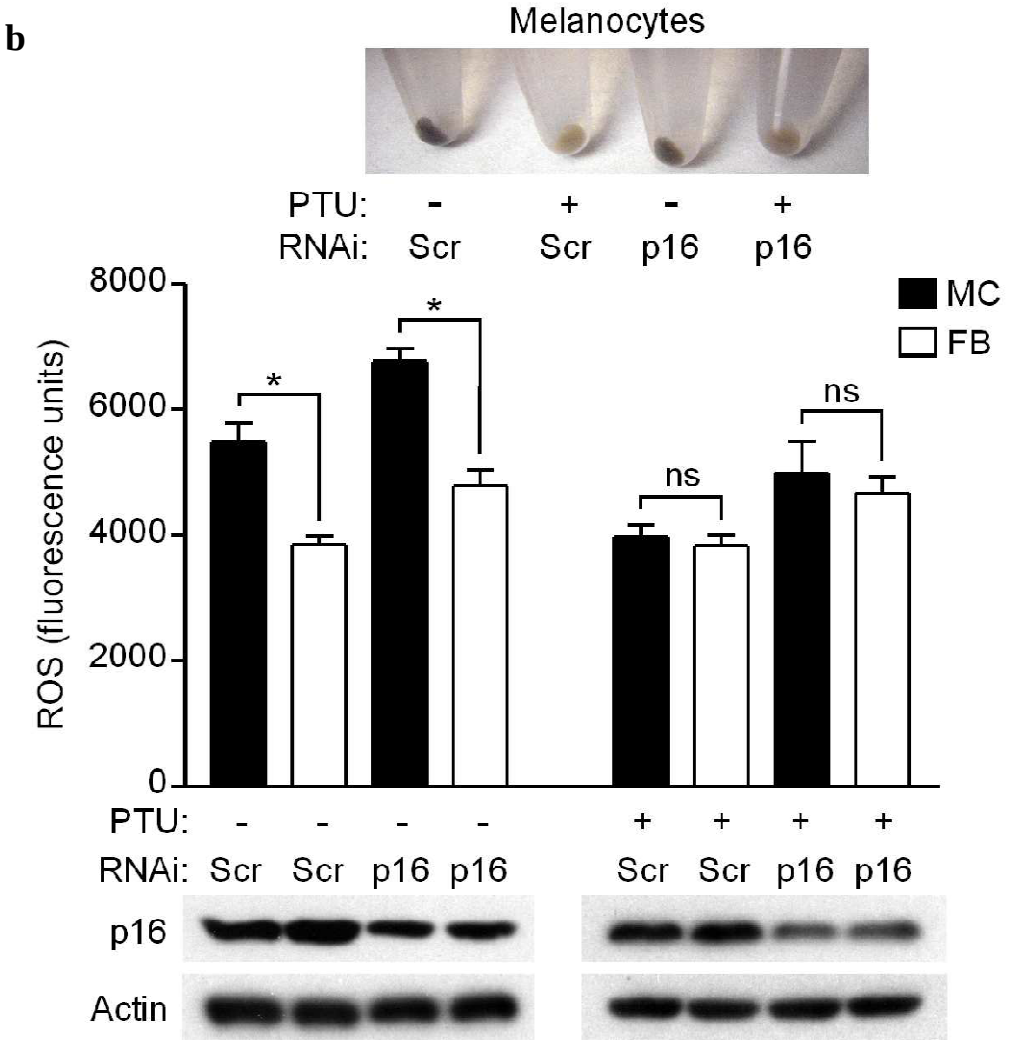
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control or siRNA specific for $p16^{INK4A}$ (Jenkins et al. 2011) to deplete endogenous $p16^{INK4A}$ protein (Figure 4.1b, lower panel). Depletion of $p16^{INK4A}$ in both cell types led to increases in intracellular ROS, with ROS levels consistently higher in melanocytes compared to fibroblasts under both controlconditions and following $p16^{INK4A}$ knockdown (Figure 4.2b, middle). Removal of melanin by PTU (Figure 4.2b, upper) was associated with reduction of ROS in melanocytes to levels comparable to fibroblasts, even under conditions of $p16^{INK4A}$ depletion (Figure 4.2b). These results implicate melanin as the cause of increased oxidative stress in normal and $p16^{INK4A}$ -depleted melanocytes.

Conclusions and Future Directions

It is established that chronic oxidative stress, and resulting oxidative damage, promotes carcinogenesis. Melanocytes are more susceptible to oxidative damage due to maintenance of higher levels of ROS (Jenkins et al. 2011). Loss of $p16^{INK4A}$ function through methylation-mediated gene silencing, mutation, or gene deletion as is commonly found in melanoma (Sharpless and DePinho, 1999), would be predicted to further increase ROS levels and correspondingly increase oxidative damage. Elevated levels of ROS in melanocytes are likely compounded by the relative deficiency of this cell type in the repair of oxidative DNA lesions (Wang et al. 2010). Both acute and chronic UV radiation induces ROS in the skin, and we have previously shown that administration of the antioxidant N-acetylcysteine prior to and following acute UV exposure delays melanoma onset in a mouse melanoma model (Cotter et al. 2007). In this same model system, loss of $p16^{INK4A}$ accelerates UV-induced melanoma development (Recio et al. 2002).

Although melanocytes may be protected by endogenous melanin which can directly absorb UV-generated photons and oxygen radicals (Riley 1997), at higher UV doses melanin can be oxidized leading to the generation of ROS (Wood et al. 2006). However, we have found in the absence of UV exposure that the pro-oxidative nature of melanin production is directly associated with higher melanocyte basal levels of intracellular ROS, which increase significantly following $p16^{INK4A}$ depletion. Thus the presence of melanin in the skin appears to be a double-edged sword: it protects melanocytes as well as neighboring keratinocytes in the skin through its capacity to absorb UV radiation, but its synthesis in melanocytes results in higher levels of intracellular ROS that may increase melanoma susceptibility. Several addition experiments can be performed to enhance our understanding of the exact role melanogenesis is playing in constitutively increasing intracellular ROS. For example, alternative methods can be used to inhibit both pheomelanin and eumelanin production, such as RNAi against tyrosinase. Additionally, one could use RNAi to inhibit TRP-1 and/or TRP-2, which are selective for the synthesis of eumelanin, in order to parse out any differential effects on intracellular ROS through the synthesis of eumelanin versus pheomelanin.

Materials and Methods

Treatment of cells with N-phenylthiourea

We derived melanocytes and fibroblasts from three separate individuals as previously described (Jenkins et al. 2011) and cells were cultured in the absence or presence of 200 μM N-phenylthiourea (PTU) (Sigma, St. Louis, MO.) for 14 days. Cells

were than harvested via trypsinization, pelleted at 1200 RPM for 5 minutes, washed with PBS and re-pelleted to be photographed.

References

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC (1996). Involvement of the cyclin-dependent kinase inhibitor p16(INK4a) in replicative senescence of normal human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **93:** 13742–13747.

Cotter MA, Thomas J, Cassidy P, Robinette K, Jenkins N, Florell SR *et al.* (2007). Nacetylcysteine protects melanocytes against oxidative stress/damage and delays onset of UVinduced melanoma in mice. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13:** 5952-5958.

Goldstein AM, Chan M, Harland M et al. (2006). High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer research.* **66:** 9818–9828.

Ito, S, and Wakamatsu, K (2008). Chemistry of mixed melanogenesis – pivotal roles of dopaquinone. *Photochem. Photobiol.* **84:** 582–592.

Jenkins, NC, Liu, T, Cassidy, P, Leachman, SA, Boucher, KM, Goodson, AG *et al.* (2011). The p16INK4A tumor suppressor regulates cellular oxidative stress. *Oncogene* **30:** 265-274.

Joosse A, De Vries E, van Eijck CH, Eggermont AM, Nijsten T, Coebergh JW (2010). Reactive oxygen species and melanoma: an explanation for gender differences in survival? *Pigment Cell Melanoma Res* **23:** 352-364.

Lukas J, Parry D, Aagaard L et al. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375:** 503–506.

Meyskens FL Jr., Farmer P, Fruehauf JP (2001). Redox regulation in human melanocytes and melanoma. *Pigment Cell Res* **14:** 148-154.

Pavel, S, van Nieuwpoort, F, van der Meulen, H, Out, C, Pizinger, K, Cetkovska, P *et al.* (2004). Disturbed melanin synthesis and chronic oxidative stress in dysplastic naevi. *Eur J Cancer* **40:** 1423–1430.

Recio JA, Noonan FP, Takayama H, Anver MR, Duray P, Rush WL *et al.* (2002). Ink4a/arf deficiency promotes ultraviolet radiation-induced melanomagenesis. *Cancer research* **62:** 6724-6730.

Riley, PA (1997). Melanin. Int J Biochem *Cell Biol* **29:** 1235-1239.

Sharpless NE, DePinho RA (1999). The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev* **9:** 22–30.

Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, Prota G, *et al.* (1994). The inherent cytotoxicity of melanin precursors: a revision. *Biochim Biophys Acta* **1221:** 272-278.

Wang HT, Choi B, Tang MS (2010). Melanocytes are deficient in repair of oxidative DNA damage and UV-induced photoproducts. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 12180-12185.

Wood SR, Berwick M, Ley RD, Walter RB, Setlow RB, Timmins G.S. (2006). UV causation of melanoma in Xiphophorus is dominated by melanin photosensitized oxidant production. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 4111-4115.

CHAPTER 5

SUMMARY AND PERSPECTIVES

Dissertation Summary

This dissertation overviews experimental designs and discoveries that were driven by the hypothesis that the familial-melanoma predisposition gene *p16*^{INK4A} functions to regulate intracellular oxidative stress independently of its canonical role as a regulator of the G1/S transition of the cell cycle. Previous work in our lab has demonstrated that reducing oxidative stress in a highly-penetrant melanoma mouse model delays the formation of melanoma (Cotter et al. 2007). Also, excessive UVR exposure, widely accepted to be the most important environmental contributing factor in melanoma, is thought to induce its melanomagenic effects through excessive ROS production (Herrling et al. 2006, Kripke 1994, Noonan et al. 2001, Meyskens et al. 2001). These previous observations led us to investigate whether this most important environmental factor was intimately associated with a very important genetic factor in melanomagensis; that is whether p16^{INK4A} was functioning to regulate aberrantly high levels of intracellular ROS. Using multiple in vitro and in vivo approaches, we observed that p16^{INK4A} depletion or deficiency leads to dysregualtion of intracellular oxidative stress, and an increase in potentially mutagenic oxidative DNA lesions (Jenkins et al. 2011). Due to the inherently pro-oxidative effect of melanin production that we observed, melanocytes seem especially sensitive to these aberrantly high levels of intracellular ROS and oxidative lesions. Taken together, these data implicate p16^{INK4A} as a possible bipartite tumor suppressor that is not only necessary to prevent the formation of potentially mutagenic lesions through this newly-discovered intracellular oxidative stress regulatory function, but also is necessary to act as a G1/S transition checkpoint of the cell cycle, allowing DNA repair mechanisms ample time to repair potentially oncogenic lesions, preventing

propagation of these errors into future cellular generations (Jenkins et al. 2011). This may also give rationale as to why melanocytes seem especially sensitive to oncogenic transformation compared to other cell types in the context of $p16^{INK4A}$ compromise. Perhaps there is a threshold of intracellular oxidative dysregulation necessary to promote oncogenesis, and the constitutively higher levels of ROS observed in melanocytes due to their unique burden of producing melanin lend them to being more easily pushed above that threshold when the antioxidant effect of $p16^{INK4A}$ is negated.

Many mutations in the $p16^{INK4A}$ coding sequence can potentially alter the sequence of both $p16^{INK4A}$ and *ARF*, yet our analysis shows that murine fibroblasts nullizygous for both these genes were able to have regulation of intracellular ROS restored upon re-expression of just $p16^{INK4A}$, signifying both its necessity and sufficiency to regulate this novel pathway. This observation seems to be recapitulated in patient samples with regard to $p16^{INK4A}$ being more important for tumor development than ARF, as specific somatic loss of $p16^{INK4A}$ has been reported in thousands of human cancers (Forbes et al. 2006). Also, unrelated kindreds that are predisposed to various forms of cancer have presented at least 56 unique germline mutations in $p16^{INK4A}$ that do not affect *ARF* (Greenblatt et al. 2003).

A high percentage of mutations associated with familial melanoma span the coding sequences that do not seem to cluster in discernible “hot spots” (Greenblatt et al. 2003). These observations combined with our novel discovery of an alternative function of this protein lead us to hypothesize that several of these mutations may differentially affect the two functions of cell-cycle and oxidative stress regulation, and in some cases these two functions may be completely uncoupled, showing an ability to either

completely restore ROS regulation or cell cycle arrest while showing a complete dysregulation of the other function. To this end, a panel of familial melanoma-associated point mutants were constructed via overlap mutagenic PCR and these constructs were expressed at consistent levels in murine fibroblasts nullizygous for *p16*^{INK4A}. Interestingly, we were able to segregate the phenotypes exhibited by these mutant constructs of *p16*^{INK4A} into four functional groups using a cutoff value of 30% of wild-type activity as qualifying as competent to restore function. We classified mutants as unable to restore either oxidative or cell cycle regulation (R87W, L97R, P81T), mutants that selectively restored oxidative regulation (R99P, V126D, R24Q), mutants that selectively restored cell cycle regulation (A36P, A57V, P114S), and mutants that restored both oxidative and cell cycle function (R24P, G35A, G35V). The cell cycle regulatory ability observed for these mutants in our system largely agreed with previous studies in the literature that involved slightly different experimental systems (McKenzie et al. 2010, Jones et al. 2007, Kannengiesser et al. 2009, Becker et al. 2001, Miller et al. 2011, Spica et al. 2006, Debniak et al. 2005, Goldstein et al. 2008). However, our system was optimized with several advantages over what has been performed previously when functionally characterizing these mutants. Whereas several previous studies utilized various cancer cell lines that were null for the entire *CDKN2A* locus (McKenzie et al. 2010, (Jones et al. 2007, Miller et al. 2011) we performed the exogenous expression of our p16^{INK4A} constructs in a primary fibroblast line selectively null for p16^{INK4A}, thereby limiting the potential for confounding effects elicited by oncogenic background mutations in the tumor lines. Careful titration work was also performed to ensure the level of p16^{INK4A} expression introduced through our lentiviral delivery system was near

physiological levels natively seen in wild-type cells as well as nearly identical between mutants. This level of consistency of expression was rarely observed in other functional studies of candidate $p16^{INK4A}$ mutations, as the proteins of interest were often grossly overexpressed. The careful, consistent expression levels of these familial melanoma-associated $p16^{INK4A}$ mutants potentially allows us to get a more accurate representation of the events involved with initiation of melanomagenesis in the affected families.

Future Directions for Classifying $p16^{INK4A}$-mediatedIntracellular Oxidative Stress Regulation as a True Novel Tumor-Suppressor Function

It is widely accepted that excessive intracellular oxidative stress is oncogenic due in a large part to oxidation of DNA, which in turn can lead to genomic instability (Waris and Ahsan 2006). Our study demonstrates through several in vitro modalities that compromise of $p16^{INK4A}$ can lead to excessively high intracellular ROS and oxidative stress (Jenkins et al. 2011). However, we have yet to show that oxidative regulatory activity of $p16^{INK4A}$ is truly tumor-suppressive in vivo. Recently, new in vivo melanoma models have been developed which may allow for the rapid validation of regulation of oxidative stress as a tumor suppressor function. For example, VanBrocklin et al. (2010) recently developed a highly penetrant melanoma mouse model that utilizes a somatic gene delivery system that facilitates the rapid validation of genetic alterations that occur during disease development. These mice are engineered to express the tumor virus A (TVA) receptor that is under the control of the dopachrome tautomerase (DCT) promoter, which ensures that only melanocytes will express the receptors for members of the avian leukosis virus. The lab uses an avian leukosis virus family member, a modified Rous

Sarcoma virus (RSV), in which the region encoding src has been replaced by a gateway cassette that can be used to express one or more oncogenic genes of interest (known as an RCAS vector). The DCT-TVA mice are crossed to *p16*^{INK4A}*/Arf*$^{lox/lox}$ mice, so that the resulting DCT-TVA/ *p16*^{INK4A}*/Arf*$^{lox/lox}$ mice are primed to have RCAS introduce linked NRAS and Cre expression into in vivo melanocytes nullizygous for p16^{INK4A} to insure a basal melanoma rate of 60-70%. We potentially could use these mice as a positive control for melanoma development that is dependent on deficiency of p16^{INK4a}, the same way we used murine fibroblasts cultures as a positive control for cell cycle and oxidative stress dysregulation in our in vitro model. We could then treat a subset of these mice with a virus that expressed an RCAS consisting of NRAS linked with Cre as well as our individual familial melanoma-associated point mutations of interest. By expressing p16^{INK4a} mutants that were found to selectively dysregulate either oxidative stress or cell cycle, we could theoretically score the individual contribution that each dysregulated phenotype has in tumorigenesis. This system would also allow us to test the p16^{INK4a} constructs that did not dysregulate either oxidative stress of cell cycle to observe whether, and to what extent, they are tumorigenic in this mouse model.

In theory, it would also be of great clinical relevance to use this newly-discovered data to observe patient samples stemming from families that harbor the familial melanoma-associated point mutations in *p16*^{INK4A}. Cells derived from such tumors could be analyzed for intracellular ROS versus cell cycle dysregulation compared to donor-matched melanocytes; this could give more accurate insight into what role each of these putative suppressor functions contributes to melanomagenesis in human patients. Also, one could analyze patient records over time to score aggressiveness of tumor growth and

metastasis in patients that harbor different germline mutations in *p16*^{INK4A}, and then compare that score with oxidative versus cell cycle phenotype. However, this type of patient analysis is not currently feasible given the extremely low number of families that have been identified with these mutations of interest, and the low amount of tumor material therefore that could be analyzed from these given families would most likely not yield enough data points for a statistically significant stratification of oxidative and cell cycle deregulatory phenotypes. Our observations, however, can easily be extended to what is most commonly seen in melanoma. Most melanoma cell lines are null for *CDKN2A*, brought about mostly through promoter methylation mediated by BMI-1 and Polycomb Repressor Complex 1 and 2 (Molofsky et al. 2003, (Lessard and Sauvageau 2003, Park et al. 2003, Jacobs et al. 1999). Melanoma lines have been observed to produce higher levels of intracellular ROS than normal melanocytes (Pavel et al. 2004), and our data would suggest that perhaps this phenotype in caused at least in part through a lack of p16^{INK4A}-mediated oxidative stress regulation that is truly independent of the Rb pathway and cell-cycle regulation, and that reestablishment of p16^{INK4A} expression in these lines could at least partially normalized cell cycle and oxidative stress regulation (Jenkins et al. 2011).

Cellular senescence is a state of permanent growth arrest that serves as a barrier against tumorigenesis. Senescence is known to involve the upregulation of p16^{INK4A} and is induced through a number of mechanisms such as DNA damage, telomere attrition, oxidative stress, and aberrant signals from known oncogenes (such as oncogenic RAS) (Collado and Serrano 2006). In light of our observations, it would be interesting to test various familial melanoma-associated p16^{INK4A} point mutants to see whether, and to what

extent, they retain their ability to induce senescence under various conditions, such as prolonged time in cell culture or expression of oncogenic RAS signaling. These studies could shed light on whether the putative tumor suppressor mechanism of senescence induced by $p16^{INK4A}$ was being compromised, and if senescence regulation could be uncoupled from the other described functions of $p16^{INK4A}$, cell cycle and oxidative stress regulation.

Future Directions to Identify Novel Binding Partners and Mechanistic Pathways of $p16^{INK4A}$

The discovery of a novel, potentially tumor-suppressive function of $p16^{INK4A}$ that is independent of its canonical cell-cycle regulatory role is very exciting in the context of better elucidating the complex signaling occurring during melanomagenesis. This work underscores the importance of continued investigation into possible alternative signaling pathways for even the most well-defined tumor suppressors.

Recent studies have presented evidence of the existence of even more potentially tumor-suppressive functions of $p16^{INK4A}$, and presents possible pathways with which $p16^{INK4A}$ interacts to regulate intracellular oxidative stress. The expression of a chromatin remodeling factor, brahma-related gene 1 (BRG-1) is frequently lost in primary and metastatic melanomas (Becker et al. 2009). It has been observed that $p16^{INK4A}$ interacts with BRG-1 (Becker et al. 2009), but the functional consequences remain unclear. As chromatin remodelers have the capacity to globally affect transcription, perhaps $p16^{INK4A}$ affects the expression levels of many different proteins through this relatively understudied interaction. The RAS-JNK-Jun-AP-1 signaling pathway is important for many types of cellular transformation, and may therefore cause

increased oxidative stress when activated. It has been observed that $p16^{INK4A}$ can bind to JNK 3, and block UV-induced phosphorylation of c-Jun that occurs through the RAS-JNK-Jun-AP-1 signaling pathway (Choi et al. 2005). One could test whether this $p16^{INK4A}$-induced inhibition of this pathway serves to regulate oxidative stress, and inhibit melanonagenesis, especially since it is occurring in the context of UV-exposure.

In breast cancer cell lines, overexpression of $p16^{INK4A}$ decreases the expression of vascular endothelial growth factor (VEGF) (Zhang et al. 2010). VEGF is an important inducer of angiogenesis, and is essential for tumors to grow beyond a microscopic size. It was found that $p16^{INK4A}$ binds to hypoxia-inducible factor-1 α (HIF-1α), a critical subunit of the transcriptional activator of the *VEGF* gene (Zhang et al. 2010), thereby potentially altering the ability of HIF-1α to transactivate VEGF expression. Potentially $p16^{INK4A}$ could be regulating intracellular oxidative stress through inhibiting HIF-1α, since several transcriptional targets of HIF-1α have the potential to increase ROS levels. It would also benefit the field to analyze whether this interaction is unique to breast cancer, or if this $p16^{INK4A}$-HIF-1α pathway is present in melanoma.

In light of our novel observation that $p16^{INK4A}$ can regulate intracellular oxidative stress, in may be of great relevance to re-analyze the role of $p16^{INK4A}$ and its interactions in these other potentially oncogenic pathways. It would also be of great interest to analyze different familial melanoma-associated point mutations in $p16^{INK4A}$ to observe any effect the compromise of different residues has on the binding of different classes of proteins and functions in different pathways.

Perspectives

The discovery of alternative, potentially anti-oncogenic roles of established tumor-suppressor proteins is an exciting area of biology. Advances in the elucidation of potential secondary or tertiary roles for these well-studied proteins that are yet to be discovered could lead to a more comprehensive understanding of the signaling networks involved in oncogenesis. Understanding mechanisms and pathways at this level would allow for the design of more potent and specific inhibitors of dysregulated pathways, as well as decreasing the potential for off-target effects. Our observation that different cases of familial melanoma involving different mutations in the predisposition gene $p16^{INK4A}$ may potentially exhibit nuanced differences in the initiating events of their cancers may eventually lead to much more effective prophylactic prevention, diagnosis, and treatment for affected individuals based on their own specific genetic disposition.

The studies presented here also potentially have much larger implications that extend well beyond the role of $p16^{INK4A}$ and melanomagenesis. As mentioned previously, the ankyrin repeat protein family contains approximately 3,600 members identified by the nonredundant SMART protein database (Mosavi et al. 2004), and are involved in a diverse array of functions as transcriptional regulation, cytoskeleton organization, cell cycle progression, cell development, and differentiation (Sedgwick and Smerdon 1999, Michaely et al. 2002). Our observations demonstrate that the target selection by ankyrin repeat proteins in general may not be as strict as previously thought, and despite the wide array of biological functions known to be performed by these proteins, perhaps many more have alternative disease suppressing functions that remain to be elucidated.

References

Becker TM, Rizos H, Kefford RF, Mann GJ (2001). Functional impairment of melanoma-associated p16(INK4a) mutants in melanoma cells despite retention of cyclin-dependent kinase 4 binding. *Clinical cancer research : an official journal of the American Association for Cancer Research* **7:** 3282-3288.

Becker TM, Haferkamp S, Dijkstra MK, Scurr LL, Frausto M, Diefenbach E *et al* (2009). The chromatin remodelling factor BRG1 is a novel binding partner of the tumor suppressor p16INK4a. *Molecular cancer* **8:** 4.

Choi BY, Choi HS, Ko K, Cho YY, Zhu F, Kang BS *et al* (2005). The tumor suppressor p16(INK4a) prevents cell transformation through inhibition of c-Jun phosphorylation and AP-1 activity. *Nature structural & molecular biology* **12:** 699-707.

Collado M, Serrano M (2006). The power and the promise of oncogene-induced senescence markers. *Nature Rev Cancer* **6:** 472-476.

Cotter MA, Thomas J, Cassidy P, Robinette K, Jenkins N, Florell SR *et al* (2007). N-acetylcysteine protects melanocytes against oxidative stress/damage and delays onset of ultraviolet-induced melanoma in mice. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13:** 5952-5958.

Debniak T, Gorski B, Huzarski T, Byrski T, Cybulski C, Mackiewicz A *et al* (2005). A common variant of CDKN2A (p16) predisposes to breast cancer. *J Med Genetics* **42:** 763-765.

Forbes S, Clements J, Dawson E, Bamford S, Webb T, Dogan A *et al* (2006). Cosmic 2005. *Br J cancer* **94:** 318-322.

Goldstein AM, Stacey SN, Olafsson JH, Jonsson GF, Helgason A, Sulem P *et al* (2008). CDKN2A mutations and melanoma risk in the Icelandic population. *J Med Genetics* **45:** 284-289.

Greenblatt MS, Beaudet JG, Gump JR, Godin KS, Trombley L, Koh J *et al* (2003). Detailed computational study of p53 and p16: using evolutionary sequence analysis and disease-associated mutations to predict the functional consequences of allelic variants. *Oncogene* **22:** 1150-1163.

Herrling T, Jung K, Fuchs J (2006). Measurements of UV-generated free radicals/reactive oxygen species (ROS) in skin. *Spectrochimica acta Part A, Molecular and biomolecular spectroscopy* **63:** 840-845.

Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* **397:** 164-168.

Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG *et al* (2011). The p16(INK4A) tumor suppressor regulates cellular oxidative stress. *Oncogene* **30:** 265-274.

Jones R, Ruas M, Gregory F, Moulin S, Delia D, Manoukian S *et al* (2007). A CDKN2A mutation in familial melanoma that abrogates binding of p16INK4a to CDK4 but not CDK6. *Cancer research* **67:** 9134-9141.

Kannengiesser C, Brookes S, del Arroyo AG, Pham D, Bombled J, Barrois M *et al* (2009). Functional, structural, and genetic evaluation of 20 CDKN2A germ line mutations identified in melanoma-prone families or patients. *Hum Mutat* **30:** 564-574.

Kripke ML (1994). Ultraviolet radiation and immunology: something new under the sun--presidential address. *Cancer research* **54:** 6102-6105.

Lessard J, Sauvageau G (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423:** 255-260.

McKenzie HA, Fung C, Becker TM, Irvine M, Mann GJ, Kefford RF *et al* (2010). Predicting functional significance of cancer-associated p16(INK4a) mutations in CDKN2A. *Hum Mutat* **31:** 692-701.

Meyskens FL, Jr., Farmer P, Fruehauf JP (2001). Redox regulation in human melanocytes and melanoma. *Pigment Cell Res* **14:** 148-154.

Michaely P, Tomchick DR, Machius M, Anderson RG (2002). Crystal structure of a 12 ANK repeat stack from human ankyrinR. *The EMBO journal* **21:** 6387-6396.

Miller PJ, Duraisamy S, Newell JA, Chan PA, Tie MM, Rogers AE *et al* (2011). Classifying variants of CDKN2A using computational and laboratory studies. *Hum Mutat* **32:** 900-911.

Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* **425:** 962-967.

Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY (2004). The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* **13:** 1435-1448.

Noonan FP, Recio JA, Takayama H, Duray P, Anver MR, Rush WL *et al* (2001). Neonatal sunburn and melanoma in mice. *Nature* **413:** 271-272.

Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL *et al* (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423:** 302-305.

Pavel S, van Nieuwpoort F, van der Meulen H, Out C, Pizinger K, Cetkovska P *et al* (2004). Disturbed melanin synthesis and chronic oxidative stress in dysplastic naevi. *Eur J Cancer* **40:** 1423-1430.

Sedgwick SG, Smerdon SJ (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* **24:** 311-316.

Spica T, Portela M, Gerard B, Formicone F, Descamps V, Crickx B *et al* (2006). The A148T variant of the CDKN2A gene is not associated with melanoma risk in the French and Italian populations. *J Invest Dermatol* **126:** 1657-1660.

VanBrocklin MW, Robinson JP, Lastwika KJ, Khoury JD, Holmen SL (2010). Targeted delivery of NRASQ61R and Cre-recombinase to post-natal melanocytes induces melanoma in Ink4a/Arflox/lox mice. *Pigment Cell Melanoma Res* **23:** 531-541.

Waris G, Ahsan H (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog* **5:** 14-21.

Zhang J, Lu A, Li L, Yue J, Lu Y (2010). p16 Modulates VEGF expression via its interaction with HIF-1alpha in breast cancer cells. *Cancer investigation* **28:** 588-597.