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Mechanisms of Hyperoxia-Induced Senescence in Primary Human Lung Fibroblasts

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ABSTRACT

Mechanisms of Hyperoxia-Induced Senescence in Primary Human Lung Fibroblasts

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Senescence, permanent, irreversible replication arrest that occurs in all primary cells studied to date, is considered a cellular model of aging. Recently, senescence has gained attention as a potential tumor-suppressing mechanism. However, despite the obvious importance of senescence, its exact mechanisms remain unclear. One current hypothesis to explain senescence postulates that it is caused by oxidative damage from reactive oxygen species (ROS). ROS are generated normally by the cell's mitochondria, and increase as the cell ages, leading to senescence. Senescence-inducing stressors may elevate ROS to critical levels, also leading to senescence. In support of this theory, previous studies have shown that replicative lifespan was longer in fibroblasts cultured in low oxygen (hypoxia) and decreased under high oxygen concentration (hyperoxia). The canonical interpretation had been that cells generate fewer ROS under hypoxia, and more under hyperoxia, extending or shortening replicative lifespan, respectively. However, other studies have shown that hypoxia paradoxically elevates ROS levels. Our group further determined that, under hypoxia, increased ROS play a signaling role to delay senescence.

This thesis further tests the oxidative theory of aging by determining the mechanisms of hyperoxia-induced senescence. The results demonstrate that exposure to 70% O_2 leads to stress-induced senescence. Hyperoxia exposure also elevates mitochondrial, but not cytosolic, ROS levels. Importantly, however, overexpression of antioxidant proteins was not sufficient to prevent hyperoxia-induced senescence. Stabilizing mitochondrial iron-sulfur cluster proteins by

frataxin overexpression also failed to prevent hyperoxia-induced senescence. Hyperoxia depletes cells of ATP and, therefore, upregulates AMPK, which can lead to senescence. However, overexpression of a kinase-dead mutant of LKB1, which prevented AMPK activation, did not prevent hyperoxia-induced senescence. Knocking down p21 via shRNA, or suppression of the p16/pRb pathway by BMI1 or HPV16-E7 overexpression, was also insufficient to prevent hyperoxia-induced senescence. However, suppressing p53 function resulted in partial rescue from senescence, suggesting that hyperoxia-induced senescence involves p53 but not p21. Suppressing both the p53 and pRb pathways resulted in almost complete protection, indicating that both pathways cooperate in hyperoxia-induced senescence. Collectively, these results indicate that hyperoxia induces senescence through a ROS-independent mechanism that involves activity of both the p53 and pRb pathways.

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Chapter 1: Introduction

Cellular senescence, or permanent, irreversible replication arrest, was first characterized in 1961, and occurs in all primary cells studied to date (Hayflick and Moorhead 1961). Senescence can occur after a certain number of cell divisions (replicative senescence), or at any point in the replicative lifespan if the cell encounters sub-lethal levels of certain stresses, such as elevation of reactive oxygen species (ROS), oncogene overexpression, or irradiation. Because of the timing mechanism inherent in replicative senescence, it is thought to be a model for cellular aging. Also, since senescence would limit proliferation of cells potentially transformed through numerous replication events or exposure to mutagenic stress, it may be a tumor suppressing mechanism.

However, despite the interest in senescence, our understanding of its underlying mechanisms is still incomplete. It is widely accepted that human cells undergo replicative senescence when telomeres, specialized structures that protect the ends of linear chromosomes, shorten during cell division, finally acting like a double-stranded (DS) DNA break to induce sustained proliferative arrest (reviewed in (de Lange 2002)). DNA damage response protein p53 is often involved in this DS break response, usually acting through its direct downstream target p21 to induce senescence (Di Leonardo, Linke et al. 1994; Beausejour, Krtolica et al. 2003). Likewise, p16, a cell cycle checkpoint protein, is often implicated through signaling to retinoblastoma protein (pRb) (Alcorta, Xiong et al. 1996; Hara, Smith et al. 1996; Stein, Drullinger et al. 1999). Telomere shortening is not usually involved in stress-induced senescence, but the p53 and/or pRb pathway play a role in all senescence pathways studied to date. However, the exact upstream events that trigger their activation remain unclear, and likely vary in different cell types and the type of senescence-inducing stressor used.

Cellular Senescence

The phenomenon of replicative senescence was originally described by Hayflick and Moorhead in 1961 (Hayflick and Moorhead 1961), who noticed that primary human fibroblasts serially cultured *in vitro* underwent irreversible growth arrest after a certain finite number of cell divisions. The cells remained viable for many weeks, but failed to divide despite optimal proliferation conditions. Since the original study in 1961, such replicative senescence has been observed in many other primary cell types from humans, mice, and other organisms. Embryonic stem cells and transformed tumor cells, on the other hand, escape senescence, hinting at its role in tumor suppression.

Cells can also senesce prematurely at any point in their replicative lifespan in response to sublethal levels of stressful exogenous stimuli, like oxidative damage, γ-irradiation, and oncogene overexpression (discussed in more detail below). Termed stress-induced or premature senescence, this process, together with replicative senescence, constitutes the broader biological program termed cellular senescence (Figure 1.1). Regardless of the stimulus, senescent cells display a characteristic phenotype, which includes flattened, enlarged cytoplasm, altered gene expression, increased lysosomal β-galactosidase activity at pH 6 (Dimri, Lee et al. 1995), overexpression of p16 (Krishnamurthy, Torrice et al. 2004), and formation of senescence-associated DNA damage foci (Takai, Smogorzewska et al. 2003). While it is unclear why b-galactosidase activity is increased in senescent cells, β-galactosidase staining remains the most commonly used and accepted marker of senescence.

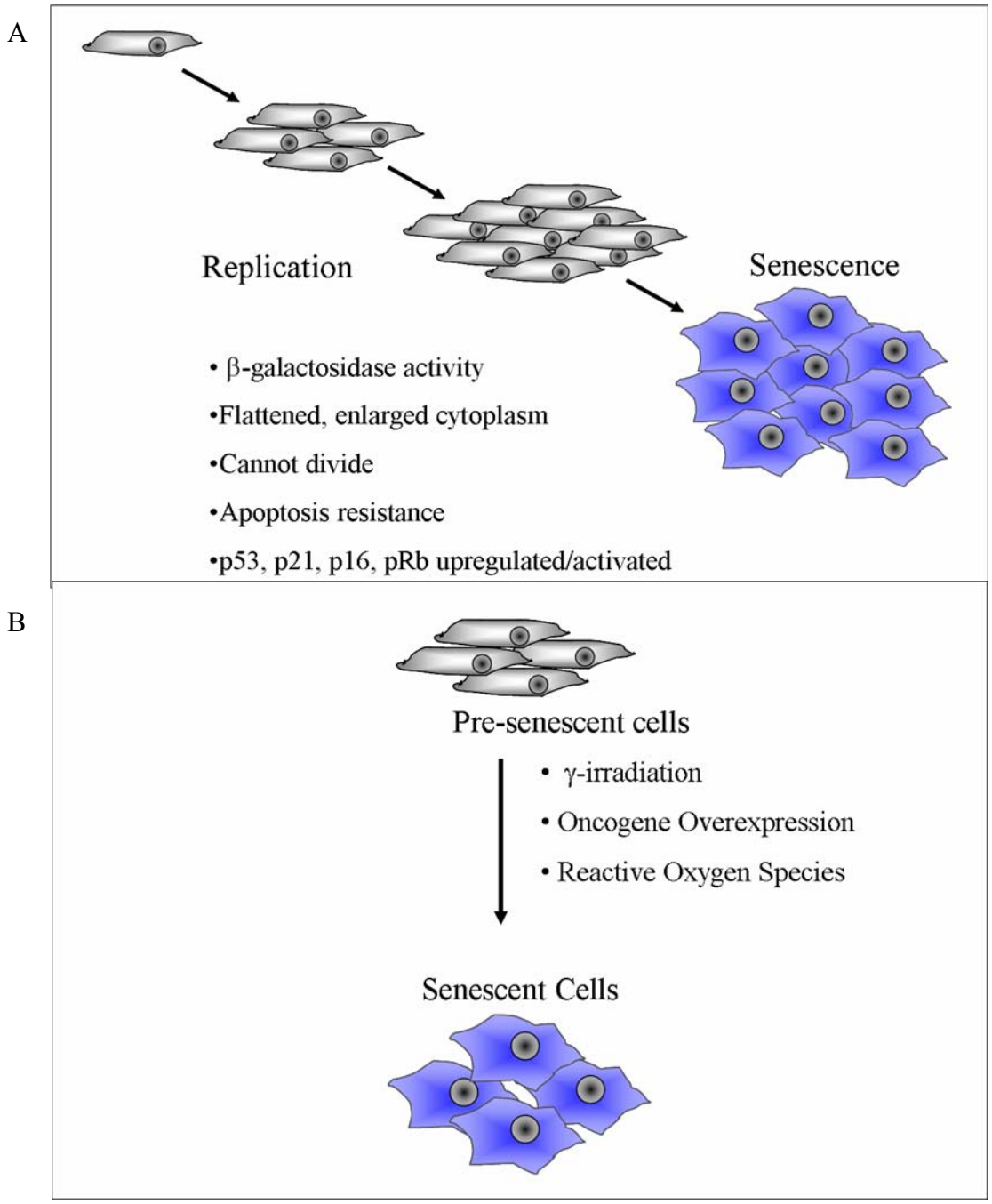

Figure 1.1. Replicative and stress-induced senescence.

A. Replicative senescence occurs after the cells have undergone a certain finite number of population doublings. At the end of replicative lifespan, cells display a characteristic phenotype, as described.

B. Premature or stress-induced senescence occurs at any point when a cell encounters sublethal levels of certain stresses, such as those listed.

Although cells are cell cycle-arrested and can no longer proliferate, they are metabolically active (Di Leonardo, Linke et al. 1994; Herbig, Jobling et al. 2004). Additionally, senescent cells are often resistant to many apoptotic stimuli (Chen, Liu et al. 2000; Hampel, Malisan et al. 2004). Since it can occur in response to diverse stimuli and has a distinct phenotype, senescence is now widely accepted as a programmed biological response.

Senescence as a Cellular Model of Aging

The "clock" inherent in replicative senescence, which arrests proliferation permanently after a certain number of population doublings, has led researchers to consider senescence as a cellular model of aging. Additionally, the cell cycle arrest observed in senescent cells may explain why aged tissue cannot regenerate after damage as easily as young, non-senescent tissues. However, cell culture conditions differ significantly from *in vivo* conditions, raising questions of whether senescence is a true biological phenomenon, or merely a product of in vitro culturing. Detection of senescent cells *in vivo* in aging human skin (Dimri, Lee et al. 1995) and liver (Paradis, Youssef et al. 2001), and at sites of age-related pathology, including atherosclerotic lesions, benign prostate hyperplasia, and pre-neoplastic lesions in liver (reviewed in (Itahana, Zou et al. 2003)) has helped to eliminate those doubts. In addition, replicative senescence has been experimentally demonstrated *in vivo*. In one study, mutant mice bearing partially eroded telomeres (discussed in more detail below) were subjected to partial hepatectomy. Rather than proliferating indefinitely, as wild-type hepatocytes, the mutant hepatocytes in these mice proliferated for a limited time before undergoing senescence (Satyanarayana, Wiemann et al. 2003). Thus, senescence appears to occur *in vivo* as well as *in*

vitro, provided that proper conditions are present. These data have lead to the widespread acceptance of senescence as an in vitro model and cellular basis of organismal aging.

Senescence as a Tumor-Suppressing Mechanism

While senescence may contribute to age-related pathologies and can thus be detrimental to an organism, it can also be beneficial by serving as a tumor suppressing mechanism. Many, if not all, of the stimuli that induce senescence are potentially transformative and oncogenic. Senescence provides a way to limit cellular proliferation, particularly when other growth-limiting mechanisms, such as apoptosis, fail. For instance, as mentioned previously, senescence can occur when apoptotic pathways are blocked (Braig, Lee et al. 2005). In addition, overexpression of potentially transformative oncogenic proteins, such as Ras, Raf, and Mek can lead to senescence, preventing oncogenic transformation by these factors. Oxidative stress and irradiation, two other stresses that can induce senescence, damage DNA, which can result in potentially tumorigenic mutations. It is therefore believed that senescence is a biological process that has evolved to protect organisms with renewable cells from tumorigenesis by preventing the proliferation of damaged and potentially tumorigenic cells (Figure 1.2).

Indeed, several *in vivo* studies have implicated senescence in tumor suppression. In mice expressing mutant Ras, pre-malignant tumors display high numbers of senescent cells and a low proliferative index, whereas malignant tumors do not, indicating that senescence acts to block full malignant transformation (Collado, Gil et al. 2005).

A B

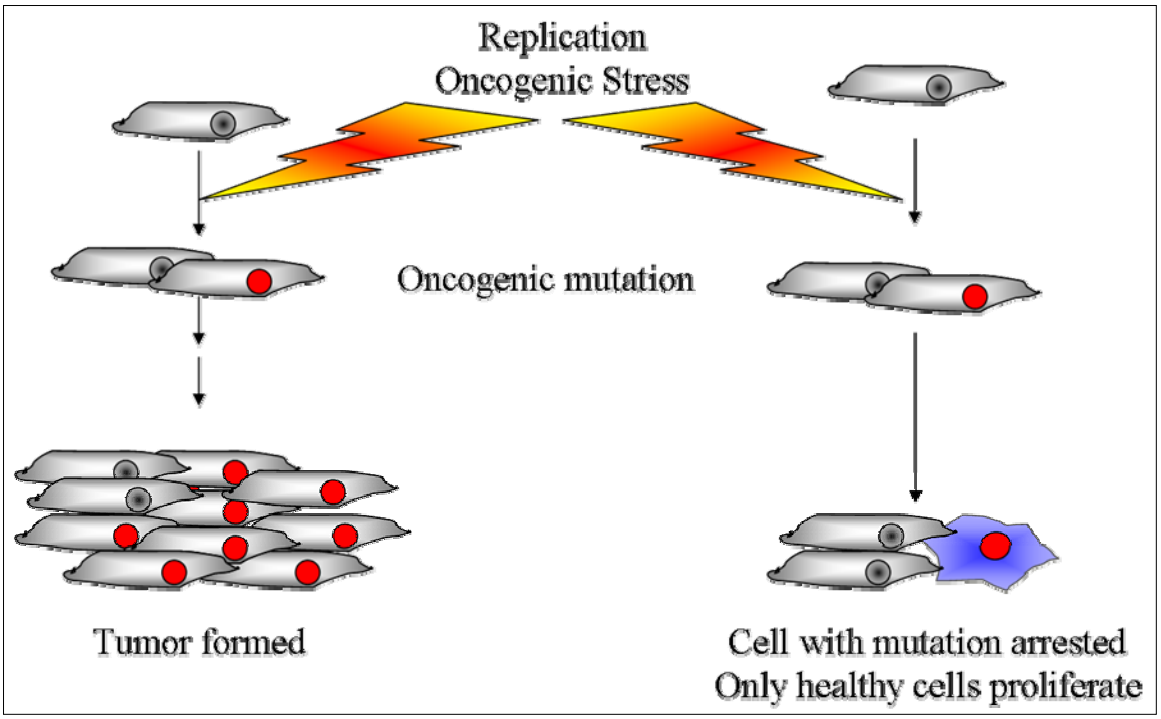

Figure 1.2. Senescence as a tumor-suppressing mechanism.

A. Oncogenic stresses may make a cell susceptible to oncogenic transformation. If allowed to proliferate, the cell may form a tumor.

B. Senescence prevents the further replication of a damaged cell, preventing tumorigenesis.

Furthermore, senescence opposes Ras-induced lymphoma (Braig, Lee et al. 2005) and mammary gland tumor (Sarkisian, Keister et al. 2007) formation in mice. Similarly, in humans, mutations in the Ras downstream effector BRAF induce nevi, benign tumors that can remain growth-arrested for decades and that display β-galactosidase activity and high levels of p16 (Michaloglou, Vredeveld et al. 2005). Loss of the tumor-suppressor PTEN in mouse prostate leads to p53-dependent senescence (discussed below) which must be overcome for full tumor progression (Chen, Trotman et al. 2005). In all of these systems, tumor progression occurred only after senescence machinery was overcome through, for instance, loss of p53 in addition to the original oncogenic stress (Chen, Trotman et al. 2005).

Recent studies further show that reactivating senescence pathways in already-malignant tumors can lead to tumor regression. Even brief reactivation of p53 in p53-deficient mouse liver carcinomas produces not only senescence, but also immune system clearing of the tumor, resulting in complete tumor regression (Xue, Zender et al. 2007). Similarly, reactivating p53 in mouse sarcoma cells leads to tumor regression through senescence (Ventura, Kirsch et al. 2007). Later studies suggest that oncogene overexpression may induce senescence by leading to DNA replication stress, which, in turn, activates p53 to result in senescence (Bartkova, Rezaei et al. 2006; Di Micco, Fumagalli et al. 2006).

The role of senescence in tumor suppression is not necessarily straightforward, however (reviewed in (Pazolli and Stewart 2008)). While senescence does prevent the further replication of a potentially mutated cell, senescent cells also release growth factors and metalloproteinases (Ray, Devaux et al. 2003; Campisi 2005). By releasing these factors, senescent fibroblasts can damage and/or induce dysplasia in neighboring epithelial cells, particularly if the epithelial cells are malignant or pre-malignant (Parrinello, Coppe et al. 2005), (Krtolica, Parrinello et al. 2001),

(Bavik, Coleman et al. 2006). Senescent fibroblasts have also recently been shown to secrete vascular endothelial growth factor (VEGF; (Coppe, Kauser et al. 2006)), a potent pro-angiogenic molecule, which may contribute to microvasculature remodeling and tumor growth. Furthermore, such stroma-tumor interaction may become a self-perpetuating cycle. For instance, in human ovarian cancer specimens, stromal fibroblasts adjacent to the epithelial cancer cells are often senescent; in turn, the cancer cells express GRO-1, which induces senescence in the stromal fibroblasts (Yang, Rosen et al. 2006). These data suggest that while senescence in the would-be tumor cell itself prevents tumor progression, senescence in stromal cells may actually lead to tumorigenic transformation. Thus, studying mechanisms of senescence in various cell types will contribute to our understanding of tumor progression.

Telomeres and Senescence

The natural timing mechanism behind replicative senescence is an intriguing question. In human cells, telomere shortening is thought to be the main biological "clock" that triggers replicative senescence. DNA replication is primed by a RNA primer, which is removed at the end of the replicative cycle, and the resulting gaps are filled in by polymerases that use the 5'-most fragment of nascent DNA as the new primer. In bacteria, which have circular chromosomes, all the gaps are filled. Eukaryotic cells, however, have linear chromosomes, and the end-most gaps cannot be filled due to the lack of a 5' primer. As the result, eukaryotic chromosomes shorten after each round of replication. The potential loss of critical DNA, fusion of chromosomal ends, and genomic instability that can result from this progressive shortening, are prevented by telomeres, specialized regions of repetitive nonsense DNA and protein that cap the ends of chromosomes (reviewed in (d'Adda di Fagagna, Teo et al. 2004)).

Telomeres are maintained by telomerase, an enzyme that uses an RNA template to resynthesize telomeric DNA lost after each round of replication (reviewed in (Collins and Mitchell 2002)). Human germline cells express constitutive levels of active telomerase, which maintains telomeres in these cells at approximately 15 kb. In contrast, most adult human somatic cells do not express telomerase, and telomeres are shorter, decreasing in length after every cycle of cell division, and finally inducing senescence (see Figure 1.3; e.g.(Harley, Futcher et al. 1990; Kim, Piatyszek et al. 1994; Effros, Dagarag et al. 2003; Masutomi, Yu et al. 2003)), seemingly through a classical DNA damage response (d'Adda di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003; Gire, Roux et al. 2004; Herbig, Jobling et al. 2004). To support the role of telomere shortening in senescence, studies have shown that driving telomerase expression is sufficient to halt senescence and increase lifespan in some human cells (Bodnar, Ouellette et al. 1998),(Vaziri and Benchimol 1998). Moreover, expression of hTERT, a catalytic component of telomerase, can lead to oncogenic transformation by leading to unchecked proliferation and immortalization. Notably, most human tumor cells, which are able to escape senescence, express active telomerase (Kim, Piatyszek et al. 1994). However, telomere shortening is not involved in most cases of stress-induced senescence (Chen, Prowse et al. 2001).

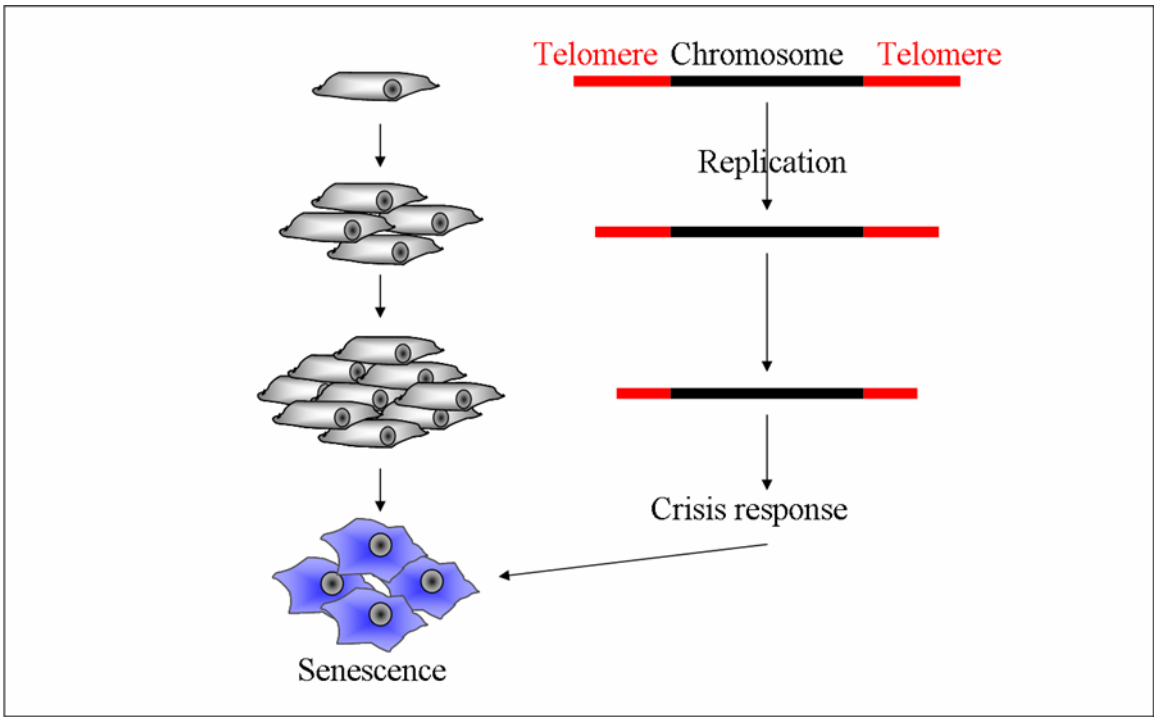

Figure 1.3. Telomere shortening leads to replicative senescence in human cells.

Telomeres, specialized structures that protect the ends of linear chromosomes, shorten with each round of DNA synthesis and cell division. Once they reach a certain critical length, a double-stranded DNA break response is activated, leading to replicative senescence.

Cell Cycle Checkpoint Factors and Senescence

To ensure that cell division does not occur unless conditions are favorable (e.g., DNA undamaged so that synthesis of a copy of the DNA can occur), numerous mechanisms exist to regulate cell cycle progress. During the G1 phase, or gap phase that precedes the DNA synthesis (S) phase, hypophosphorylated (active) retinoblastoma protein (pRb) sequesters and thereby inactivates E2F transcription factors, which regulate transcription of genes necessary for entry into the S phase. Once conditions are favorable for entry into the S phase, cyclin-dependent kinases (CDK) become activated by binding to cyclin proteins. CDKs 4 and 6 are activated by binding to cyclin D, and CDK2 is activated by cyclins E and A. The active cyclin-CDK complexes phosphorylate and inactivate pRb, causing it to release E2F, which is then available to bind DNA and initiate transcription of S phase genes. CDK inhibitors (CKIs) work to prevent pRb phosphorylation and inactivation if conditions for S phase entry are unfavorable. The CIP/KIP family of CKIs includes p21, p27, and p57, while the INK4 family consists of p15INK4b, p16INK4a, p18INK4c, and p19INK4d. For reviews, see (Sherr and Roberts 1999; Vidal and Koff 2000).

To date, nearly all senescent cells have been shown to have elevated levels of either p21 or p16 CKIs, or both. The cell-cycle checkpoint factor p21 is generally regulated by p53, which senses DNA damage and signals to arrest cell cycle (Di Leonardo, Linke et al. 1994), ((Beausejour, Krtolica et al. 2003). In fact, cells deficient in the activity of p53 and p21 are able to escape senescence. In addition, some senescent cells, including fibroblasts, display increased levels of the CDK4 inhibitor p16 that occurs after the increase in p21 (Hamilton, Guo et al. 2001). The p53 and p16 pathways are thought by some to constitute distinct senescence pathways (Beausejour, Krtolica et al. 2003), as loss or bypass of p16 pathway allows cells to

bypass senescence, in a manner independent of p53 (Beausejour, Krtolica et al. 2003). The retinoblastoma tumor suppressor protein, pRB, is also thought to play a role in senescence, and certain senescent cells display increased levels of hypophosphorylated (activated) pRb. Loss of pRb can also allow bypass of senescence (Park, Qian et al. 2003).

p53 is activated by DNA damage through p53 protein stabilization or modification, including phosphorylation (Xu 2003). It then signals to multiple pathways, including activating p21, which, in turn, inactivates CDK4 and 6 to induce cell cycle arrest. Indeed, disruption of p53 using various methods, including expression of simian virus large T antigen 40 (SV40) (Ide, Tsuji et al. 1983; Tsuji, Ide et al. 1983) or human papilloma virus protein E6 (Shay, Pereira-Smith et al. 1991); dominant negative p53 mutant (Bond, Wyllie et al. 1994); and p53 deletion (Bischoff, Yim et al. 1990) can delay or even reverse replicative senescence, demonstrating the importance of p53 in senescence. p53 is phosphorylated (activated) and its DNA binding activity is enhanced in aging fibroblasts, though its protein levels are unchanged (Rittling, Brooks et al. 1986; Atadja, Wong et al. 1995; Bond, Haughton et al. 1996; Vaziri, West et al. 1997; Webley, Bond et al. 2000; Itahana, Dimri et al. 2002; Herbig, Wei et al. 2003; Herbig, Jobling et al. 2004). Accordingly, p21 levels are elevated in senescent cells that have increased p53 phosphorylation, but not in cells lacking functional p53 (Noda, Ning et al. 1994; Tahara, Sato et al. 1995; Medcalf, Klein-Szanto et al. 1996; McConnell, Starborg et al. 1998; Dulic, Beney et al. 2000). These studies suggest that senescence involves p53 activation and its signaling through p21.

Stresses that result in double-stranded DNA breaks, like telomere attrition and exposure to radiation, generally induce senescence by activating p53. However, senescence can also proceed independently of p53/p21. The CKI p16 competes with D cyclins for binding to CDK4

and 6, resulting in cell cycle arrest, and it, too, can induce senescence. For example, overexpressing p16 in human fibroblasts induces senescence (Kato, Miyazawa et al. 1998; McConnell, Starborg et al. 1998). It can be activated after the p53 pathway is induced by stress (Stein, Drullinger et al. 1999; Jacobs and de Lange 2004), or induce senescence on its own. For example, RAS activation induces p16, leading to senescence (Ohtani, Zebedee et al. 2001). Additionally, certain cell types require p16 inactivation for immortalization and bypass of senescence (Kiyono, Foster et al. 1998; Dickson, Hahn et al. 2000). Furthermore, unlike p53-induced senescence, senescence induced by sustained p16 expression is not reversible even after p16 inactivation (Beausejour, Krtolica et al. 2003). Thus, the p53/p21 and the p16/pRb pathways are the most well-known regulators of senescence, and are activated separately or jointly in all instances of senescence studied to date (Figure 1.4).

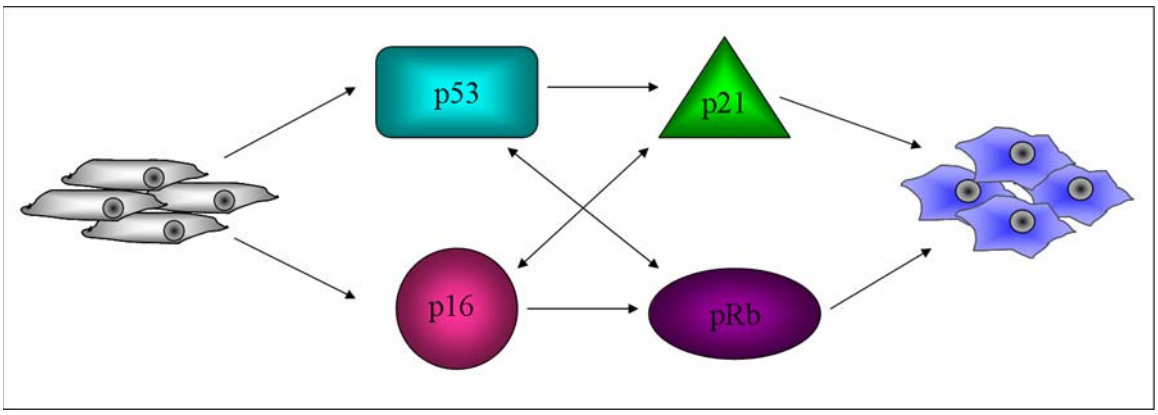

Figure 1.4 p32 and p16 regulate senescence.

Certain stresses, such as telomere shortening, activate p53, which signals to induce senescence by activating p21. In other situations, p16 maintains retinoblastoma protein pRb in its active form, leading to senescence. While the p53 and p16 pathways can induce senescence independently, crosstalk between the two pathways also often occurs.

Free Radical Theory of Aging

While telomere attrition is clearly a major trigger of replicative senescence in human cells, it does not explain stress-induced senescence, as it usually occurs without telomere involvement (Chen, Prowse et al. 2001; de Magalhaes, Chainiaux et al. 2004; Guney, Wu et al. 2006), though exceptions do exist (Duan, Duan et al. 2005). Thus, a different mechanism must be in place to induce senescence prematurely after stress exposure. The free radical theory of aging postulates that organisms age because of the deleterious effects of accumulating levels of reactive oxygen species (ROS) on cellular components (reviewed in (Beckman and Ames 1998)). Much evidence exists to suggest that ROS can cause cellular senescence, as most of the senescence-inducing exogenous stressors act through the elevation of intracellular ROS levels. For instance, as previously mentioned, addition of sublethal concentrations of H_2O_2 causes cells to rapidly senesce in a p21-dependent manner, displaying the characteristic growth arrest, flattened, enlarged cell morphology, and increased β-galactosidase activity (Chen and Ames 1994; Chen, Fischer et al. 1995; de Magalhaes, Chainiaux et al. 2002; Ray, Devaux et al. 2003; de Magalhaes, Chainiaux et al. 2004).

While exposure to exogenous ROS is rare *in vivo*, cells normally generate a low level of ROS, particularly through the electron transport chain (Figure 1.5). The complexes of the electron transport chain generate superoxide into the mitochondrial matrix, where it can damage mitochondrial proteins or DNA, or into the intermembrane space, from which ROS can pass into the cytosol and react with cellular components and nuclear DNA. The vast majority of ROS generated within the mitochondria is rapidly neutralized by ROS scavenger proteins, but a low percentage may escape to react with

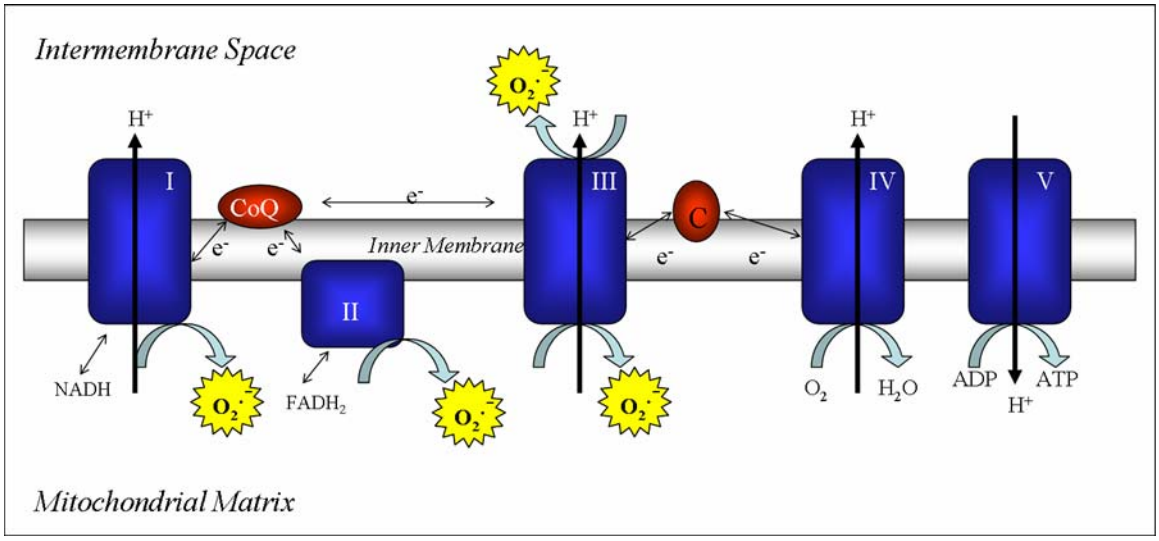

Figure 1.5. Mitochondrial electron transport chain generates ROS.

Through normal functioning, the mitochondrial electron transport chain generates low levels of ROS. Complexes I, II, and III all generate superoxide into the mitochondrial matrix, while complex III is also capable of releasing superoxide into the intermembrane space. Various senescence-inducing stressors increase mitochondrial ROS generation, possibly leading to senescence.

cellular components. Various stressors increase mitochondrial ROS generation. For instance, overexpression of oncogenic or mitogenic factors, like Ras, Raf, or Mek leads to rapid senescence (Saretzki, Feng et al. 1998; Collado, Gil et al. 2005; Wajapeyee, Serra et al. 2008). Oncogenic stress may actually cause senescence through oxidative damage, as Ras overexpression increases ROS production by the mitochondria, (Irani, Xia et al. 1997; Lee, Fenster et al. 1999; Deng, Liao et al. 2004), and neutralizing the ROS with hydrogen peroxide scavengers reversed the senescent phenotype (Lee, Fenster et al. 1999). Even overexpression of senescence regulating factors p21 and p53 elevates intracellular ROS levels, and senescence induced by the overexpression of these factors can be prevented or delayed by adding antioxidants (Macip, Igarashi et al. 2002; Macip, Igarashi et al. 2003).

It is clear, therefore, that oxidative stress is involved in stress-induced senescence. In fact, even replicative, telomere-driven senescence may occur as the result of ROS generation. Despite neutralization of the majority of ROS generated in the cell, a low percentage may react with telomeres, leading to ever-accumulating damage. Indeed, treating human fibroblasts with antioxidant α-phenyl-t-butyl-nitrone (PBN) reduces intracellular ROS levels and delays telomere shortening and onset of senescence (von Zglinicki, Pilger et al. 2000). Overexpression of superoxide dismutase or treatment with the mitochondrial antioxidant MitoQ also delays telomere shortening and extended replicative lifespan (Saretzki, Murphy et al. 2003; Serra, von Zglinicki et al. 2003). Thus, plentiful evidence exists for the role of ROS in replicative and stress-induced senescence and, assuming that senescence is the mechanism behind aging, to support the free radical theory of aging.

Senescence and Oxygen Levels

According to the free radical theory of aging, high oxygen levels (hyperoxia) should elevate intracellular ROS production and induce senescence. Indeed, many researchers have found elevated ROS production in cells exposed to hyperoxia, and human fibroblasts cultured under 40% O_2 senesce more quickly than their normoxic counterparts (von Zglinicki, Saretzki et al. 1995; Saretzki, Feng et al. 1998). In the first study, WI-38 fibroblasts were only able to undergo 1-3 population doublings before becoming senescent when cultured under 40% O_2 (von Zglinicki, Saretzki et al. 1995). Morphologically, these cells resembled fibroblasts that underwent replicative senescence under normoxia, and their telomeres rapidly shortened under hyperoxia. Skin fibroblasts also underwent rapid senescence under hyperoxia, becoming unable to proliferate after 4-6 weeks under 40% O_2 (Saretzki, Feng et al. 1998). Moreover, treatment with the mitochondrial antioxidant MitoQ was sufficient to reduce telomere shortening and prolong replicative lifespand under 40% O_2 in MRC-5 fibroblasts (Saretzki, Murphy et al. 2003). The results of these studies suggested that telomere-driven replicative senescence occurs because of oxidative damage that shortens telomeres and leads to senescence.

To further support the free radical theory of aging, it was shown that reducing oxygen levels (hypoxia) extends replicative lifespan (Packer and Fuehr 1977; Saito, Hammond et al. 1995). IMR-90 fibroblasts underwent delayed senescence when cultured under 1% O_2 compared to the same cells cultured under 20% O_2 (Saito, Hammond et al. 1995). Furthermore, fibroblasts from individuals with the premature aging disorder, Werner's disease, also underwent delayed senescence under hypoxia. The effect of culturing fibroblasts under hypoxia was cumulative over the lifespan of the cell, as placing aged fibroblasts from normoxia to hypoxia did not delay senescence. This suggested that some sort of cumulative damage occurs under 21% O_2 that is

reduced under 1% O_2. The canonical interpretation of the hypoxia and hyperoxia studies has been that, under hyperoxia, more ROS are produced, leading to more damage to the cell and accelerated senescence, while hypoxia produces fewer ROS, leading to reduced damage and delayed senescence.

Our group recently demonstrated that ROS levels increase, rather than decrease, in human lung fibroblasts cultured under hypoxia (Bell, Klimova et al. 2007b). These ROS are generated by the Q_o site of mitochondrial complex III into the mitochondrial intermembrane space and appear in the cytosol (Bell, Klimova et al. 2007a). Furthermore, we showed that this increase in ROS activates the transcription factor hypoxia inducible factor (HIF). Addition of the mitochondrial antioxidant MitoQ prevents ROS generation and HIF stabilization under hypoxia. HIF is a transcription factor involved in hypoxic signaling, regulating the expression of hundreds of genes that allow the cell to adapt to the hypoxic environment. One of the genes regulated by HIF is hTERT gene, a catalytic component of the enzyme telomerase. Under hypoxia, therefore, the resulting increase in telomerase activity extends replicative lifespan of fibroblasts, thus resolving the apparent discrepancy that increased levels of ROS can increase lifespan. This finding indicates that the connection between ROS levels and senescence is not as direct as it had previously been assumed, and that a closer exploration of the free radical theory is needed. To further examine the theory, this study focuses on the mechanisms of senescence under hyperoxia (defined here as 70% O_2).

Results:

Chapter 2: Hyperoxia induces premature senescence, but not death, in primary human lung fibroblasts.

While previous studies of the effects of hyperoxia on senescence have been conducted, they utilized moderate hyperoxia (40%O_2) and time periods of several weeks, observing an accelerated replicative senescence-like phenotype (Saretzki, Feng et al. 1998). To attempt to induce senescence more quickly, we cultured primary adult human lung fibroblasts (PHLFs) in either 21% O_2 (approximately physiological oxygen concentration for these cells) or 70% O_2 for 72 hrs. Unlike PHLFs grown in 21% O_2, those cultured in 70% O_2 displayed a marked growth arrest as indicated by reduced number of cells seen per field, and the enlarged, flattened phenotype associated with senescence (Figure 2.1A). Senescence was further confirmed by staining with X-gal (5-bromo-4-chloro-3-indolyl-b”-D-galactopyranoside) to test for β-galactosidase activity at pH 6, a well-established marker of senescence (Dimri, Lee et al. 1995). Approximately 10% of untreated cells stained blue, while 80% stained blue in the PHLF population exposed to 70% O_2 (Figure 2.1B). Moreover, the phenotype of PHLFs exposed to hyperoxia was similar to that of PHLFs treated with a sub-lethal concentration of H_2O_2 (100μM for 2hr), a stimulus widely used to induce senescence (Figure 2.1). Together, these data indicate that 70% O_2 induces premature senescence in PHLFs.

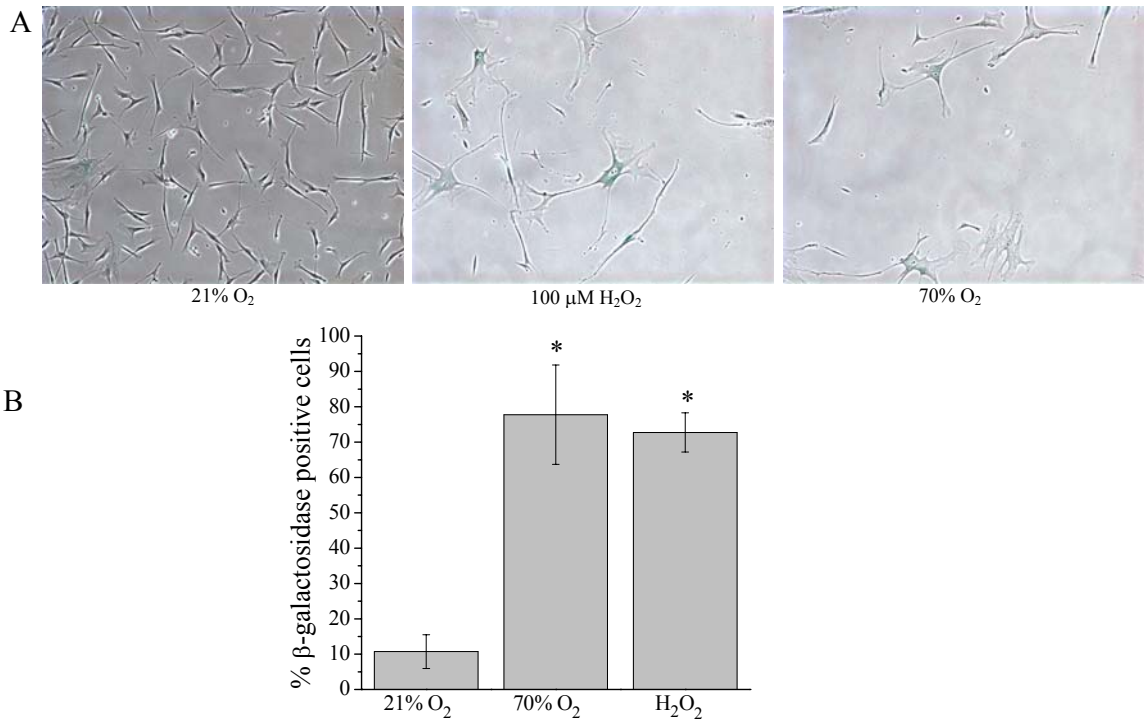

Figure 2.1. Hyperoxia induces premature senescence in primary human lung fibroblasts.

A. Representative phase microscopy images of PHLFs exposed to 21% or 70% O_2 for 72 hrs, or 100μM H_2O_2 for 2 hrs, and stained with X-gal to show senescence-associated β-galactosidase activity.

B. Percentage of β-galactosidase positive cells after exposure to 21% O_2, 70% O_2, or 100 μM H_2O_2. * indicates $P<0.05$ for H_2O_2- or 70% O_2-treated PHLFs compared to 21% O_2 controls (P=0.00010 and 0.00000, respectively). N=4.

As previously mentioned, hyperoxia-exposed PHLFs displayed a marked decrease in cell number compared to normoxic counterparts. Such a decrease could certainly be accounted for by senescence, since normoxic cells continue to proliferate while the cells that senesce due to stress exposure are unable to proliferate. However, high O_2 levels could also be lethal (Budinger, Tso et al. 2002). As senescent cells are more resistant to apoptotic stimuli, the high percentage of cells with senescent phenotype seen after hyperoxia exposure could therefore be due to the dying off of normal cells rather than an increase in senescence. To ensure that the comparative reduction in cell number after 70% O_2 exposure was not due to increased cell death, we measured cell death through lactate dehydrogenase (LDH) release. LDH is an intracellular enzyme released from dying cells, and serves as a global indicator of apoptotic and necrotic cell death. While 70% O_2 exposure doubled the percentage of LDH release compared to controls, cell death levels were still at around 10% of total cells, indicating that cell death was likely not the main factor in the reduction of cell number (Figure 2.2). 100μM H_2O_2 was also insufficient to increase cell death to more than 15% of total cells. By contrast, a 2hr treatment with an established lethal dose of H_2O_2 (1 mM) elevated LDH release to over 60% of total cells (Figure 2.2). We therefore conclude that 70% O_2 induces senescence, but is insufficient to cause cell death.

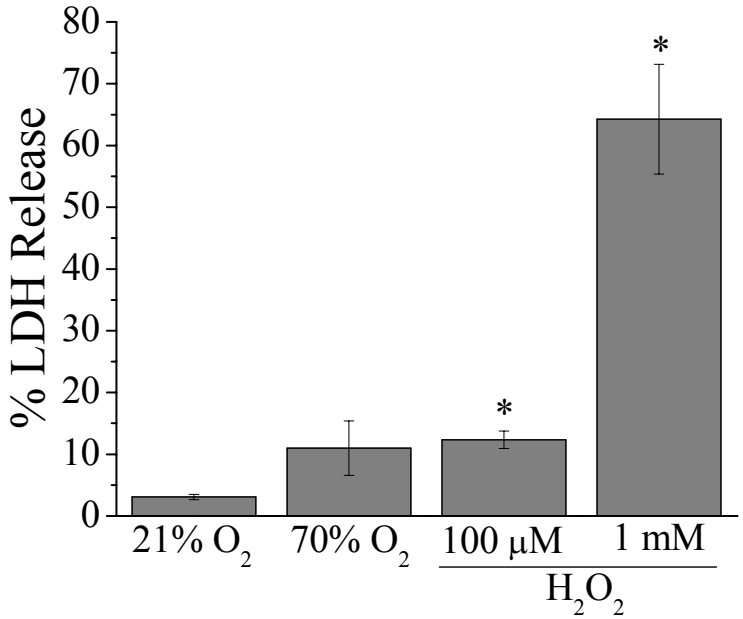

Figure 2.2. Hyperoxia does not induce death in PHLFs.

Cell death measured by percent lactate dehydrogenase released from PHLFs exposed to 21% O_2 or 70% O_2 for 72hr, or to 100μM H_2O_2 or 1mM H_2O_2 for 2hrs (LDH release measured 72 hrs after initial exposure). * indicates $P<0.05$ for sample compared to 21% O_2 control (P=0.00072 for 100μM H_2O_2; P=0.00047 for 1mM H_2O_2). N=4.

Chapter 3: Telomerase overexpression does not prevent hyperoxia induced senescence.

Previous studies of hyperoxia-induced senescence used relatively low oxygen levels (40% O_2) to induce replicative-like senescence prematurely (von Zglinicki, Saretzki et al. 1995; Saretzki, Feng et al. 1998). As previously discussed, replicative senescence and stress-induced senescence proceed through distinct mechanisms, with replicative senescence being driven by telomere shortening or attrition. Previous studies characterized hyperoxia-induced senescence as a form of replicative senescence, as it involved accelerated telomere shortening (von Zglinicki, Saretzki et al. 1995). Most stresses that induce rapid senescence, however, proceed independently of telomere length. To determine whether senescence induced by 70% O_2 is telomere dependent, we overexpressed the catalytic component of telomerase, hTERT, in PHLFs. hTERT overexpression is sufficient to extend replicative lifespan indefinitely in many cell types, including fibroblasts (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998). Indeed, hTERT overexpression allowed PHLFs to continue proliferating indefinitely under 21% O_2 (Figure 3.1), indicating that hTERT was functional. On the other hand, PHLFs expressing the empty retroviral vector ceased to proliferate after approximately 30 days post-infection. However, upon exposure to 70% O_2, hTERT-overexpressing PHLFs senesced in similar numbers compared to vector controls (Figure 3.2). This indicates that hyperoxia-induced senescence is telomere independent and likely proceeds through a stress-induced senescence mechanism.

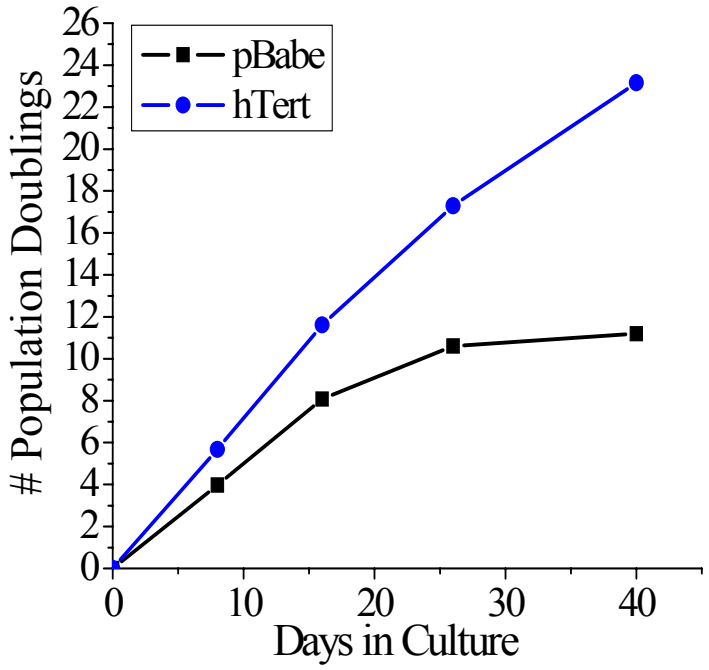

Figure3.1. hTERT overexpression prevents replicative senescence in PHLFs.

Population doublings in culture of PHLFs stably expressing empty vector (pBabe; squares) or pBabe-hTERT (hTERT; circles) after retroviral infection.

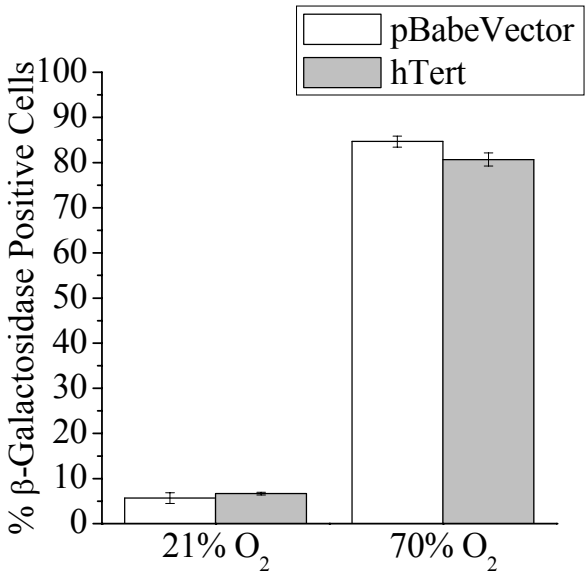

Figure 3.2. Hyperoxia-induced senescence is telomere-independent.

Percentage of β-galactosidase positive PHLFs expressing empty vector (pBabe; white) or hTERT (gray) after 72hr exposure to 21% O_2 or 70% O_2. N=3.

Chapter 4. Hyperoxia induces senescence independently of an increase in mitochondrial and cytosolic ROS generation.

To date, the vast majority of senescence-inducing stressors have been shown to act through increased ROS generation. To determine whether hyperoxia induced ROS production (as had previously been demonstrated in other systems, e.g. (Fridovich 1998)), we quantified intracellular ROS levels with redox-sensitive green fluorescent protein (roGFP; Figure 4.1). This probe consists of GFP containing two additional cysteine thiols (S147C and Q204C) located on the outer surface (Dooley, Dore et al. 2004; Hanson, Aggeler et al. 2004). The position of these residues allows for dithiol formation or breakage, depending on whether the environment of the cell is oxidizing or reducing. roGFP emission at 525 nm is assessed via flow cytometry at excitation wavelengths at 400 nm and 490 nm. When the probe is oxidized, the emission ratio (400nm/490nm) increases, and decreases when the probe is reduced (Dooley, Dore et al. 2004; Hanson, Aggeler et al. 2004). This ratiometric behavior allows the probe to be calibrated to the redox state and renders the redox signal independent of the protein expression level. In our study, we utilized untargeted roGFP (cytosolic), and roGFP targeted to the mitochondrial matrix (Figure 4.2). While untargeted roGFP is distributed throughout the cell, the mitochondrially targeted roGFP strongly colocalizes with the mitochondrial marker tetramethylrhodamine (TMRE) and is not seen in the cytosol.

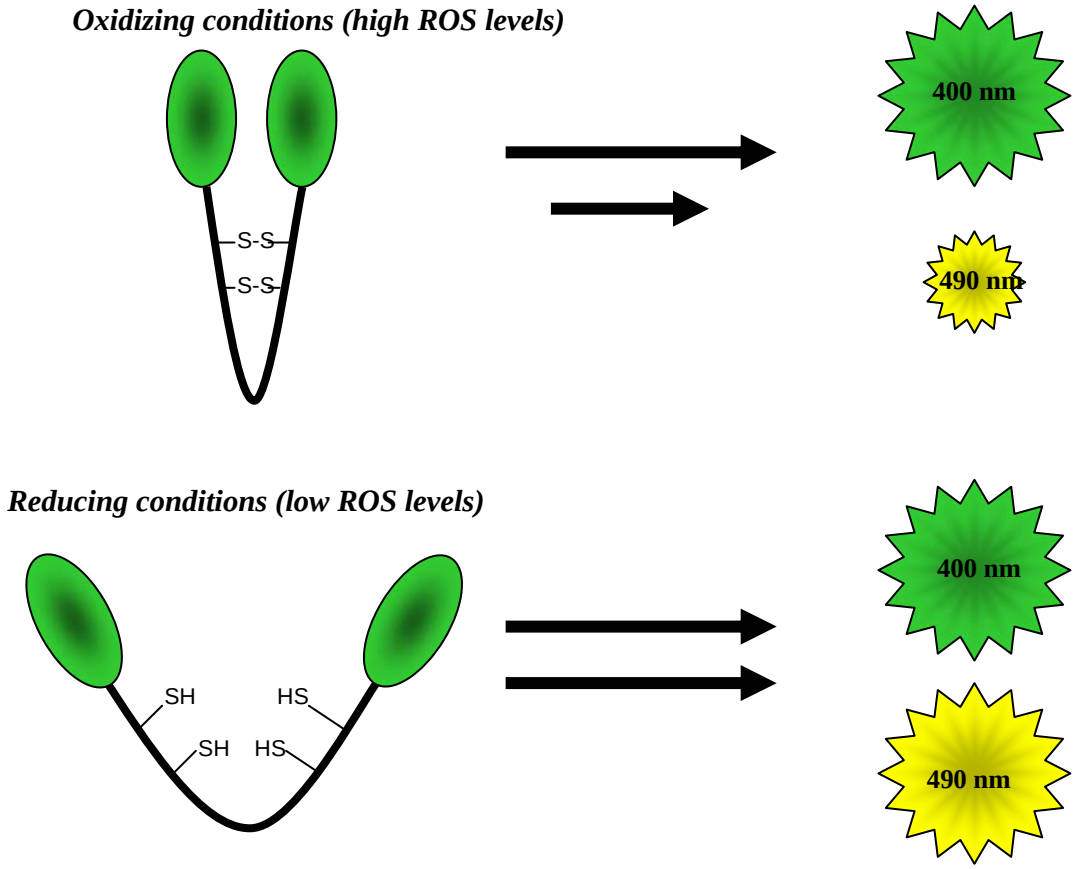

Figure 4.1. Redox-sensitive GFP probe responds to the redox state of the cell.

Cysteine residues allow for formation and dissolution of disulfide bonds, depending on the ROS levels in the cell. The conformational change affects the ratio of the excitation wavelengths of the probe, which can be monitored with flow cytometry.

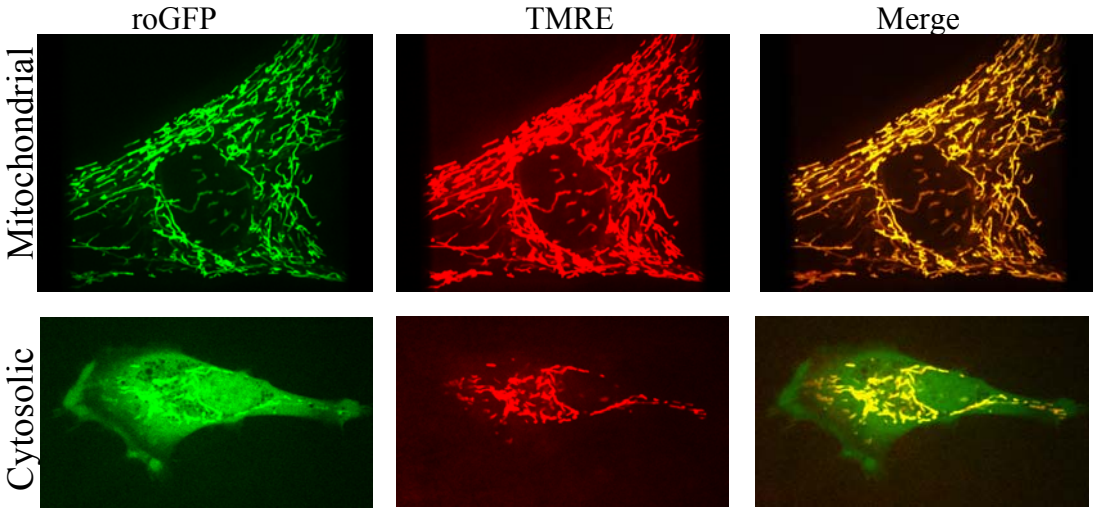

Figure 4.2. roGFP can be subcellularly targeted.

Fluorescent microscopy demonstrating subcellular localization of roGFP (green channel) delivered by adenovirus and targeted to the mitochondria or the cytosol. TMRE used to stain mitochondria is in red; colocalization indicated by yellow.

PHLFs infected with roGFP were placed in 21% O_2 or 70% O_2 for 18 hrs before determining probe oxidation cytometrically. roGFP was internally calibrated by treatment with 1mM H_2O_2 to full oxidize the probe and 10mM DTT to fully reduce it. Additionally, to ensure that the change in roGFP oxidation was in fact due to ROS, we overexpressed mitochondrial ROS scavenger enzymes MnSOD and catalase via adenoviral infection. Manganese superoxide dismutase is an enzyme native to the mitochondrial matrix; it converts the superoxide produced as a by-product by the electron transport to hydrogen peroxide. Catalase, which neutralizes hydrogen peroxide to water, is not native to the mitochondrial matrix, but was tagged with a mitochondrial localization sequence. Both enzymes were overexpressed efficiently in PHLFs (Figure4.3).

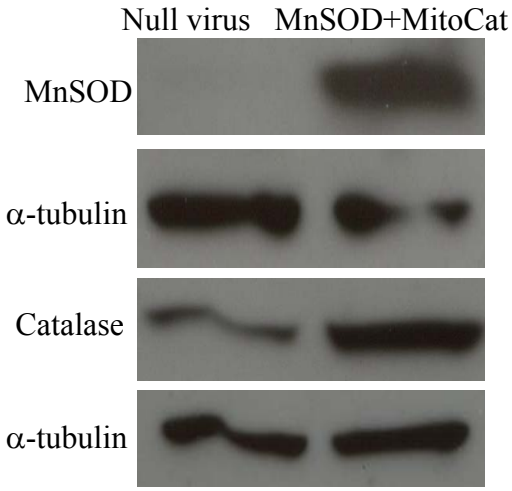

Figure 4.3. MnSOD and catalase protein overexpression using adenovirus.

MnSOD and catalase levels in whole-cell lysates of PHLFs infected with sham adenovirus or adenovirus expressing MnSOD and mitochondrial catalase.

Exposure to 70% O_2 for 18 hours increased mitochondrial matrix roGFP oxidation (Figures 4.4). On the other hand, the untargeted or cytosolic roGFP showed little to no increase in oxidation after hyperoxia exposure (Figure 4.5); the oxidation increase that was observed could be due to the partial localization of this probe to the mitochondrial matrix as well as the cytosol. This indicates that hyperoxia increases mitochondrial matrix ROS generation, but that these ROS are confined to that compartment, as no corresponding increase in ROS is seen in the cytosol. Overexpression of MnSOD and catalase lowered mitochondrial roGFP oxidation to normoxic levels (Figure 4.4) and also neutralized the small increase in oxidation of the cytosolic roGFP. This indicated that mitochondrial matrix antioxidants are capable of fully neutralizing ROS produced under hyperoxia. However, after hyperoxia exposure, PHLFs overexpressing MnSOD and mitochondria-targeted catalase displayed numbers of β-galactosidase-positive cells comparable to PHLFs infected with sham virus (Figure 4.6) despite efficient overexpression of these proteins (Figure 4.3) and their ability to effectively neutralize mitochondrial matrix ROS (Figure 4.4). Collectively, these results indicate that hyperoxia-induced senescence occurs independently of mitochondrial matrix ROS generation.

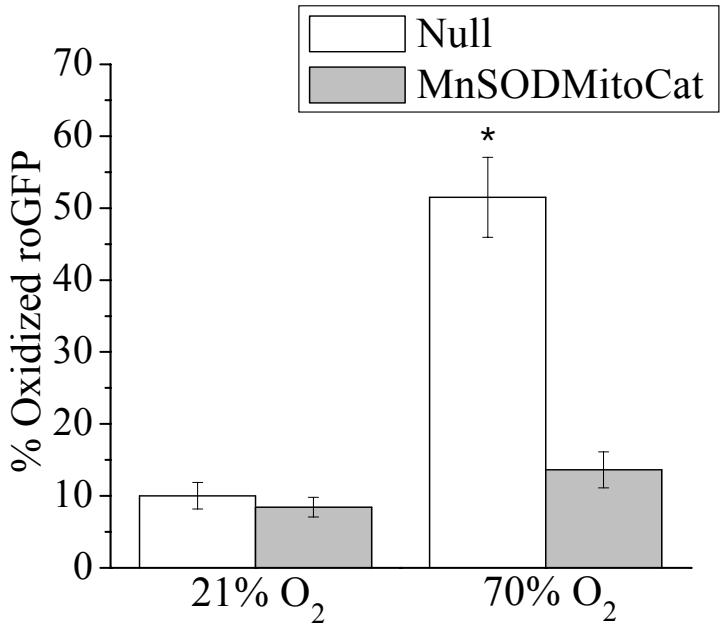

Figure 4.4. Hyperoxia elevates mitochondrial ROS production.

ROS levels detected after 16 hrs in 21% O_2 or 70% O_2 using roGFP targeted to the mitochondrial matrix, expressed via adenovirus in PHLFs. ROS levels are represented as percentage of roGFP maximally oxidized by treating PHLFs with 1mM H_2O_2 and maximally reduced by addition of 10mM DTT for 5 min. PHLFs were co-infected with null virus (white) or MnSOD and mitochondrially-targeted catalase (gray). *indicates $P<0.05$ for samples compared to null virus-infected PHLFs at 21% O_2 (P=0.00039). N=4.

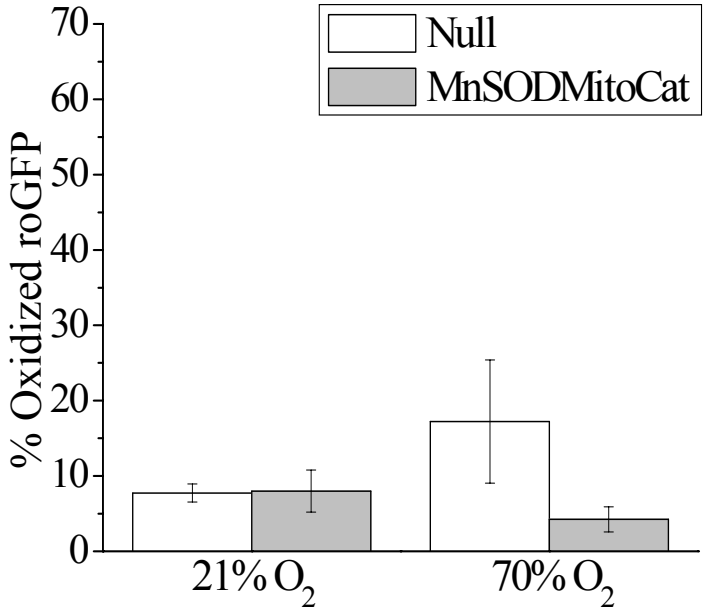

Figure 4.5. Cytosolic ROS levels are unaffected by hyperoxia.

ROS levels detected after 16 hrs in 21% O_2 or 70% O_2 using cytosolic roGFP, expressed via adenovirus in PHLFs. ROS levels are shown as percentage of roGFP maximally oxidized by treating PHLFs with 1mM H_2O_2 and maximally reduced by addition of 10mM DTT for 5 min. PHLFs were co-infected with null virus (white) or MnSOD and mitochondrially-targeted catalase (gray). N=4.

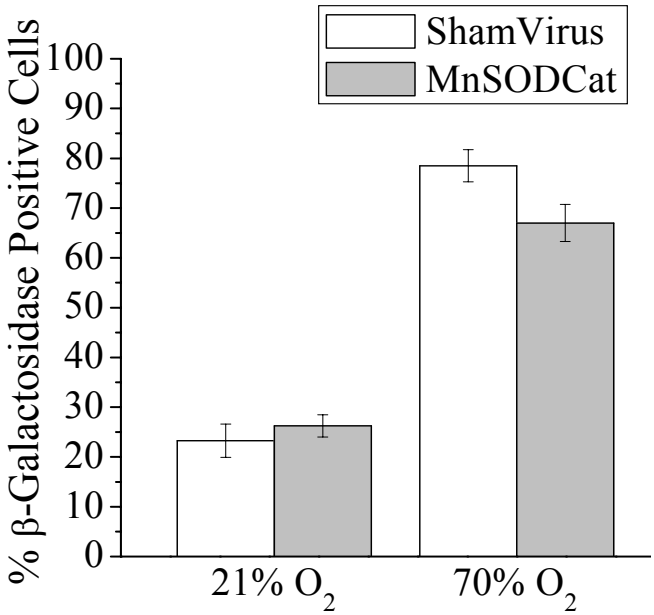

Figure 4.6. Hyperoxia-induced senescence is ROS independent.

Percent β-galactosidase positive PHLFs expressing null virus (white) or MnSOD and mitochondrial catalase together (gray) after 72 hrs in 21% O_2 or 70% O_2. N=4

Chapter 5. Frataxin overexpression is insufficient to prevent hyperoxia-induced senescence.

Hyperoxia has long been known to destabilize the numerous iron-sulfur cluster-containing proteins of the mitochondria. A particularly sensitive target of hyperoxia is mitochondrial aconitase (ACO2) (Gardner, Nguyen et al. 1994), a citric acid cycle dehydratase that contains a 4Fe-4S center in its active site. Superoxide generation by the mitochondria is known to disrupt ACO2. However, ACO2 Fe-S cluster destabilization under hyperoxia can occur independently of ROS generation, possibly because of the increased levels of oxygen itself (Gardner, Raineri et al. 1995). Other mitochondrial Fe-S cluster proteins may be similarly affected by hyperoxia. Fe-S cluster destabilization may affect electron transport and, thus, the energy available to the cell, which can lead to stress responses, including senescence. Indeed, disrupting Fe-S clusters of certain mitochondrial proteins with desferoxamine (DFO) has been shown to lead to senescence (Yoon, Byun et al. 2003).

Frataxin (protein mutated in *Fr*iedrich's *atax*ia) contributes to iron metabolism and is a global chaperone for mitochondrial Fe-S cluster proteins (Bulteau, O'Neill et al. 2004; Zhang, Lyver et al. 2006). Knocking down frataxin by shRNA reduces activity of aconitase and other Fe-S cluster proteins like succinate dehydrogenase (Bulteau, O'Neill et al. 2004; Stehling, Elsasser et al. 2004). Furthermore, knocking down the *C. elegans* frataxin homolog is sufficient to reduce this organism's lifespan (Vazquez-Manrique, Gonzalez-Cabo et al. 2006), suggesting that frataxin and/or Fe-S cluster proteins may play a role in senescence and making Fe-S cluster proteins intriguing candidates for regulating hyperoxia-induced senescence. Thus, to globally stabilize mitochondrial Fe-S cluster proteins, we overexpressed frataxin in PHLFs. Frataxin was efficiently overexpressed in PHLFs via retroviral infection (Figure 5.1A), but was insufficient to protect against hyperoxia-induced senescence (Figure 5.1B). This suggests that frataxin

overexpression, and, thus, increased Fe-S cluster stability, is insufficient to prevent hyperoxia-induced senescence.

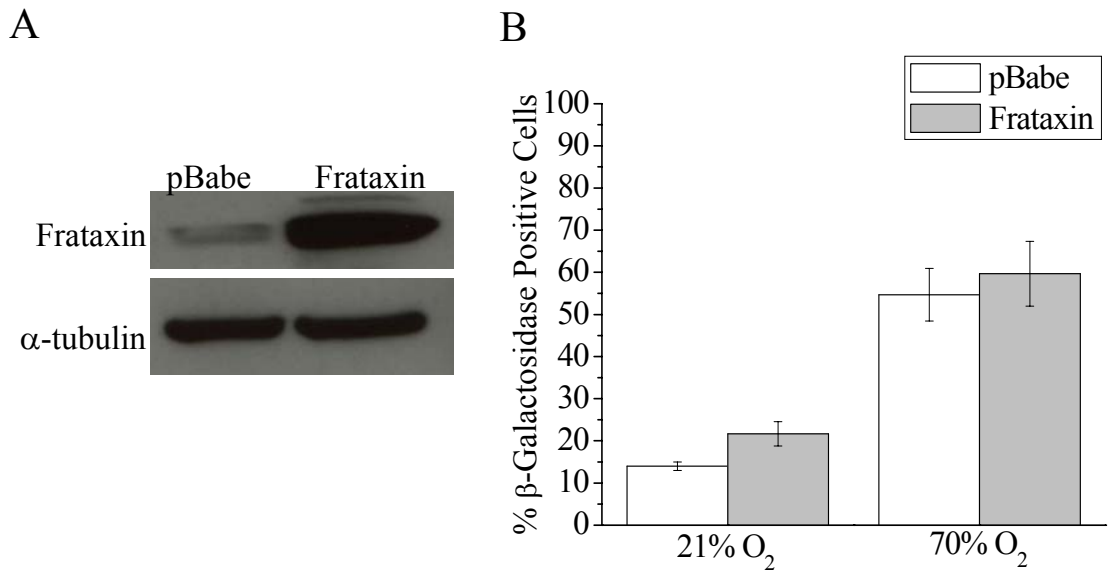

Figure 5.1. Frataxin does not protect against hyperoxia-induced senescence.

A. Frataxin protein levels in PHLFs retrovirally infected with empty vector (pBabe) or frataxin.

B. Percent β-galactosidase positive PHLFs expressing pBabe (white) or frataxin (gray) after 72hr exposure to 21% O_2 or 70% O_2. N=4.

Chapter 6. Hyperoxia-induced senescence does not involve AMP activated protein kinase

Part 1: AMPK activation is sufficient to induce senescence.

Hyperoxia leads to a metabolic crisis by disrupting oxidative phosphorylation (Kimura, Thulin et al. 1983; Schoonen, Wanamarta et al. 1990). As cells continue to utilize energy without being able to produce more ATP, the AMP to ATP ratio will increase. The rise in the AMP/ATP ratio activates AMP-activated protein kinase (AMPK), which signals to reduce cellular energy consumption by shutting down such energy-costly processes as fatty acid synthesis, and to increase energy production. AMPK can also signal to both p53 and pRb and induce senescence (Wang, Yang et al. 2003; Jones, Plas et al. 2005). Furthermore, LKB1, a well-known regulator of AMPK, can induce senescence in certain types of human tumor cell lines (Gurumurthy, Hezel et al. 2008). Indeed, treatment with AICAr, an AMP mimetic and known activator of AMPK, is sufficient to activate AMPK (Figure 6.1A) and induce senescence in PHLFs (Figure 6.1B and C) under normal oxygen conditions.

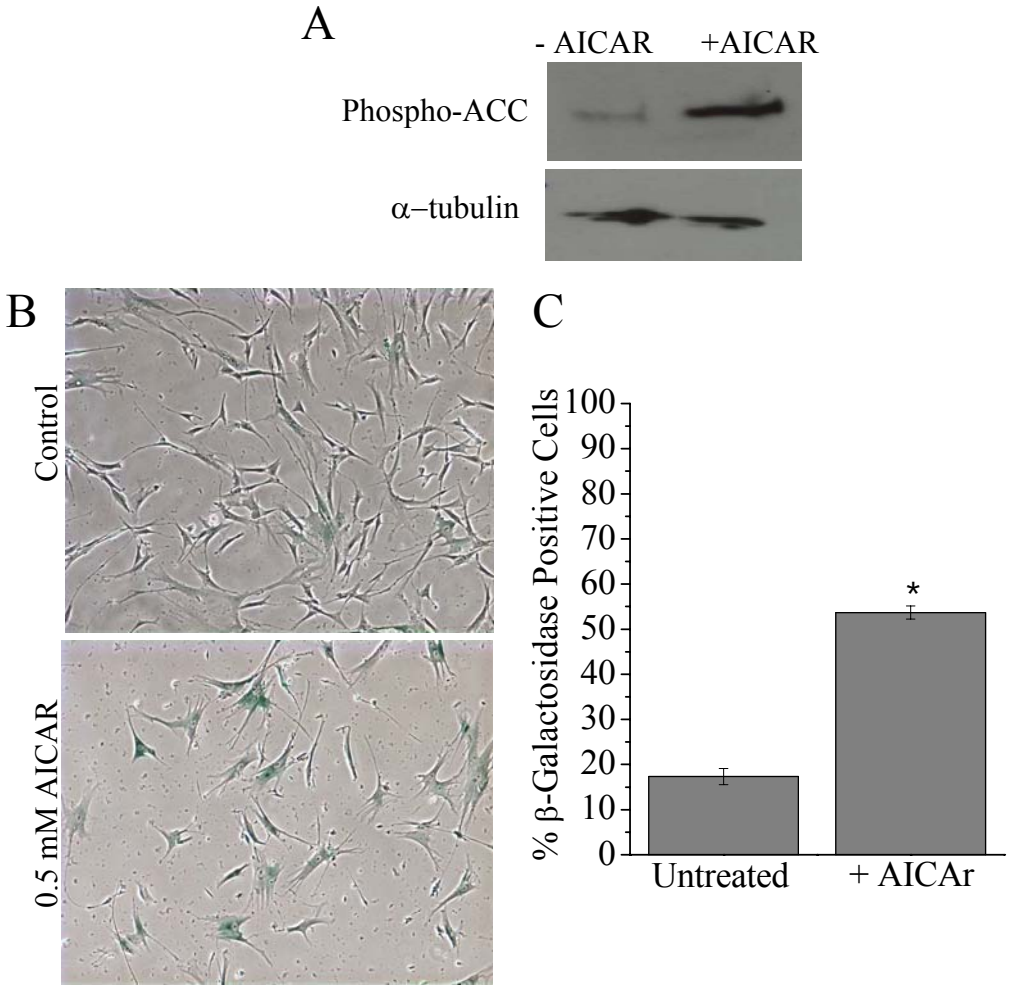

Figure 6.1. AMPK activation is sufficient to induce senescence under normoxia.

A. Phosphorylated ACC and α-tubulin levels in PHLFs treated with AMPK activator AICAr (0.5 mM) for 30 min.

B. Representative phase microscopy image of PHLFs stained with X-gal to show β-galactosidase activity. N=3. PHLFs were untreated for 72hr or treated with 0.5mM AICAr for 16 hr at 21% O_2 and allowed to develop senescent phenotype for 72hr before staining.

C. Percent β-galactosidase positive PHLFs after treatment with 0.5mM AICAr as above. * indicates $P<0.05$ for AICAr-treated PHLFs compared to untreated controls. N=3.

Part 2. AMPK inactivation is not sufficient to prevent hyperoxia-induced senescence.

AMPK can be activated by various stressors, including 100 μM H_2O_2 . 70% O_2 exposure also rapidly activates phosphorylation of acetyl carboxylase (ACC), a direct phosphorylation target of activated AMPK (Figure 6.2). Since we showed that AMPK can induce senescence, and is activated by hyperoxia exposure, it represented a likely candidate to regulate hyperoxia-induced senescence. To test this, we overexpressed wild-type or kinase-dead (KD) LKB1 in PHLFs. LKB1 is a well known activator of AMPK; after AMP binds to AMPK, LKB1 recognizes it for phosphorylation and activation. Both WT and KD-LKB1 were overexpressed in PHLFs using retrovirus (Figure 6.3A). Upon treatment with 100μM H_2O_2 or 70% O_2, levels of phosphorylated ACC increased significantly in PHLFs expressing WT-LKB1 (Figure 6.3B). However, KD-LKB1 overexpression prevented the increase in phosphorylated ACC, indicating that KD-LKB1 prevents AMPK activation under H_2O_2 and hyperoxia (Figure 6.3B). Surprisingly, both WT-LKB1 and KD-LKB1-expressing PHLFs underwent senescence at similar levels to control cells after hyperoxia exposure (Figure 6.3C). Thus, we conclude that AMPK is not required for hyperoxia-induced senescence.

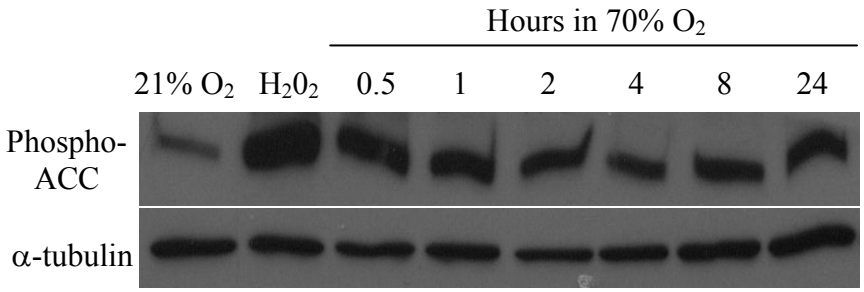

Figure 6.2. Hyperoxia activates AMPK.

Levels of phosphorylated and total ACC after treatment with 70% O_2 for indicated time. Treatment with 100μM H_2O_2 for 5 min serves as a positive control. Blot representative of 3 independent experiments.

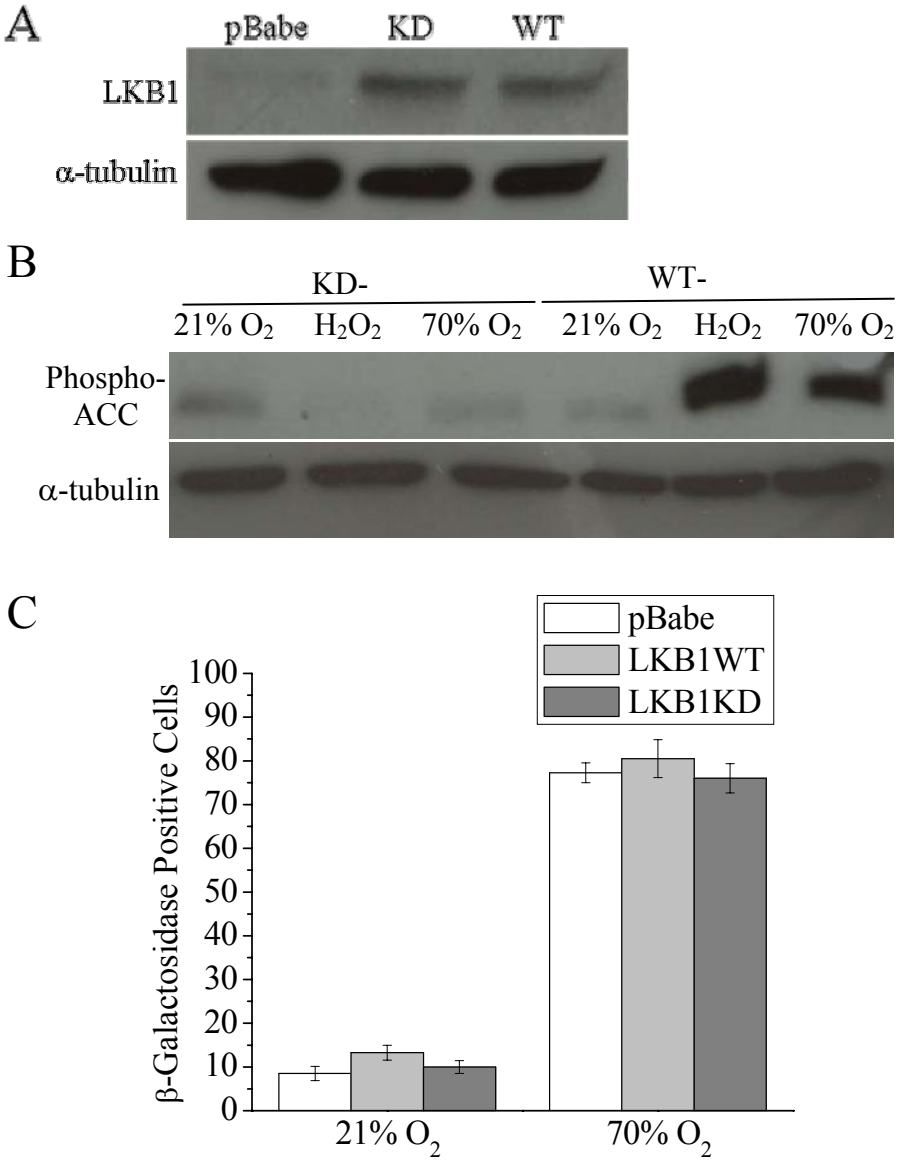

Figure 6.3. Hyperoxia-induced senescence is AMPK-independent.

A. Levels of LKB1 protein in PHLFs infected with retrovirus expressing empty pBabe vector or wild-type or kinase dead (KD) LKB1.

B. Levels of phosphorylated and total ACC and AMPK in PHLFs overexpressing empty vector, wild-type LKB1, or kinase dead (KD) LKB1. Cells were placed in fresh media for 1 hr before treatment with 70% O_2-preequilibrated media for 30 min, or with 100μM H_2O_2 for 5 min, and harvested.

C. Percent β-galactosidase positive PHLFs overexpressing empty vector, wild-type LKB1 or KD-LKB1 after exposure to 21% O_2 or 70% O_2 for 72hr. N=4.

Chapter 7. Hyperoxia-induced senescence requires cooperation between the p53 and pRb pathways.

Part 1. BMI1 overexpression is insufficient to prevent hyperoxia-induces senescence.

p16, which signals to retinoblastoma protein (pRb) to maintain cell cycle arrest, is a widely known regulator of senescence (Hamilton, Guo et al. 2001). In many cases of senescence, the p16/pRb pathway acts independently of the p53/p21 pathway, though cooperation can also occur (Beausejour, Krtolica et al. 2003). p16 is often thought to be activated by stress; indeed, 72-hr exposure to 70% O_2 elevated p16 levels in PHLFs (Figure 7.1), suggesting involvement of p16 in hyperoxia-induced senescence. Recently, members of the Polycomb group proteins, particularly BMI1 and CBX7, have been shown to suppress p16 expression (Jacobs and de Lange 2004). While CBX7 affects both the p16 and p21 pathways, BMI1 appears to act only on p16 (Jacobs, Kieboom et al. 1999; Jacobs and de Lange 2004). Overexpressing BMI1 can be sufficient to prevent senescence (Jacobs, Kieboom et al. 1999; Liu, Andrews et al. 2006). Additionally, aging cells reduce BMI1 levels (Sasaki, Ikeda et al. 2006); some other senescence-inducing stimuli also reduce BMI1 levels, leading to senescence (Itahana, Zou et al. 2003; Guney, Wu et al. 2006). However, 70% O_2 exposure does not reduce BMI1 levels (Figure 7.2).

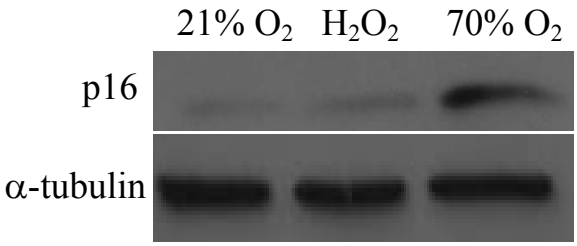

Figure 7.1. Hyperoxia increases p16 expression.

p16 protein levels after exposure to 21% O_2 or 70% O_2. Cells were kept below 70% confluence to prevent p16 activation by overcrowding. Blot representative of 4 independent experiments.

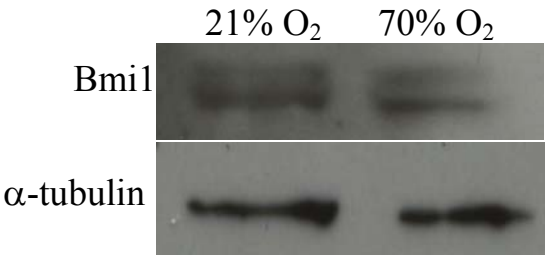

Figure 7.2. Hyperoxia does not reduce BMI1 levels.

BMI1 protein levels after exposure to 21% O_2 or 70% O_2. Cells were kept below 70% confluence to prevent p16 activation by overcrowding. Blot representative of 4 independent experiments.

Since BMI1 is a transcriptional repressor that blocks p16 expression, its activity may be modulated at the level of DNA binding; thus, it may still be involved in hyperoxia-induced senescence even though its levels under hyperoxia are unaffected. To test its involvement, we overexpressed BMI1 in PHLFs (Figure 7.3A). BMI1 overexpression suppressed p16 protein levels in overconfluent cells (overconfluence activates p16 expression), but not p21, as expected (Figure 7.3A). Despite p16 suppression, however, the majority of PHLFs overexpressing BMI1 still senesced under hyperoxia (Figure 7.3B), indicating that BMI1 overexpression and subsequent suppression of the p16/pRb pathway alone is not sufficient to protect against hyperoxia.

To confirm this finding, we overexpressed the human papilloma virus 16 protein E7, which downregulates pRb function (Boyer, Wazer et al. 1996). E7-overexpressing PHLFs underwent senescence in similar numbers to PHLFs expressing vector control (Figure 7.4). This further supported our finding that hyperoxia-induced senescence does not rely solely on the p16/pRb pathway.

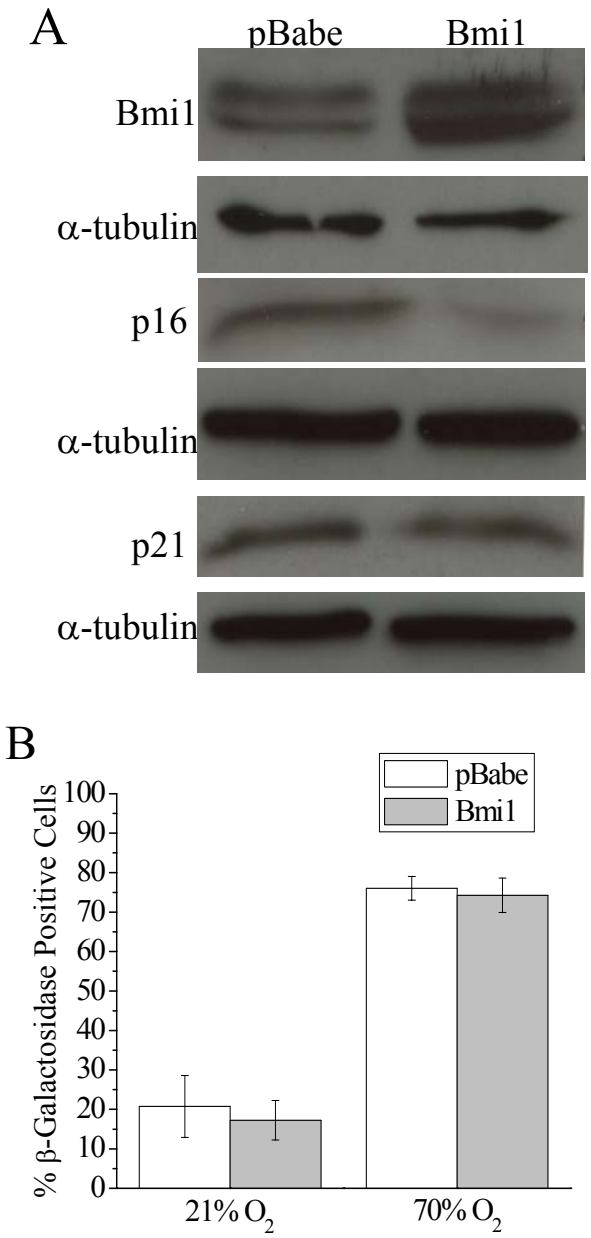

Figure 7.3. BMI1 does not protect against hyperoxia-induced senescence.

A. BMI1, p16, and p21 protein levels in PHLFs overexpressing BMI1 or expressing empty vector. Cells were allowed to reach overconfluence to activate p16.

B. Percent β-galactosidase positive PHLFs expressing empty vector or BMI1 after exposure to 21% O_2 or 70% O_2 for 72 hrs. N=4.

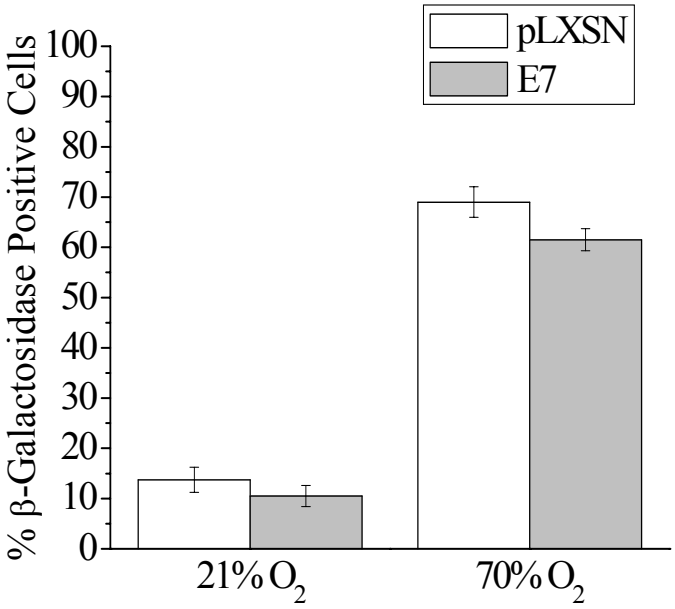

Figure 7.4. Overexpression of E7 does not prevent hyperoxia-induced senescence.

Percent β-galactosidase positive cells expressing empty vector or the human papillomavirus type 16 protein E7 after exposure to 21% O_2 or 70% O_2 for 72 hr. N=4.

Part 2. *p21 knockdown is insufficient to protect against hyperoxia-induced senescence.*

p53 is a known regulator of senescence, usually acting through p21 activation (Di Leonardo, Linke et al. 1994; Beausejour, Krtolica et al. 2003). To begin determining whether the p53/p21 pathway is responsible for hyperoxia-induced senescence, we used short hairpin RNA to knock down p21 expression. p21 expression was sufficiently suppressed using this shRNA despite growing cells to overconfluence to activate p21 (Figure 7.4A). However, under hyperoxia, p21-shRNA-expressing PHLFs senesced in similar numbers to controls expressing a scrambled shRNA, indicating that p21 suppression is insufficient to prevent hyperoxia-induced senescence (Figure 7.4B).

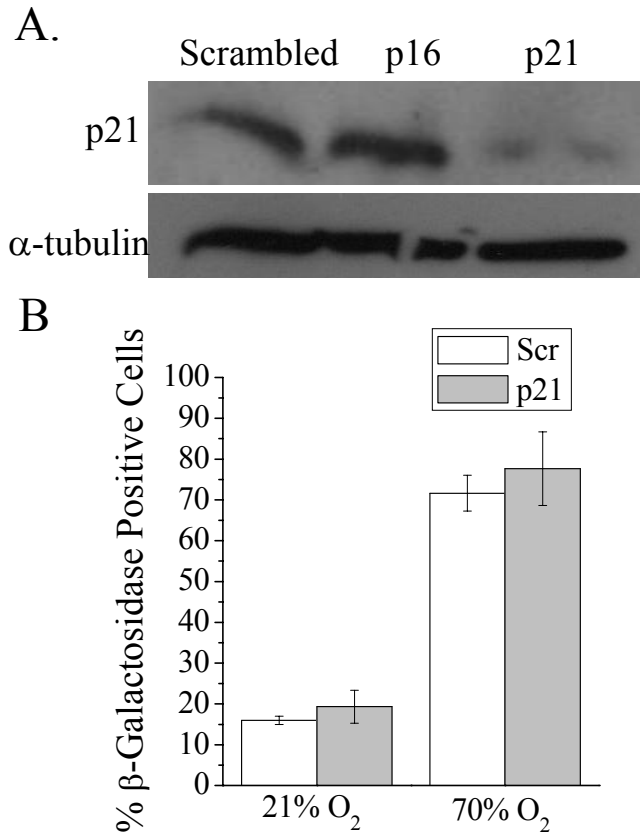

Figure 7.4. Suppressing p21 is not sufficient to prevent hyperoxia-induced senescence.

A. p21 protein levels in PHLFs expressing scrambled shRNA or shRNA targeted against p21.

B. Percent β-galactosidase positive PHLFs expressing scrambled shRNA (Scr) or p21 shRNA after 72 hr exposure to 21% O_2 or 70% O_2. N=3.

Part 3. Inactivating the p53 pathway partially protects against hyperoxia-induced senescence.

p53 has many downstream targets besides p21 that can induce senescence (Qian, Zhang et al. 2007), though p21 is the most well-known p53 target in this role. To determine whether p53 is involved, we suppressed p53 function by overexpressing a dominant negative p53 in a retroviral vector, which resulted in effective suppression of p21 expression (Figure 7.5A), indicating suppression of p53 function. PHLFs overexpressing DN-p53 showed a significant decrease in the number of β-galactosidase positive cells after 70% O_2 exposure compared to PHLFs expressing empty vector (Figure 7.5B). These data indicate that the p53 pathway is involved in hyperoxia-induced senescence, but not through p21 activation. Additionally, since incomplete suppression was observed, p53 may act in cooperation with another protein.

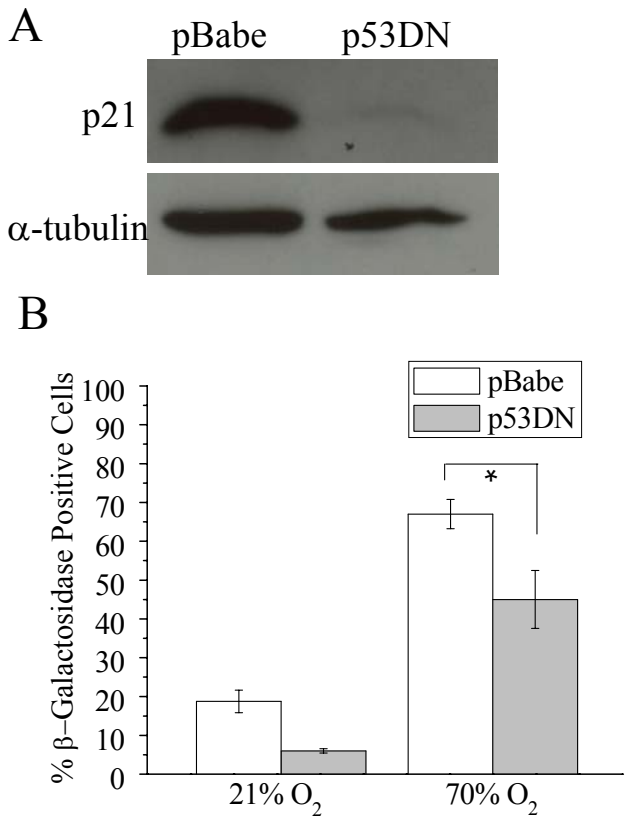

Figure 7.5. Inhibiting the p53 pathway partially protects against hyperoxia-induced senescence.

A. Immunoblot demonstrating suppression of p21 protein expression by overexpression of DN-p53 in PHLFs.

B. Percent β-galactosidase positive PHLFs expressing vector control or DN-p53 after 72 hr exposure to 21% O_2 or 70% O_2. * indicates $P<0.05$ for p53-DN samples at 70% O_2 compared to pBabe vector samples at 70% O_2 (P=0.03876). N=4.

Part 4. Hyperoxia-induced senescence involves cooperation between the p53 and pRb pathways.

Most cells senesce via either the p53/p21 or the p16/pRb pathway, but the pathways can act on each other and cooperate to induce senescence. For example, p21 can also maintain pRB in its hypophosphorylated (active) form. Furthermore, suppressing the p16/pRb pathway can increase p21 expression (as previously reported and seen in our own system), indicating that one pathway can compensate for the lack of the other. To test for the requirement of both the p53/p21 and p16/pRb pathways under hyperoxia, we overexpressed the HPV proteins E6 (which suppresses p53 function (Scheffner, Huibregtse et al. 1993)) and E7 together in the same cells. While approximately 70% of the PHLFs expressing vector controls senesced under 70% O_2, only 20% of E6/E7 expressing PHLFs underwent senescence (Figure 7.6), indicating that both the p53 and pRb pathways participate in hyperoxia-induced senescence. This demonstrates that hyperoxia-induced senescence proceeds through genetically coded and previously well-described senescence pathways.

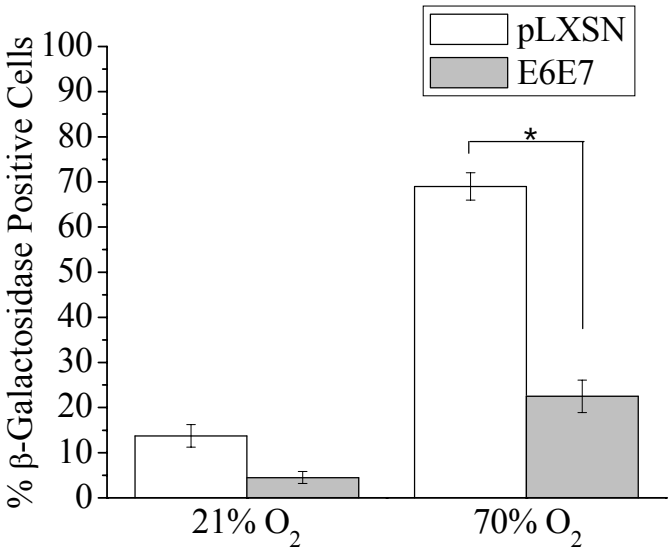

Figure 7.6. Hyperoxia-induced senescence requires both the p53/p21 and p16/pRb pathways.

Percent β-galactosidase positive PHLFs expressing empty vector or HPV16/18 E6 and E7 after 72hr exposure to 21% O_2 or 70% O_2. * indicates $P<0.05$ for E6E7 PHLFs at 70% O_2 compared to vector control PHLFs at 70% O_2 ($P=0.00006$). N=4

Chapter 8: Discussion.

Despite the immense importance of senescence, its mechanisms remain incompletely understood. A prominent model suggests that senescence occurs in response to damage to cellular components by increased levels of ROS. Our data demonstrate that hyperoxia upregulates mitochondrial matrix ROS generation, and that hyperoxia induces telomere-independent senescence. Contrary to the free radical theory of aging, though, we show that neutralizing mitochondrial matrix ROS is insufficient to prevent hyperoxia-induced senescence. Evidence against a straightforward relationship between ROS and senescence is growing, however. For example, one recent study also reported the onset of premature senescence occurring independently of ROS generation (Stockl, Zankl et al. 2007). Furthermore, our current data are consistent with our previous finding that hypoxia extends replicative lifespan despite an upregulation in mitochondrial ROS production (Bell, Klimova et al. 2007b). In that system, the mitochondrial ROS were generated not in the matrix, but into the intermembrane space by complex III (Bell, Klimova et al. 2007a). Thus, hypoxia-induced mitochondrial ROS can escape easily into the cytosol. However, we showed that the levels of ROS that escape into the cytosol in hypoxia are below the threshold to induce damage and activate senescence pathways. In fact, under hypoxia, the ROS work as signaling molecules to activate HIF-dependent hTERT expression to extend replicative lifespan.

In the present study, we used a redox-sensitive GFP (roGFP) protein probe to measure intracellular oxidant stress (Dooley, Dore et al. 2004; Hanson, Aggeler et al. 2004). Using roGFP is advantageous over the use of other measures of ROS because it can be calibrated to permit comparisons between experiments. Furthermore, it is a protein and, therefore, a relevant biological target of ROS. Finally, roGFP can be targeted to subcellular compartments, like the

mitochondrial matrix, by the addition of a localization tag. This probe allowed us to measure ROS levels that would be sufficient to alter cellular components, and, furthermore, to determine ROS levels specifically in the mitochondrial matrix or the cell overall. Because of the localization specificity, we can study the effects not only of ROS levels, but ROS location, as well. We show that during hyperoxia, ROS levels increase specifically in the mitochondrial matrix, but not in the cytosol. Collectively, our studies suggest that ROS levels must exceed a threshold in the cytosol to cause senescence. Thus, the levels and localization of ROS are likely to determine whether the ROS serve as adaptive signaling molecules or damaging, senescence-inducing molecules.

A potential mechanism for hyperoxia-induced senescence is the destruction of the electron transport chain by the reaction of molecular oxygen with the iron-sulfur centers of the ETC machinery proteins. A previous study demonstrated that the disruption of aconitase, a hyperoxia-sensitive protein, may occur independently of ROS generation (Gardner, Raineri et al. 1995). Therefore, aconitase and other Fe-S cluster proteins in the ETC may be susceptible to hyperoxic disruption even in the presence of antioxidants. However, overexpressing frataxin, a mitochondrial chaperone of Fe-S cluster proteins, did not protect against hyperoxia-induced senescence. Further experiments are needed to determine whether Fe-S cluster stabilization is not sufficient to protect against hyperoxia-induced senescence, or if overexpressing frataxin in our system is not sufficient to stabilize Fe-S proteins.

AMPK made an attractive potential regulator of hyperoxia-induced senescence, as it is activated by various stresses and can signal to p53 and pRb to induce senescence. Indeed, AMPK activation by AICAR led to senescence in our PHLFs, and hyperoxia led to rapid and sustained AMPK activity. However, suppressing AMPK by expressing a kinase-dead LKB1 mutant, while

effective at suppressing AMPK activation, did not prevent senescence after hyperoxia. It is possible that AMPK acts coordinately with another pathway to induce senescence under hyperoxia exposure; however, that pathway remains unidentified.

Cellular senescence is ultimately regulated by the p53 and/or p16/pRb pathways. We found that the p16/pRb pathway does not independently cause hyperoxia-induced senescence, while p53 inactivation suppresses hyperoxia-induced senescence incompletely. The inactivation of both the p53 and pRb pathways leads to almost complete prevention of hyperoxia-induced senescence, demonstrating that hyperoxia-induced senescence proceeds through well-known senescence-inducing pathways. This was consistent with other studies showing that p53 and p16 pathways can interact to induce senescence. It is generally thought that p53 activation signals to senescence, while p16 activation maintains cells in the senesced state. This may be the case in our system, where p16 activation is apparent, yet p16 inactivation alone is insufficient to prevent senescence. Interestingly, however, p53 may be acting independently of p21 to induce senescence, as simply knocking down p21 expression does not prevent hyperoxia-induced senescence, while using dominant negative p53 does. It may simply be that the knock down of p21 was insufficient, and that only a small amount of p21 is enough to drive senescence, while DN-p53 resulted in more efficient p21 suppression. However, p53-induced senescence can indeed occur independently of p21, for example, through DEC1 (Qian, Zhang et al. 2007). It remains to be determined how p53 and/or pRb pathways are initiated during hyperoxia. It is possible that the pathways studied in this work cooperate to activate p53 and pRb; for example, AMPK activation may work coordinately with iron-sulfur cluster protein destabilization. Additionally, hyperoxia may lead to the destabilization of numerous proteins within the cell, leading to ER stress, which, in turn, may induce senescence. Also, heat shock proteins may be

involved in attempting to repair the damage caused by hyperoxia, signaling to p53 and/or pRb activation and senescence.

Many researchers argue that cell culture conditions, which maintain oxygen levels at 21%, actually place cells in hyperoxia, which causes stress and leads to senescence. To demonstrate the effect of hyperoxia on cellular lifespan, moderate levels of hyperoxia (40%) have been used previously in other studies. In these studies, cells were cultured for several weeks under those conditions, simulating a replicative senescence-like phenotype. However, we used lung fibroblasts, which are normally exposed to approximately atmospheric oxygen concentrations, and therefore, 21% O_2 is physiological normoxia for these cells.

Clinical studies show that elevated oxygen levels are a life-saving measure for patients with heart and/or lung failure, but prolonged exposure to hyperoxia leads to inflammation and pulmonary edema. Extreme hyperoxia appears to induce these ill effects by causing cell death (Budinger, Tso et al. 2002), but 70% O_2, a concentration used to treat patients, does not cause cell death (as we showed) while still being sufficient to cause lung damage. Our study suggests that the cause of this lung damage may be cellular senescence. While most studies on hyperoxia-induced lung damage have focused on epithelial cells, lung fibroblasts may be no less important. As previously mentioned, senescent fibroblasts can secrete tissue-remodeling factors (Krtolica, Parrinello et al. 2001). Indeed, several studies demonstrate an interdependence between lung fibroblasts and epithelial cells, wherein stimuli that inhibit fibroblast growth also prevent proper repair of epithelial cells (Adamson, Hedgecock et al. 1990; Bowden, Young et al. 1994; Ray, Devaux et al. 2003). Moreover, senescent fibroblasts secrete tissue-remodeling factors, including growth factors like keratinocyte growth factor (KGF) and metalloproteinases (Ray, Devaux et al. 2003; Campisi 2005). By releasing these factors, senescent fibroblasts can damage

and/or induce dysplasia in neighboring epithelial cells (Parrinello, Coppe et al. 2005) (also reviewed in (Krtolica and Campisi 2002)). Hyperoxia-induced senescence specifically of lung fibroblasts may therefore be very important to hyperoxia-induced lung damage, and determining ways to prevent this senescence will improve patient care.

In summary, our data indicate that hyperoxia utilizes p53 and pRB pathways to execute senescence independently of an increase in ROS or an increase in AMPK. Our data therefore suggest that oxidative stress is not always causal in the induction of senescence. While not induced in senescence, hyperoxia-induced ROS may still activate genes to counteract the stress of high oxygen. However, during hyperoxia exposure, other mechanisms are also invoked which ultimately lead to p53/pRB induced senescence. Nevertheless, our current data on hyperoxia, along with our previously published data on hypoxia, indicate that the relationship between increases in ROS and senescence are not correlative.

Chapter 9: Materials and Methods:

Cell Culture

Normal primary diploid fibroblasts from adult human lung (PHLF) were purchased from Cambrex. They were cultured in Fibroblast Growth Media (FGM-2; Cambrex), which consisted of Fibroblast Basal Media supplemented with 2% fetal bovine serum, insulin and penicillin-streptomycin. PHLFs were maintained at 37°C in 21% O_2/5% CO_2 (defined here as normoxia) humidified incubators and passaged upon reaching confluence.

Hyperoxia Exposure

For senescence studies, low-passage (passage 5 or earlier) PHLFs were seeded into 35-mm dishes at $3x10^4$ cells/plate, or approximately 25% confluence. Hyperoxia exposure (70% O_2, 5% CO_2, balanced with N_2) for 72 hr occurred in a humidified chamber, with gas delivery controlled by Oxyxycler model C42 chamber and software (Biospherix). Alternatively, hyperoxic conditions were achieved in a humidified Plexiglass chamber supplied with a gas mixture of 70% O_2/5% CO_2/balance N_2. As a positive control, PHLFs split into 35mm dishes as above were treated with 100uM H_2O_2 for 2 hrs. Media was then changed to fresh FGM-2 and cells were allowed to develop the senescent phenotype for 72 hrs at normoxia. Untreated negative control PHLFs were left at 21% O_2 for 72 hrs. After 72 hrs, PHLFs were removed from hyperoxia chamber, given fresh FGM-2, and kept at normoxia for 24 hrs before determining senescence-associated β-galactosidase activity.

Senescence-associated β-galactosidase staining

To determine senescence-associated (SA) β-galactosidase activity in PHLFs, PHLFs were exposed to conditions as above and fixed and stained at pH 6.0 using the Senescence-Associated β-Galactosidase Staining Kit (Cell Signaling). Twenty-four hours after staining, phase microscopy images were obtained with a Nikon Eclipse TE200 inverted microscope at magnification of X10. Five random fields were captured per plate, and the numbers of total and β-galactosidase-positive cells manually counted.

Lactate dehydrogenase assay

To determine whether reduced cell number after 70% O_2 exposure is the result of cell death rather than senescence, PHLFs were plated to 50% confluence in 60mm dishes and exposed to 70% O_2 or 100μM or 1mM H_2O_2 as above. Normoxic controls were plated to 25% confluence to prevent overconfluence at 72 hrs. 72 hrs later, cell death was determined using the Lactate Dehydrogenase Assay kit (Roche) following manufacturer's protocol.

Plasmids and constructs

pBabe-Puro retroviral expression vector (Clontech) was used to overexpress human hTert (Addgene). YCpLAC22-HFA (frataxin) yeast expression construct was generously donated by Dr. G. Isaya (Mayo Clinic). From this construct, the frataxin coding sequence was excised using BamHI and cloned into pBabe-Puro retroviral expression vector. pBabe-Puro-BMI1 retroviral expression construct was a kind gift from Dr. G. Dimri (Northwestern University). pBabe-puro-FLAG-LKB1 WT and KD were generated by Dr. L. Cantley and purchased from Addgene. pSiren-RetroQ retroviral short hairpin (shRNA) expression vector (Clontech) was used to

express shRNA sequences for p21 (5'-TGTCAGAACCGGCTGGGGATT-3') and a scrambled control shRNA (5'-AGCGCGATTTGTAGGATTCGT-3'). These sequences were previously published (p21: (Li, Tzeng et al. 2005); p16 and scrambled shRNA: (Bond, Jones et al. 2004)).

Stable cell lines

Stable cell lines were generated in early-passage PHLFs by retroviral infection, using PT67 packaging cell line (Clontech). At approximately 50% confluence, early passage PT67 cells were transfected with 10 μg plasmid using Transit-LT1 (Mirus) according to manufacturer's protocol. Specifically, 100ml unsupplemented DMEM was incubated with TransIT LT-1 reagent (2.5 μl/μg plasmid) for 15 minutes at room temperature. Plasmid was then added and the mixture incubated for an additional 30 minutes before being added dropwise to PT67 cells grown in 100mm dishes in full DMEM supplemented with 25 μM chloroquine. Twenty-four hrs later, PT67s were washed with PBS and placed in DMEM containing appropriate selection antibiotics. PT67s were maintained in antibiotic-containing media until a control plate of untransfected PT67s had died; at this point, it was assumed that surviving PT67s stably integrated the plasmid. For infection of PHLFs, PT67 cells stably expressing the plasmid were washed once with PBS and placed in 4ml FGM-2 media overnight. Twenty-four hrs later, virus-containing media was supplemented with 8μg/ml polybrene (Sigma) and applied to PHLFs. PHLFs stably expressing plasmid of interest were selected with appropriate antibiotic as above. PHLFs overexpressing LXSN-HPV-E7, LXSN-HPV-E6 and E7 together, and empty LXSN vector were generated by the same general protocol, but using stable PA317 packaging cells; these were generated and donated by Dr. L.A. Laimins (Northwester University).

Measuring Replicative Lifespan

PHLFs expressing hTERT or pBabe controls were seeded at $4x10^5$ cells/flask and allowed to reach approximately 70% confluence. They were then trypsinized, counted, and reseeded at $4x10^5$ cells. Population doublings were calculated with the formula: $\log_2(D/D_o)$, where D_o = number cells seeded and D = number of cells counted at confluence. The plateau point when cells ceased to undergo further population doublings was deemed replicative senescence.

Adenoviral infection

PHLFs plated in appropriate size dishes (35mm for SA-β-galactosidase staining; 100mm for immunoblotting) were treated with a low volume of serum-free Fibroblast Basal Media. 75pfu/cell of each virus was used (the amount of null virus used in controls was equal to the sum of all the adenoviruses used to infect experimental cells), and PHLFs were incubated at 37^oC for 4-6hrs. Fully supplemented FGM-2 media was then added and cells were allowed to rest overnight before being placed in conditions. MnSOD and mitochondrial catalase adenoviruses were obtained from Iowa Vector Core. roGFP adenoviruses were obtained from Dr. Paul T. Schumacker.

Immunoblotting

PHLFs were plated in multiple 100 mm dishes and exposed to conditions. For hyperoxia treatment, FGM-2 was preincubated at 70% O_2 for a minimum of 2 hrs before being applied to PHLFs. Treated PHLFs were harvested using cell lysis buffer (Cell Signaling) supplemented with 100 μM PMSF. Additionally, buffer used to harvest PHLFs for analyzing phosphorylated proteins was supplemented with phosphatase inhibitors NaF (50mM) and Na_3VO_4 (2mM). Cell

lysates were frozen, thawed on ice, and centrifuged for 3 minutes to pellet cellular debris. 50-70μg samples were resolved on sodium dodecyl sulfate-acrylamide gels (7.5% for ACC; 12% for all other proteins). Protein was then transferred to nitrocellulose membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad) at 15 V (45 min for p16 and p21; 80 min for ACC; 50 min for all other proteins). Membranes were blocked for a minimum of 1hr in 5% milk in TBS-0.1% Tween (TBS-T), and primary antibody applied overnight in milk. Antibodies to phosphorylated proteins were diluted in 5% BSA in TBS-T. The following day, the membranes were washed thrice for 10 min with 5% milk/TBS-T and appropriate secondary antibody applied for 1 hr before membranes were washed twice for 45 min in TBS-T. Antibody was visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce) or ECL Plus (GE).

Antibodies

Antibodies used were: p16 (BD Biosciences), 1:200; p21 (BD Biosciences), 1:200; MnSOD (BD Biosciences), 1:500; catalase (Abcam), 1:1000; ACO2 (Abgent), 1:200; frataxin (MitoSciences), 1:200; phosphorylated and total ACC (Cell Signaling), 1:500; phosphorylated and total AMPK (Cell Signaling), 1:500; LKB1 (D-19; Santa Cruz), 1:200. α-tubulin (Sigma), 1:2000, served as a loading control for all samples.

Fluorescent microscopy

To verify correct localization of roGFP probes, $3x10^4$ PHLFs were plated on glass bottom micro well dishes (MakTek Corp.) and infected with 75pfu/cell of adenovirus encoding cytosolic or mitochondrial roGFP probe as above. After 6 hr infection in serum-free media, FGM-2 was

added and cells were left at 21% O_2 overnight. The following day, cells were treated with 50nM tetramethylrhodamine (TMRE) to show mitochondrial localization and imaged with Yokogawa spinning disc confocal microscope from Perkin Elmer, fitted on a Nikon TE2000-U fluorescent inverted microscope using the 100X objective. Excitation at 488nm was used to visualize roGFP and 568nm to visualize TMRE. MetaMorph software was used to process images.

ROS measurement

To determine whether 70% O_2 elevated mitochondrial ROS production, we utilized the redox-sensitive GFP (roGFP) construct targeted to the mitochondria or cytosol. PHLFs were infected with 75 pfu/cell of adenovirus encoding roGFP, along with 75 pfu/cell each of adenovirus encoding MnSOD and mitochondria-targeted catalase, or with 150 pfu/cell of sham virus. After a 6hr infection in serum-free media, FGM-2 was added and cells were placed in 70% O_2 or left in normoxia for 18hr. After exposure, PHLFs were harvested in trypsin and analyzed with a CyanADP flow cytometry analyzer (Dako). As internal controls, samples were fully reduced with 10 mM DTT and full oxidized with 1 mM H_2O_2. The mean fluorescent channel for the ratio of violet excitable to blue excitable signal was determined with Summit software 4.2 (Dako). A minimum of 1500 cells was counted per sample. Percent oxidized probe was determined with the equation $(R-R_{DTT})/(R-R_{H2O2})$, where R is sample without DTT or H_2O_2 added; R_{DTT} fully reduced sample, and R_{H2O2} is fully oxidized.

AICAr treatment

PHLFs were plated at $3x10^4$ cells in 35mm plates and exposed to 0.5mM AICAr for 16 hr. Media was then replaced and cells were allowed to recover for 72 hrs before staining for SA-β-galactosidase activity as above. For determining AMPK activation, PHLFs grown in 100mm plates were treated with fresh media for 1hr before being treated with 0.5mM AICAr for 30 min and harvested. AMPK activation was then tested by immunoblotting for phosphorylated ACC.

Statistical analysis

Data are presented as means ± standard error of mean. One-way analysis of variance was performed in Origin 7 to determine the presence of significant differences in the data. When analysis of variance indicated that a significant difference was present, two-sample Student's t-tests were performed to compare experimental data with appropriate controls (as indicated in each figure legend). Statistical significance was determined at a value of $P<0.05$.

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Mentor: Navdeep S. Chandel
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NIH T32 CA009560
National Cancer Institute
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Mentor: Kathy Green
9/05-9/06

Publications (in reverse chronological order):

Research Papers:

1. **Tatyana A. Klimova**, Eric L. Bell, Emelyn H. Shroff, Goberdan P. Dimri, Paul T. Schumacker, and Navdeep S. Chandel. Hyperoxia-induced premature senescence requires p53 and pRb, but not mitochondrial matrix ROS. In preparation.

2. Eric L. Bell, **Tatyana A. Klimova**, James Eisenbart, Paul T. Schumacker, and Navdeep S. Chandel. Mitochondrial ROS trigger HIF-dependent extension of replicative lifespan during hypoxia. Mol Cell Biol. 2007 Jun 11.

3. Eric L. Bell, **Tatyana A. Klimova**, James Eisenbart, Carlos T. Moraes and Navdeep S. Chandel. The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via ROS production. J Cell Biol. 2007 Jun 18; 177(6):1029-36

4. Jeremy A. Lavine, Ashley J. Rowatt, **Tatyana A. Klimova**, Aric J. Whitington, Emmeline Dengler, Catherine Beck, Wade H. Powell. Aryl hydrocarbon receptors in the frog *Xenopus laevis*: two AhR1 paralogs exhibit low affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicol. Sci. 2005, 88(1):60-72

Review Articles:

1. Eric L. Bell, **Tatyana A. Klimova**, and Navdeep S. Chandel. Targeting the mitochondria for cancer therapy: Regulation of hypoxia inducible factor by mitochondria. Antioxidant and Redox Signaling.

2. Eric L. Bell, **Tatyana A. Klimova**, and Navdeep S. Chandel. Mitochondrial oxygen sensing: Regulation of hypoxia inducible factor by mitochondrial generated reactive oxygen species.

Abstracts:

1. **Tatyana A. Klimova**, G.R. Scott Budinger, and Navdeep S. Chandel. Mitochondria reactive oxygen species are not required for cellular senescence under hyperoxia. European Bioenergetics Conference 2006.

2. **Tatyana A. Klimova**, G.R. Scott Budinger, and Navdeep S. Chandel. Hyperoxia-induced senescence is independent of mitochondrial ROS. Keystone Symposia 2006

3. Jeremy A. Lavine JA, Blythe H. Philips, Thomas C. Susman, Ashley J. Rowatt, Aric J. Whitington, **Tatyana A. Klimova**, and Wade H. Powell. Molecular mechanisms of dioxin insensitivity in *Xenopus laevis* embryos and tadpoles. Society of Toxicology 2004.

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6. **Tatyana A. Klimova**, and Wade H. Powell. Expression and biochemical characterization of aryl hydrocarbon receptors 1α and 1β (AHR1α and AHR1β) in *Xenopus laevis.* Kenyon College Summer Science Poster Session 2002.

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