Title
Signaling pathways that regulate cellular senescence

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Publication Date
2010

Peer reviewed|Thesis/dissertation

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by

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Dr. Judith Campisi, co-chair
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Fall 2010

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ABSTRACT

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Chronic inflammation is associated with aging and plays a causative role in several age-related diseases such as cancer, atherosclerosis, and osteoarthritis. Studies have shown that cellular senescence, a tumor suppressive stress response that is also associated with aging, entails a striking increase in the secretion of pro-inflammatory proteins, termed the senescence-associated secretory phenotype (SASP), which might be an important contributor to chronic inflammation. Little is known about pathways that regulate the SASP, or how those pathways overlap with the pathways that regulate the senescence growth arrest, such as p53 and $p16^{INK4A}$. We previously showed that DNA damage response (DDR) signaling is essential but not sufficient to establish and maintain the SASP. Additionally, p53, while required for senescence growth arrest, is not required for the SASP and in fact restrains the phenotype.

In this dissertation, I delineate a crucial pathway for regulating the SASP and its relationship to the classic DDR and p53. I show, in normal human fibroblasts, that senescence-inducing stimuli such as ionizing radiation or oncogenic RAS activate p38MAPK with kinetics that parallel the development of the SASP. p38MAPK was required for the majority of SASP expression, and constitutive activation of p38MAPK was sufficient to induce a robust SASP. Moreover, p53 restrained p38MAPK activation such that p38MAPK was more active in p53-deficient cells, and the amplified SASP caused by p53 deficiency was p38MAPK dependent. Further, p38MAPK activation was independent of the DDR and constitutive p38MAPK activation induced a SASP without inducing DDR signaling. Mechanistically, p38MAPK induced the SASP at the mRNA level by increasing NF-κB transcriptional activity. These findings assign p38MAPK a novel role in SASP regulation – one that is independent of previously described pathways.

I also examined how the p38MAPK/NF-κB pathway affected the senescence growth arrest. p38MAPK was required for oncogene-induced growth arrest, however it was not required for DNA damage-induced growth arrest, and NF-κB was not required for growth arrest in either context or for p38MAPK-induced senescence. Thus, p38MAPK regulates the SASP but not growth arrest via NF-κB, demonstrating a bifurcation in the growth arrest/SASP pathways downstream of p38MAPK. These findings demonstrate how the SASP and growth arrest can be independently regulated, suggesting possibilities for mitigating the deleterious effects of the SASP without adversely affecting the tumor suppressive growth arrest. Additionally, these data have implications for our understanding of growth arrest regulation in oncogenic backgrounds.

Lastly, I identified lamin B_1 loss as a novel biomarker of senescence that is independent of other senescence regulatory pathways and may serve as a useful tool for identifying senescent cells in multiple contexts.

DEDICATION

For my parents

Thank you. So much.

ACKNOWLEDGMENTS

"Silent gratitude isn't much use to anyone"
-G.B. Stern

Writing a scientific dissertation provokes more self-reflection than one might think. It is, from an operational perspective, primarily the act of revisiting years-old data, generated by experimental paradigms that no longer apply, to test hypotheses that are now known to be false. Consequently, I often found myself asking, not ruefully, but with true curiosity, "what *was* I thinking?" And in examining my past mental landscapes, I was constantly reminded of the many, many people who have helped me complete this Ph.D., and who help me still.

I must thank, most directly, all the members of my lab, particularly Chris Patil for making every day an intellectual venture, Jean-Philippe Coppé for training me in the ways of science, Francis Rodier for virtually my entire protocol book, Rémi-Martin Laberge for being a wellspring of collaborative spirit and side-bets, and Arturo Orjalo, Albert Davalos, Chris Wiley, Peter de Keizer, Michael Velarde, Pierre Desprez, and Shruti Waghray for their minds, hands, reagents, and most of all, years of support. Judy Campisi forever has my gratitude for accepting me into her lab – the only place I wanted to do my doctoral research – based on little more than my enthusiasm for the subject, and for supporting me (both intellectually and financially) for the last 5 years.

I owe no small measure of my sanity to my colleagues at UC Berkeley, all of whom made the last five years an excellent time of my life. I raise a particularly full glass to James Fraser, Sean Cater, Nadine Jahchan, Becky Pferdehirt, Veronica Zepeda, Veronica Anania, Rachel Zunder, Seemay Chou, and Te-Wen Lo, for being both colleagues and good friends.

Outside of science, my single most time-consuming activity of the last five years (not including the bar) was wushu, and I offer up blood and sweat to everyone in the wushu community for giving me an outlet from the rest of the world. I particularly salute Jeff Lee, James Yang, Justin Leong, Amanda Lam, Emily Hsu, Eleanor Yang, John Nguyen, and Talia Moore for always raising me up, both on and off the carpet.

There are no words to express my gratitude to my mother and father. Any success I may have is primarily a reflection of the quality of their parenting and guidance. They have given from every aspect of themselves, and without the opportunities they have afforded me, no part of this would have been possible. I will never know exactly how much of myself I owe to my siblings, Kurt and Andrea, but they have been there for every stage of my life, and it is the confidence that they will always be there that makes the world a far less turbulent place. Penultimately, I honor my accomplices Vasiliy Zhulin and Ethan Steele; any description of their role in my life would be emphatically inadequate, but suffice to say they are the other two legs of a triad. And lastly, I owe my happiness to Emily Crane. I have made several discoveries over the last five years, but finding her was the most important of all.

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OVERALL INTRODUCTION

“Regular naps prevent old age, especially if you take them while driving”
-Unknown

The role of inflammation in aging and age-related disease

Acute inflammation

External signs of inflammation – pain, redness, heat and swelling – were known long before biologists began to investigate their molecular and cellular mechanisms. We now know that the external signs of inflammation are caused by the dilation of blood vessels and action of phagocytes at the site of injury. Phagocytes, in turn, produce pro-inflammatory factors such as cytokines and chemokines, which attract leukocytes to deal with the presence of foreign organisms or particles. Normally, the inflammatory response ceases within hours or days, once the foreign objects have been removed, and damaged tissue then begins to heal. This type of inflammation is known as acute inflammation.

Chronic inflammation correlates with aging and age-related diseases

Chronic inflammation, by contrast, is the continued presence (sometimes over many years) of pro-inflammatory factors at levels higher than baseline, but many fold lower than those found in acute inflammation. Chronically inflamed tissues are characterized by the presence of infiltrating lymphocytes and macrophages, abundant blood vessels, fibrosis, and often, tissue necrosis (Nathan, 2002; Sarkar & Fisher, 2006). Chronic inflammation, as measured by the serum levels of pro-inflammatory mediators near sites of pathology, is associated with many age-related pathophysiologic processes and diseases, including Alzheimer's, diabetes, atherosclerosis, osteoarthritis, and cancer, among others (Ferrucci et al, 2004; Vasto et al, 2007) (Figure Intro-1). Chronic inflammation is also associated with normal aging. For example, on average, there is a 2-4 fold increase in serum levels of pro-inflammatory mediators (e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)α) in aged individuals (>50 years of age), compared to younger individuals (Bruunsgaard, 2006; Maggio et al, 2006). Moreover, individuals who age unusually well – for example, healthy centenarians – typically have a lower inflammatory profile than frail centenarians (Franceschi et al, 2007) (or individuals that display obvious signs of aging and age-related disease). The inflammatory profile of a tissue or plasma profile is determined by a balance between pro- and anti-inflammatory factors. For example, although both frail and healthy centenarians often have plasma levels of pro-inflammatory mediators that are higher than young individuals, healthy centenarians often also have increased levels of anti-inflammatory mediators (for example, cortisol and IL-10) and, overall, reduced chronic inflammation (Franceschi et al, 2007).

Although the correlation between inflammation and aging is well established, it is difficult to demonstrate a causal connection. This difficulty stems from both the systemic, diffuse nature of chronic inflammation, and the lengthy times that are required for definitive studies. Nonetheless, it is now clear that chronic inflammation plays an important role in the initiation and/or progression of several age-related diseases, including atherosclerosis, Alzheimer's disease, osteoarthritis, and cancer (Brennan et al, 1995; Brod, 2000; Caruso et al, 2004). Important outstanding questions remain, though. What is the relationship between chronic inflammation and normal aging? Does aging drive chronic inflammation, or does something else cause chronic inflammation, which in turn drives aging? Are aging and chronic inflammation too intricately intertwined to be neatly separated? There are, as yet, no definitive answers to these questions, but in the following section we describe several cellular mechanisms by which chronic inflammation could drive age-associated pathologies.

Chronic inflammation might propel basic aging processes

Chronic inflammation might contribute to general aging in several ways. First, the continual presence of circulating pro-inflammatory factors may keep the immune system in a state of chronic low-level activation. Eventually, this chronic immune activation will cause immunosenescence, commonly defined as the functional decline of the adaptive immune system with age. Immunosenescence is caused primarily by an exhaustion of the pool of naïve T cells, clonal expansion among T and B cells, and the consequent shrinkage of "immunological space"; together, these phenomena reduce the body's ability to respond to new antigens (Franceschi et al, 2000; McElhaney & Effros, 2009). In addition to causing immunosenescence, some inflammation-associated factors may degrade tissue microenvironments (Campisi, 2005); for example, matrix metalloproteinase (MMP)-3 (stromelysin) has been shown to disrupt normal branching morphogenesis by mammary epithelial cells (Parrinello et al, 2005). Additionally, cytokines such as IL-6 and IL-8, are potent attractors and activators of innate immune cells, which can destroy tissue environments by virtue of the oxidizing molecules they release (designed to kill pathogens) (Prelog, 2006).

Chronic inflammation can also disrupt stem cell function. This disruption can be direct, as inflammatory mediators can drive stem cell differentiation (Carlson & Conboy, 2007; Gopinath & Rando, 2008; Huang et al, 2009; Mourkioti & Rosenthal, 2005; Seita et al, 2008). It can also be indirect because proteases and the destructive activities of immune cells can destroy stem cell niches, for example, by thickening the basal lamina around muscle satellite cells by extracellular matrix deposition, impeding satellite cell function (Gopinath & Rando, 2008). These effects may well be tissue and cell context-specific. For example, breast cancer stem cells are maintained by a positive feedback loop of which IL-6 is a critical component (Iliopoulos et al, 2009).

Age-related chronic inflammation is often attributed to the immune system (Caruso et al, 2004; Franceschi et al, 2000; Vasto et al, 2007). As we age, we accumulate an "antigenic burden," the sum of all the antigenic stresses (both internal and external) that we unavoidably encounter throughout life, which causes the progressive activation of macrophages and other immune-cell types. This low-level chronic activation leads to the continuous production of inflammatory factors such as cytokines and chemokines, which raises basal levels of these factors throughout the body. This process, termed "inflammaging", has been thoroughly described elsewhere and is supported by a substantial body of data (Franceschi et al, 2000; Vasto et al, 2007). However, though the immune system plays a major role in modulating the levels of pro- and anti-inflammatory signaling, it is not the only source of these factors - other cell types can produce cytokines, chemokines, etc in response to a variety of signals and stresses. In particular, cells that have entered a state of permanent growth arrest, termed cellular senescence, produce and secrete pro-inflammatory factors at levels many fold higher than normal, proliferating cells (Coppe et al, 2010a; Freund et al, 2010). These senescent cells may be an important contributor to chronic inflammation.

Cellular senescence and inflammation

An introduction to cellular senescence

The evolution of multicellularity provided organisms with a range of potential traits and characteristics absent in the unicellular world. However, in order for a multicellular organism to be viable, the cells of that organism must respond to cellular signals and correctly structure themselves within a somatic framework. Because DNA is most likely to develop spontaneous mutations during replication (Busuttil et al, 2006), this regulation is particularly precarious in

multicellular organisms with renewable tissues, such as mammals. If such DNA mutations cause even a single cell to proliferate without regard to somatic signals, that cell can quickly outcompete its conformist neighbors and endanger the viability of the entire organism.

Consequently, complex multicellular organisms have evolved a series of tumor suppressing mechanisms designed to sense potentially oncogenic changes in cells and prevent uninhibited proliferation. On a cell autonomous level, these mechanisms fall into two major categories: caretakers and gatekeepers. Caretaker mechanisms prevent the cell from acquiring potentially oncogenic changes. Most often, this takes the form of proteins that either prevent or repair DNA damage, such as DNA proofreading machinery during replication or DNA damage response proteins such as ATM/ATR (Campisi, 2005).

Gatekeeper mechanisms, on the other hand, are activated when the caretaker mechanisms fail or are overwhelmed by the extent of damage. Rather than attempting repair, gatekeeper mechanisms simply remove the option for proliferation. This removal can take three forms: transient arrest, apoptosis (death), or senescence (irreversible growth arrest) (Campisi, 2005). Transient arrest allows the caretaker mechanisms to attempt repair of the insult, which can be effective and eliminate the damage, but it also can allow for cancer if the repair is incomplete or incorrect. Cellular senescence and apoptosis, by contrast, are permanent cell fate decisions. As such, they are some of the most powerful tumor suppressing mechanisms in the body.

Cellular senescence is a state of essentially irreversible proliferative arrest caused by stresses that are potentially oncogenic. Like apoptosis, senescence prevents the runaway proliferation of cells that have ceased to correctly respond to mitogenic (and anti-mitogenic) signals. However, unlike apoptosis, which leaves behind a dead cell, the senescence program creates a cell that remains metabolically active (Campisi & d'Adda di Fagagna, 2007). Thus, senescent cells have the capacity to undergo widespread gene expression changes and alter their environment for as long as they persist.

Cellular senescence was originally observed as a response to the gradual loss of DNA at the ends of chromosomes (telomeres), an unavoidable consequence of the biochemistry of DNA replication, which results in telomere shortening with successive cellular division (in the absence of the telomere-repairing enzyme, telomerase) (Bodnar et al, 1998; Harley et al, 1990; Hayflick, 1965). Once telomeres reach a critically short length, they initiate a persistent DNA damage response (DDR) that activates the senescence program (d'Adda di Fagagna et al, 2003). Interpreted in light of senescence as a tumor-suppressing mechanism, this “replicative senescence” may be a failsafe to prevent unrestrained proliferation if earlier barriers fail. Further studies demonstrated that almost any inducer of genotoxic stress and the consequent DDR, such as DNA double strand breaks, oxidative damage, or epigenomic rearrangement, can induce senescence, provided that the damage is sufficiently great and that the damage signaling is persistent (Di Leonardo et al, 1994; Nakamura et al, 2008; Ogryzko et al, 1996).

Senescence growth arrest can also be induced by the activation of oncogenic pathways, either by the expression of oncogenes such as RAS, BRAF, or MEK (Acosta et al, 2008; Michaloglou et al, 2005; Serrano et al, 1997), or the loss of tumor suppressor genes such as PTEN or VHL (Alimonti et al, 2010; Young et al, 2008). While the extent to which replicative senescence occurs *in vivo* is questionable, the evidence strongly suggests that oncogene-induced senescence is an important *in vivo* tumor suppressive mechanism that prevents cancer progression in both humans and mice (Braig et al, 2005; Collado & Serrano, 2010; Michaloglou et al, 2005).

The senescence growth arrest is regulated primarily by the activation of two pathways: the p53/p21 pathway and the $p16^{INK4A}$ pathway. Mutations in either pathway greatly increase cancer susceptibility in mice (Collins & Sedivy, 2003; Lowe & Sherr, 2003), and almost all human cancer cells have mutations in one or both of these pathways (Gil & Peters, 2006; Ohtani et al, 2004). The p53 pathway is a critical mediator of many cellular responses to genotoxic stress, including the senescence response (Bargonetti & Manfredi, 2002). Once active (both by increased expression and post-translational modifications), p53 increases the expression of the cyclin dependent kinase inhibitor (CDKI), p21. p21 binds to and inhibits cyclins D and E and activates the retinoblastoma protein (pRb), which in turns inhibits the transcription factor E2F, preventing cells from entering S phase (Beausejour et al, 2003; Sherr, 2005). In some senescence contexts, specifically when $p16^{INK4A}$ expression is low, genetic inactivation of p53 or p21 can reverse the senescence growth arrest (Beausejour et al, 2003; Brown et al, 1997).

The $p16^{INK4A}$ pathway, in contrast, induces an arguably stronger form of growth arrest. Like p21, $p16^{INK4A}$ is a cyclin dependent kinase inhibitor that mediates growth arrest by activating pRb and preventing E2F activity (Sherr & McCormick, 2002). However, unlike the p53/p21 pathways, $p16^{INK4A}$ locks the cell into a state of growth arrest that cannot be reversed by p53, p21, pRb, and/or $p16^{INK4A}$ depletion at a later timepoint, though this seems to require multiple days of continued $p16^{INK4A}$ expression and initially requires pRb (Beausejour et al, 2003; Dai & Enders, 2000; Gil & Peters, 2006). This suggests that the $p16^{INK4A}$-mediated activation of pRb regulates additional effectors of irreversible growth arrest, potentially involving permanent epigenetic silencing of loci required for cell cycle progression. Interestingly, the levels of $p16^{INK4A}$ increase with age in multiple tissues and that increase is associated with a general decline in cell and tissue function (Janzen, 2006; Krishnamurthy et al, 2006; Krishnamurthy et al, 2004; Molofsky et al, 2006; Ohtani et al, 2010; Ressler, 2006; Zindy et al, 1997).

Though we have described them as separate pathways, it is important to note that there is substantial crosstalk between p53 and $p16^{INK4A}$ (Ohtani et al, 2010; Yamakoshi et al, 2009). The level of activation of these pathways and their crosstalk is dependent on cell type and the nature of the senescence-inducing insult (Campisi, 2005; Courtois-Cox et al, 2008).

While growth arrest is a necessary condition for a cell to be labeled senescent, it is not sufficient. Many cells and tissues in the body, such as differentiated neurons and heart and skeletal muscle cells, are incapable of proliferation; however, these cells are considered post-mitotic (or terminally differentiated) rather than senescent (Campisi & d'Adda di Fagagna, 2007). Additionally, many other cell types, such as stem and progenitor cells, exist in a state of quiescence – not dividing, but capable of bursts of proliferation given the proper extracellular signals (Campisi & d'Adda di Fagagna, 2007). Consequently, lack of division is not limited to the senescent state, and additional biomarkers are required to identify senescence cells. Unfortunately, no exclusive markers of senescence have been discovered. Instead, senescent cells are generally identified by an accumulation of multiple pseudo-markers or features, the combination of which defines senescence, though not all senescent cells express all possible senescence markers.

In no particular order, some of the most common features of senescence are:

1) Irreversible growth arrest. Irreversible in this context means irreversible by extracellular stimuli. No mitogenic signals or addition of growth factors can induce

a senescent cell to proliferate. However, genetic inactivation of the p53 tumor suppressor can cause a senescent cell to reenter the cell cycle, provided the cell does not also express $p16^{INK4A}$ (Beausejour et al, 2003).

2) Expression of β-galactosidase. Senescence-associated β-galactosidase (SA-βgal) is visible by histochemical staining in most senescent cells (Dimri et al, 1995), though it can also be induced by confluence of cells in culture. While the mechanism leading to β-gal expression is incompletely understood, the β-gal derives from the lysosomes and probably reflects the increase in lysosome biogenesis at senescence (Lee et al, 2006). Unfortunately, SA-βgal detection can only be performed on freshly prepared or snap-frozen samples (due to rapid degradation of the enzyme), and even then it is particular difficult to detect in tissue sections.

3) Enlargement in size and morphology. This may be more relevant in culture than *in vivo,* but senescent cells contain roughly 1.5-2 times as much protein as presenescent cells and generally cover more than twice the surface area (Hayflick, 1965).

4) Expression of $p16^{INK4A}$. While not all senescent cells express the cyclin-dependent kinase inhibitor, $p16^{INK4A}$ (Beausejour et al, 2003; Itahana et al, 2003), its expression is probably the closest thing to an exclusive senescence marker that has been identified so far. It prevents phosphorylation of pRb, causing permanent cell cycle arrest in all cells examined with the exception of tumor cells that have lost pRb function (Gil & Peters, 2006), though its expression may not be confined to senescence *in vivo*, as it seems to increase globally with age in some tissues, rather than in a subset of cells (Krishnamurthy et al, 2004; Ohtani et al, 2010; Zindy et al, 1997).

5) Formation of heterochromatic foci. Global alteration of the chromatin is initiated by pRB, leading to the permanent repression of proliferation-associated genes. These senescence-associated heterochromatic foci (SAHF) can be visualized by DAPI staining and contain markers of heterochromatin (Narita et al, 2003). SAHF have been visualized *in vivo* (Braig et al, 2005; Collado et al, 2005), however, their formation may be cell type dependent and they are a poor marker of senescence in mouse cells, which contain pericentromeric foci that can easily be mistaken for SAHF (Cerda et al, 1999).

6) Presence of DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). While cells can senescence in the absence of DNA damage (e.g. by the ectopic expression of $p16^{INK4A}$), cells that senescence due to persistent DDR signaling are characterized by the presence of DNA-SCARS (Rodier et al, 2010). DNA-SCARS are nuclear foci that contain activated DDR proteins such as ATM, CHK2, γH2A.X, 53BP1, and PML (Rodier et al, 2010), as well as dysfunctional telomeres (d'Adda di Fagagna et al, 2003; Herbig et al, 2004). Importantly, these foci are distinguishable from transient DNA damage-induced foci that do not induce senescence.

Additionally, there are widespread gene expression changes associated with senescence, many of which have unknown functional importance and specificity. Expression profiling has identified many of these gene expression changes, leading to the idea of a

"senescence score" – a rating of senescence based on a combination of senescence-associated gene expression changes and phenotypes (Lafferty-Whyte et al, 2010).

Senescent cells as a source of inflammatory factors

Among these gene expression changes at senescence is a striking increase in the expression and secretion of 40-80 factors that participate in intercellular signaling (Coppe et al, 2010b; Coppe et al, 2008; Young & Narita, 2009). Secretion of this set of factors has been termed the "senescence-associated secretory phenotype", or SASP (Coppe et al, 2008) (Table Intro-1). SASP proteins are generally induced at the level of mRNA (Coppe et al, 2008) and include a wide range of growth factors, proteases, chemokines and cytokines. Proteins that are known to stimulate inflammation, including IL-6, IL-8, IL-1, granulocyte macrophage colony-stimulating factor (GM-CSF), growth regulated oncogene (GRO)α, monocyte chemotactic protein (MCP)-2, MCP-3, MMP-1, MMP-3, and many of the Insulin-like growth factor (IGF)-binding proteins (Coppe et al, 2008; Kumar et al, 1992; Wang et al, 1996), are among the most robustly induced and secreted of these factors (Table Intro-1). While the list of specific SASP factors and their level of induction depends on both cell type and the senescence-inducing stimulus, a conserved core of factors has been seen in *in vitro* and *in vivo*, in both humans and mice, and from various cells and tissues including fibroblasts, keratinocytes, melanocytes, monocytes, liver stellate cells, endothelial cells, epithelial cells of the retinal pigment, mammary gland, colon, lung, pancreas, and prostate cells (Chang et al, 2002; Chen et al, 2002; Collado et al, 2005; Coppe et al, 2010c; Coppe et al, 2008; Csiszar et al, 2003; Eman et al, 2006; Kamino et al, 2003; Kortlever et al, 2006; Krizhanovsky et al, 2008; Kuilman et al, 2008; Lu et al, 2006; Schnabl et al, 2003; Schwarze et al, 2002; Schwarze et al, 2005; Shelton, 1999; Untergasser et al, 2002; Wajapeyee et al, 2008; Zhang, 2004; Zhang et al, 2003). Additionally, upregulated SASP factors were detected after the treatment of cancer patients with DNA damaging chemotherapy (Coppe et al, 2008).

Interestingly, the SASP appears to be chronic – it is maintained for as long as senescent cells persist in culture, even if the senescence-inducing stimulus is an acute burst of DNA damage (such as X-radiation) that is mostly resolved by DDR proteins (Rodier et al, 2009). This suggests that senescence-inducing stimuli establish permanent, self-perpetuating signaling mechanisms that maintain the pro-inflammatory phenotype, though these mechanisms are yet to be fully understood (see below for a discussion of identified SASP regulators).

Effects of the senescence-associated secretory phenotype

The SASP has many paracrine effects – some beneficial, but some deleterious if left unchecked, as expected for pro-inflammatory molecules. Senescent cells can disrupt normal tissue structure and function in mammary gland culture models (Parrinello et al, 2005), accelerate the invasion of transformed cells in a Boyden chamber assay via an epithelial to mesenchymal transition (Coppe et al, 2008), stimulate both endothelial cell invasion in a Boyden chamber assay and angiogenesis in a xenograft model (Coppe et al, 2006), and promote the proliferation of premalignant or malignant epithelial cells in culture and *in vivo* (Krtolica et al, 2001; Liu & Hornsby, 2007) (Figure Intro-2). Further, senescent endothelial cells and fibroblasts are sometimes found adjacent to malignant tumors in humans (Charalambous et al, 2007; Studebaker et al, 2008), and tumor cells themselves can senesce *in vivo* in human patients treated with DNA-damaging chemotherapy agents or in mice forced to express the potent tumor suppressor protein p53 (Coppe et al, 2008; Xue et al, 2007).

In addition to these tumor-promoting effects, there is correlative *in vivo* evidence that senescent cells are present near, and thus may contribute to, age-related pathologies (other than cancer). First, cells that express senescence markers accumulate with age in a variety of vertebrates, including zebrafish, rodents, non-human primates and humans, especially in renewable tissues such as the stroma, hematopoietic system, and epithelial organs (Dimri et al, 1995; Jeyapalan et al, 2007; Krishnamurthy et al, 2004). Second, senescent cells such as chondrocytes and endothelial cells are found at sites of age-related pathologies. These pathologies include degenerative conditions such as atherosclerosis, osteoarthritis, venous ulcers, and eroded vertebral discs (Erusalimsky & Kurz, 2005; Price et al, 2002; Roberts et al, 2006; Stanley & Osler, 2001; Vasile et al, 2001). They also include hyperproliferative diseases associated with aging, such as benign prostatic hyperplasia (Castro et al, 2003; Choi et al, 2000) and melanotic naevi (Michaloglou et al, 2005). Although the cell type has not been identified in all cases, there is strong evidence that senescent cells, and in some cases the accompanying SASP, increase with age and in many age-related pathologies.

Although the age-related results are only correlative, they suggest that, similar to chronic activation of the immune system, the senescence response, and in particular the SASP, may reduce fitness by promoting both the generalized inflammation associated with aging as well as the development of specific age-related diseases. At first glance, this conclusion seems paradoxical, considering that the SASP originates from a fitness-promoting tumor-suppressive response. However, the paradox is consistent with the evolutionary theory of antagonistic pleiotropy, which states that because of the high level of extrinsic mortality in most natural populations, there is little selective pressure for any trait that promotes fitness past the age when an organism will probably have already died from external causes (Campisi & d'Adda di Fagagna, 2007). Therefore, as long as a trait has a beneficial function early life, it can have a neutral or even deleterious function in late life without being negatively selected.

Applied to senescence, antagonistic pleiotropy allows that the senescence response, with the potentially long-term deleterious consequences of the SASP, might persist because its tumor-suppressing function keeps young organisms cancer-free. However, this explanation implies that the SASP itself has a beneficial role in early life. If the SASP were strictly deleterious, or even neutral (given the energy cost of maintaining high secretory activity), selective pressure should remove the SASP from the senescence response, leaving only the growth arrest phenotype.

Indeed, recent evidence suggests the SASP has at least two beneficial roles. First, certain key SASP factors such as IL-6, IL-8, GROα, and IGFBP-7 act in an autocrine feedback loop to reinforce the senescence growth arrest (Acosta et al, 2008; Kuilman et al, 2008; Wajapeyee et al, 2008; Yang et al, 2006) (Figure Intro-2). These factors cooperate with the p53 and pRb tumor suppressor pathways to reduce the risk of oncogenic transformation in a cell-autonomous manner. Second, the SASP might signal to the immune system to clear senescent cells (Figure Intro-2). In a mouse model of liver carcinoma, reactivation of p53 in tumor cells induces a senescence response *in vivo*; this response is followed by increased expression of several inflammatory cytokines, which stimulate an infiltrating immune response to clear senescent tumor cells (Xue et al, 2007). Lastly, the SASP might promote local tissue repair; in mouse models of liver fibrosis and wound healing, the presence of senescent cells curbed the accumulation of fibrotic tissue; this was likely due to matrix metalloprotease (MMP) production by the senescent cells (Jun & Lau, 2010; Krizhanovsky et al, 2008) and the subsequent clearance of the senescent cells by natural killer (NK) cells (Krizhanovsky et al, 2008).

These findings suggest that the SASP is important, especially early after senescence induction, for ensuring efficient growth arrest, preventing the accumulation of damaged tissue, and eventually for stimulating the immune system to clear senescent cells. However, despite the ability of the innate immune system to remove them, senescent cells accumulate with age *in vivo* (Campisi, 2005; Erusalimsky & Kurz, 2005). Thus, either immune clearance is not 100% efficient or the rate at which senescent cells are produced outpaces the rate of clearance. Consequently, the deleterious chronic-inflammatory effects of the SASP might only become apparent with time.

The SASP may contribute to aging by disrupting tissue structure and function directly, or indirectly, by attracting the immune system. However, given that the SASP may also have beneficial effects that include reinforcement of the senescence growth arrest (which would be vital for efficient tumor suppression), it is important to determine how the SASP and the senescence growth arrest are regulated, and whether the potentially deleterious effects of the SASP can be mitigated without interfering with the beneficial effects of the senescence phenotype.

Molecular mechanisms that control the senescence-associated secretory phenotype

Although our understanding of how the SASP is controlled remains incomplete, several key features of SASP regulation have been elucidated. First, it appears that the SASP is caused primarily by genotoxic stress rather than by proliferative arrest *per se*: genotoxic senescence inducers, such as ionizing radiation, hyperproliferation caused by oncogene activity, or dysfunctional telomeres, induce a SASP (Coppe et al, 2008; Rodier et al, 2009). However, the induction of senescence without genotoxic stress – for example, by overexpressing the pRb regulator $p16^{INK4A}$ – does not induce a SASP (Coppe et al, 2010b). Further, p53, one of the central mediators of the senescence growth arrest, is not required for the SASP. In fact, p53 inactivation in senescent cells enhances the expression and secretion of many SASP factors, though the mechanism of that enhancement remains unknown (Coppe et al, 2008). Because neither p16 nor p53 are required for the SASP, at least some of the pathways that regulate the SASP must be distinct from the pathways that regulate the senescence growth arrest. We describe the known SASP regulatory pathways in the following sections.

Transcriptional regulation

Most components of the SASP are upregulated at the level of mRNA abundance (Coppe et al, 2008). Moreover, the increase in mRNA levels of some factors depends on the transcription factors NF-κB and C/EBPβ, which have increased activity in senescent cells (Acosta et al, 2008; Kuilman et al, 2008). Depletion of C/EBPβ substantially diminishes the expression of both IL-6 and IL-8, which are among the most strongly upregulated SASP cytokines (Kuilman et al, 2008), and inhibition of NF-κB significantly decreases the levels of ENA-78, NAP-2, MCP-1, MCP-4, MIP-3a, and the GRO family members (Acosta et al, 2008). The activities of C/EBPβ and NF-κB are regulated by a plethora of pathways depending on the cellular context, so experimentation is required to determine which pathways are particularly important in the senescence response. Additionally, it is likely that there are other inflammation-associated transcription factors that contribute to the transcription of SASP-encoding genes, though they have yet to be identified.

DNA damage response

The DNA damage response (DDR) and several key DDR proteins are required for the expression of a subset of SASP factors, including IL-6 and IL-8 (Rodier et al, 2009). The DDR is a signal-amplification cascade that senses DNA damage, induces cell cycle arrest, and initiates DNA damage repair. If the extent of DNA damage is severe, cells undergo either apoptosis or senescence, depending on the cell type and/or level of damage. In the case of senescence, cells arrest growth and maintain chronic low-level DDR signaling (d'Adda di Fagagna, 2008). This persistent low-level DDR is necessary for a robust SASP; depletion of upstream components of the DDR cascade by RNA interference, specifically ATM, NBS1, or CHK2, prevents the increased expression of SASP factors such as IL-6, IL-8, and GRO family members, among others (Rodier et al, 2009).

IL-1α

Like many cytokine networks, the SASP also has an important positive feedback component. IL-1α is a cytokine that regulates its own synthesis through an autocrine, receptor-mediated, positive feedback loop that entails activation of NF-κB; this has been observed in culture in human myeloid and pancreatic cancer cells (Hiscott et al, 1993; Niu et al, 2004). IL-1α is also a key positive regulator of IL-6 and IL-8 expression by senescent human cells in culture (Orjalo et al, 2009). Loss of IL-1α signaling in senescent cells, whether by interfering with IL-1α expression or IL-1α receptor activity, markedly reduces the levels of IL-6 and IL-8, demonstrating that sustained IL-1R stimulation by surface-bound IL-1α is required to maintain senescence-associated IL-6 and IL-8 secretion (Orjalo et al, 2009). Reduction of IL-1α signaling also decreases NF-κB and C/EBPβ transcriptional activities. IL-1α activates NF-κB activity via the Toll-like receptor pathway (Orjalo et al, 2009). Thus, IL-1α triggers the formation of a complex between IL-1R and its co-receptor, activating a signaling cascade that ultimately permits nuclear translocation of NF-κB (Naugler & Karin, 2008). IL-1α may activate C/EBPβ activity indirectly via its regulation of IL-6 expression: depletion of IL-6 decreases C/EBPβ transcript levels (Kuilman et al, 2008). These positive feedback loops sustain the SASP, reinforcing its expression and the senescence growth arrest.

microRNAs

MicroRNAs also play a role in SASP regulation. Thus far, two microRNAs, miR-146a and miR-146b (miR-146a/b), have been demonstrated to negatively regulate the senescence-associated secretion of IL-6 and IL-8 (Bhaumik et al, 2009). Senescent human fibroblasts with a strong SASP upregulate these microRNAs, which inhibit the production of inflammatory cytokines. These microRNAs target IRAK1, which is a positive regulator of NF-κB (Taganov et al, 2006). Indeed, overexpression of miR-146a/b in senescent human fibroblasts markedly reduces IRAK1 levels, along with reducing the secretion of IL-6 and IL-8. In addition, blockage of IL-1R signaling prevents the upregulation of miR-146a/b, consistent with these microRNAs being part of the NF-κB feedback loop (Taganov et al, 2006).

Chromatin organization

Although the SASP is at least partly regulated by the activation of transcription factors, the global gene expression profile acquired at senescence probably entails large-scale changes in chromatin conformation, which is a feature of senescent cells (Adams, 2007b; Funayama & Ishikawa, 2007; Mehta et al, 2007; Narita, 2007). Consistent with this idea, a number of genes that encode SASP proteins are physically clustered in the human and mouse genomes. Among

these clusters are loci that contain MMP genes (MMP1, MMP3, MMP10 and MMP12) or CXCL and CCL cytokine family members. These loci are roughly the size of chromatin loops, which are an important unit of chromatin organization and transcriptional control (Horike et al, 2005). Senescent cells also develop large heterochromatic structures termed senescence-associated heterochromatin foci (SAHFs) (Funayama & Ishikawa, 2007). SAHFs physically contain, and most likely repress the expression of, several proliferation-promoting genes (Adams, 2007a). Virtually nothing is known about how senescence-causing stimuli bring about changes in chromatin organization, but it is likely that such changes are important for both the senescence-associated growth arrest and the SASP.

Summary

When a cell acquires characteristics or damage that is potentially oncogenic, a network of tumor suppressing pathways sense and integrate these signals to determine the cell's fate. One potential fate, cellular senescence, is a state of irreversible growth arrest that prevents uncontrolled proliferation. Unlike apoptotic cells (another potential fate), senescent cells remain metabolically active and, in addition to being growth arrested, are characterized by widespread morphological gene expression changes. One category of change is the marked increase in the expression and secretion of pro-inflammatory signaling molecules; a response termed the senescence-associated secretory phenotype (SASP). The SASP has well-documented paracine effects that can drive oncogenic phenotypes, and because senescent cells seem to accumulate with age *in vivo*, it may play an important role in triggering or sustaining age-associated chronic inflammation. A growing body of evidence suggests that chronic inflammation plays a causative role in at least some age-related diseases, and possibly in aging as a whole. Consequently, it is important to understand how the SASP is regulated, its *in vivo* effects, and the extent to which its potentially deleterious consequences can be mitigated without affecting the beneficial role of senescence as a tumor suppressing mechanism.

The data thus far suggest that the SASP is activated primarily at the transcriptional level by transcription factors such as NF-κB and C/EBPβ. This transcriptional activity is indirectly regulated (through unknown mechanisms) by several pathways during senescence: the DNA damage response pathway, an IL-1α positive feedback loop, and possibly by large-scale chromatin reorganization. Transcription activity is also subject to negative feedback during senescence: miR-146a/b act to inhibit NF-κB activity, restraining the production of IL-6 and IL-8. However, despite our understanding of these pathways, there remain many unanswered questions about SASP regulation:

1. The above players are only known to regulate a handful of SASP factors such as IL-6 and IL-8 – no pathways have been identified that regulate the majority of the SASP.
2. The DDR is activated immediately after damage, whereas the SASP takes days to develop, demonstrating that DDR proteins, though necessary for some SASP factors, are not sufficient for SASP activity. There must be at least one other, DDR-independent pathway that cooperates with the DDR to induce a SASP.
3. The SASP is an inflammatory response, but it is chronic rather than acute, suggesting that, though there may be regulatory overlap between the two types of inflammatory response (such as NF-κB activation), there must be a distinct set of pathways or a distinct mode of induction that prevents the SASP from initiating and resolving quickly, as occurs in acute inflammation.

4. The SASP can reinforce the senescence growth arrest in at least some contexts, but $p16^{INK4A}$ and p53 are not required for the SASP, suggesting that the pathways regulating the two phenotypes (SASP and growth arrest), though initiated by the same stimulus, must diverge. How this divergence occurs remains unknown.

In this dissertation I examine the above questions. In Chapter 1, I identify the p38MAPK pathway as a novel, DDR-independent pathway that regulates the vast majority of the SASP. p38MAPK was necessary and sufficient for the SASP, and I found that p38MAPK regulated transcription of SASP factors by modulating NF-κB activity, providing a mechanistic link between the upstream and downstream regulators of the SASP. The p38MAPK pathway is activated with slow kinetics after DNA damage, partially explaining why the SASP takes several days to develop. Additionally, p38MAPK activation after damage is restrained by p53, providing an explanation for how p53 restrains the SASP.

In Chapter 2, I discuss the role of the p38MAPK/NF-κB pathway in growth arrest regulation. p38MAPK was required for the senescence growth arrest by oncogenic RAS expression, but NF-κB was not. Given that p38MAPK is known to activate both the p53 and $p16^{INK4A}$ growth arrest pathways, these data suggest that p38MAPK may act as the node of divergence between the growth arrest pathways and the SASP regulatory pathways.

In Chapter 3, I switch focus and examine the loss of lamin B_1, an important component of the nuclear lamina, as a novel biomarker of senescence. Lamin B_1 was decreased by all senescence-inducing stimuli tested except $p16^{INK4A}$ expression, but not by induction of quiescence. The loss was regulated at the mRNA level rather than by caspase cleavage (as it is during apoptosis) and was independent of senescence-regulating factors like p38MAPK, NF-κB, or ATM. Additionally, lamin B_1 loss was not prevented by inhibition of RAS-induced growth arrest or senescence-associated morphological changes, suggesting that it is not simply a byproduct of cell cycle arrest or changes to nuclear morphology. Interestingly, preliminary data suggests that the prevention of lamin B_1 loss in senescent cells (by lamin B_1 overexpression) decreases DNA damage foci and increases the fraction of cells in S-phase, suggesting that lamin B_1 loss at senescence may reinforce DNA damage signaling and growth arrest. If true, this functional effect may be a result of lamin B_1 loss increasing nuclear plasticity and allowing chromatin rearrangement, or by increasing ROS signaling and sensitivity to oxidative stress (Malhas et al, 2009; Mehta et al, 2007; Narita et al, 2003). While further work needs to be done to identify the mechanism of lamin B_1 loss and its functional role at senescence, its specificity and applicability as a senescence biomarker may be helpful in identifying senescent cells in multiple contexts.

INTRODUCTION ACKNOWLEDGMENTS

Parts of this introduction were previously published in *Trends in Molecular Medicine* (Freund et al, 2010). I thank my co-authors of that work, Arturo Orjalo, Pierre Desprez, and Judith Campisi, for their valuable input.

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INTRODUCTION FIGURE LEGENDS

Figure Intro-1. Chronic inflammation is associated with most age-related diseases. There is an extensive range of conditions and diseases that are associated with chronic inflammation or that have an inflammatory component. Chronic inflammation lies at the root of heart disease, cancer, osteoporosis, Alzheimer's, diabetes and many other age-related diseases.

Figure Intro-2. Effects of the SASP on tissue homeostasis. The response of cells to the SASP depends on cell type and cell context. The SASP affects the original senescent cell by stimulating clearance by NK cells and reinforcing the senescence growth arrest. The SASP affects surrounding non-immune cells as well; it increases the proliferation of nearby epithelial and stromal cells, promotes invasion of any nearby preneoplastic or neoplastic cells via an epithelial to mesenchymal transition, stimulates angiogenesis by stimulating endothelial cell migration and invasion, and disrupts normal tissue structures and function.

Table Intro-1. SASP factors. The SASP is a complex, plastic phenotype that varies with cell type and mode of senescence induction. The SASP factors are categorized according to the fold change in secreted protein level over presenescent controls. The categories are approximate by necessity, as each cell type has different fold changes of each factor. The "senescence inducers" increase the expression of a given factor. "+" indicates well-documented pro-inflammatory proteins; "-" indicates well-documented anti-inflammatory proteins. Factors lacking + or - might also have pro- or anti-inflammatory activity, but these activities are either not well documented or highly context-dependent. Abbreviations: OIS, oncogene-induced senescence; DDIS, DNA-damage-induced senescence; REP, replicative senescence; RAS, oncogenic RAS overexpression; MEK, oncogenic MEK overexpression; XRA, high dose X-irradiation; BLEO, bleomycin treatment; ETOP, etoposide treatment. Cell types: HCA2, BJ – human foreskin fibroblasts; Wi-38, IMR-90 – human embryonic lung fibroblasts; PrECs – normal human prostate epithelial cells; BPH1, RWPE1, PC3 – transformed human prostate epithelial cells; PSC27, PSC31, PSC32 – human prostate fibroblasts. The last names in the reference column refer to the following papers: Acosta: (Acosta et al, 2008); Bavik: (Bavik et al, 2006); Coppe: (Coppe et al, 2010c; Coppe et al, 2008); Rodier: (Rodier et al, 2009); Wajapeyee: (Wajapeyee et al, 2008); Liu: (Liu & Hornsby, 2007); Krizhanovsky: (Krizhanovsky et al, 2008); Parrinello: (Parrinello et al, 2005); West: (West et al, 1996).

Figure Intro-1

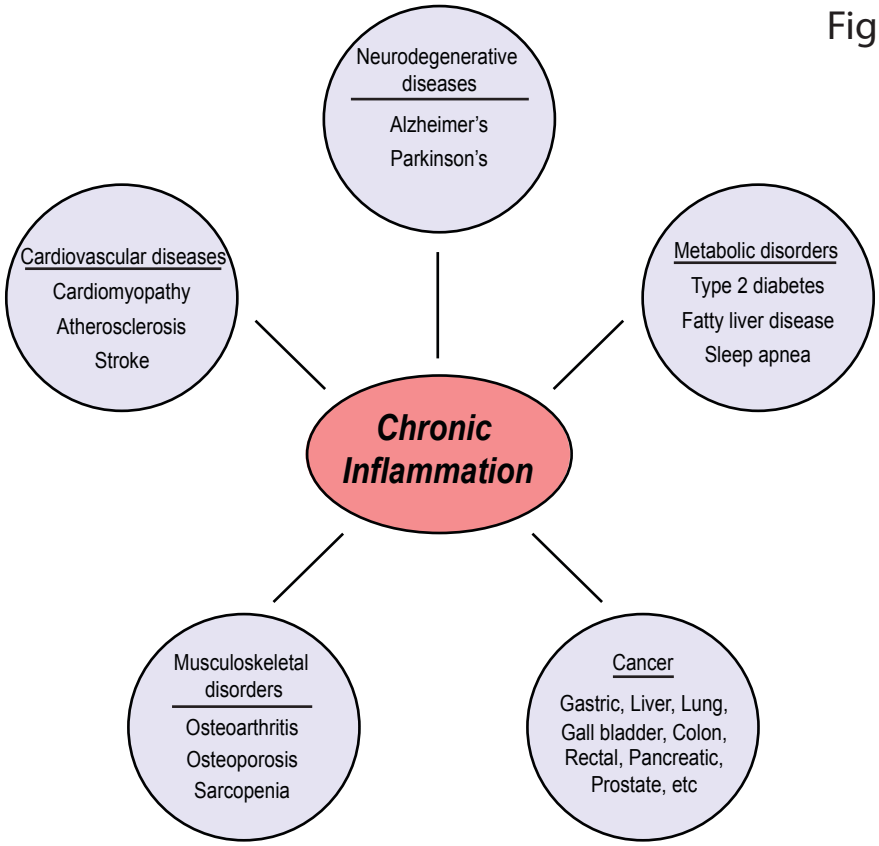

Figure Intro-2

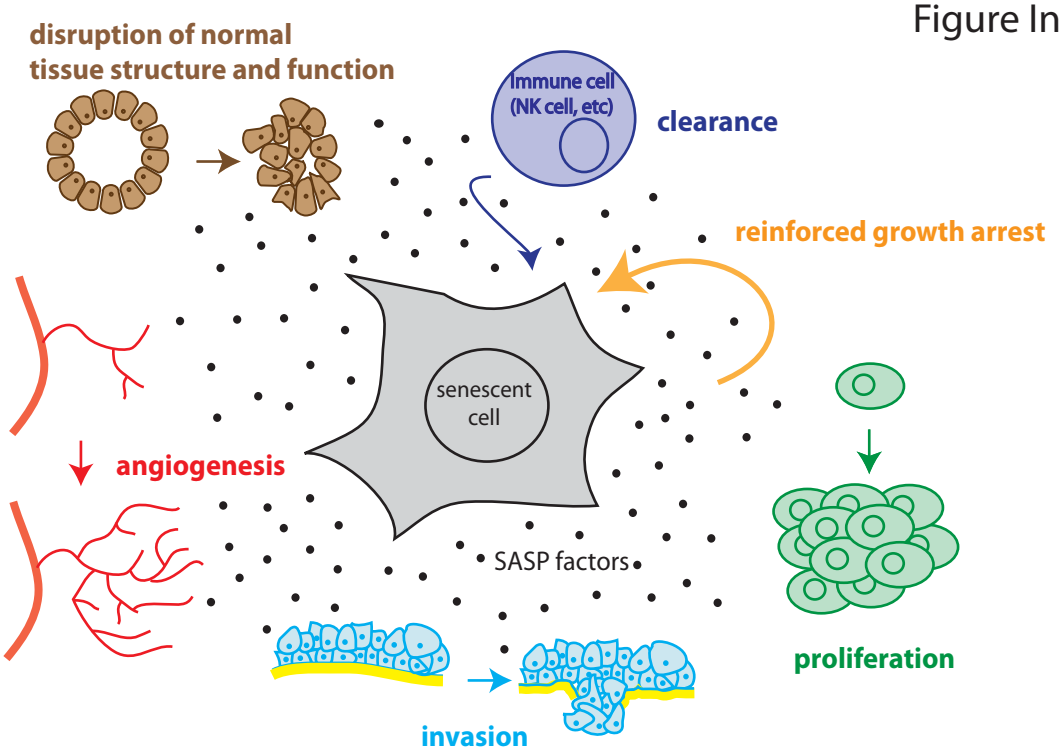

Table Intro-1

High increase (4+ fold)

Factor	Senescence inducer		Cell type	Reference
GM-CSF	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Coppe
GROα	OIS (RAS, MEK), DDIS (XRA, BLEO), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3, prostate fibroblasts (PSC27, PSC31, and PSC32)	Acosta, Coppe, Bavik
GROα,β,γ	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Rodier, Coppe
IGFBP-7	OIS (BRAF)	+	melanocytes	Wajapeyee
IL-1α	OIS (RAS, MEK), DDIS (XRA, BLEO)	+	IMR-90, HCA2, PrECs, BPH1, RWPE1, PC3	Acosta, Coppe, Liu
IL-6	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Rodier, Coppe
IL-7	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ	Coppe
IL-8	OIS (RAS, MEK), DDIS (XRA, BLEO), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3, prostate fibroblasts (PSC27, PSC31, and PSC32)	Acosta, Rodier, Coppe, Bavik
MCP-1	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Rodier, Coppe, Liu
MCP-2	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ	Coppe
MIP-1α	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
MMP-1	OIS (RAS), DDIS (XRA, BLEO), REP		IMR-90, HCA2, WI-38, BJ	Coppe, Liu
MMP-10	OIS (RAS), DDIS (XRA, BLEO, ETOP), REP		IMR-90, HCA2, WI-38, BJ, hepatic myofibroblasts	Coppe, Krizhanovsky
MMP-3	OIS (RAS), DDIS (XRA, BLEO), REP		IMR-90, HCA2, WI-38, BJ	Coppe, Liu, Parrinello

Intermediate increase (2-4 fold)

Amphiregulin	OIS (RAS), DDIS (XRA, BLEO)		PrECs, BPH1, RWPE1, PC3, prostate fibroblasts (PSC27, PSC31, and PSC32)	Coppe, Bavik
ENA-78	OIS (RAS, MEK), DDIS (XRA)	+	IMR-90, PrECs, BPH1, PC3	Acosta, Coppe
Eotaxin-3	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2	Coppe
GCP-2	OIS (RAS), DDIS (XRA)	+	HCA2, PrECs, RWPE1, PC3	Coppe
GITR	OIS (RAS), DDIS (XRA)		HCA2, PrECs, BPH1, RWPE1, PC3	Coppe
HGF	OIS (RAS), DDIS (XRA, BLEO), REP		IMR-90, HCA2, WI-38, BJ, prostate fibroblasts (PSC27, PSC31, and PSC32)	Coppe, Bavik, Liu
ICAM-1	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
IGFBP-2	DDIS (XRA, BLEO), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3, prostate fibroblasts (PSC27, PSC31, and PSC32)	Rodier, Coppe, Bavik
IGFBP-4	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ	Coppe
IGFBP-5	DDIS (BLEO), REP	+	prostate fibroblasts (PSC27, PSC31, and PSC32)	Bavik
IGFBP-6	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
IL-13	OIS (RAS), DDIS (XRA), REP	+	IMR-90, WI-38	Coppe
IL-1β	OIS (RAS), DDIS (XRA, BLEO), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe, Liu
MCP-4	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Coppe
MIF	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
MIP-3α	OIS (RAS, MEK), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Acosta, Coppe
MMP-12	DDIS (XRA, ETOP)		IMR-90, HCA2, WI-38, BJ, hepatic myofibroblasts	Coppe, Krizhanovsky
MMP-13	DDIS (XRA, ETOP)		IMR-90, HCA2, WI-38, BJ, hepatic myofibroblasts	Coppe, Krizhanovsky
MMP-14	DDIS (XRA)		IMR-90, HCA2, WI-38, BJ	Coppe
NAP2	OIS (MEK)		IMR-90	Acosta
Oncostatin M	OIS (MEK), DDIS (XRA)		IMR-90, WI-38	Acosta, Coppe
Osteoprotegerin	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
PlGF	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
RANTES	DDIS (BLEO)		HCA2	Liu
sgp130	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
TIMP-2	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
TRAIL-R3	DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ	Rodier, Coppe

Small increase (below 2 fold)

Acrp30	DDIS (XRA)		PrECs	Coppe
Angiogenin	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
Axl	DDIS (XRA), REP		WI-38	Coppe
bFGF	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
BLC	OIS (RAS)	+	HCA2, BJ	Coppe
BTC	DDIS (XRA)		PrECs	Coppe
CTACK	DDIS (XRA)	+	RWPE1, PC3	Coppe
EGF-R	DDIS (XRA)		PrECs, BPH1, PC3	Coppe
Fas	DDIS (XRA), REP		WI-38	Coppe
FGF-7	DDIS (XRA, BLEO), REP		HCA2, WI-38, BJ, prostate fibroblasts (PSC27, PSC31, and PSC32)	Coppe, Bavik
G-CSF	OIS (RAS)	+	HCA2, BJ	Coppe
GDNF	DDIS (XRA)		PrECs	Coppe
HCC-4	OIS (RAS), DDIS (XRA), REP	+	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
I-309	OIS(RAS), DDIS (XRA)	+	HCA2	Coppe
IFN-γ	OIS (RAS)	+	HCA2, BJ	Coppe
IGFBP-1	DDIS (XRA), REP	+	HCA2	Coppe
IGFBP-3	DDIS (XRA, BLEO), REP	+	HCA2, prostate fibroblasts (PSC27, PSC31, and PSC32)	Rodier, Bavik
IL-1 R1	DDIS (XRA)		HCA2	Rodier
IL-11	DDIS (XRA), REP	-	HCA2, BJ	Coppe
IL-15	DDIS (XRA), REP		IMR-90, HCA2, BJ	Coppe
IL-2R-α	DDIS (XRA)		PrECs, PC3	Coppe
IL-6 R	OIS (RAS), DDIS (XRA)		PrECs, BPH1, RWPE1, PC3	Coppe
I-TAC	OIS (RAS), DDIS (XRA)		PrECs, BPH1, RWPE1, PC3	Coppe
Leptin	DDIS (XRA), REP		IMR-90, HCA2, BJ	Coppe
LIF	OIS (MEK)		IMR-90	Acosta
MMP-2	DDIS (BLEO), REP		prostate fibroblasts (PSC27, PSC31, and PSC32)	Bavik
MSP-a	DDIS (XRA)		RWPE1, PC3	Coppe
PAI-1	REP		IMR-90, BJ, JAS-3, HUVEC	West
PAI-2	REP		IMR-90, BJ, JAS-3	West
PDGF-BB	DDIS (XRA)		BPH1, RWPE1, PC3	Coppe
SCF	DDIS (XRA), REP		IMR-90, HCA2, BJ	Coppe
SDF-1	OIS (RAS), DDIS (XRA)	+	prostate fibroblasts (PSC27, PSC31, and PSC32)	Bavik
sTNF RI	OIS (RAS), DDIS (XRA), REP	-	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
sTNF RII	DDIS (XRA), REP	-	IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Coppe
Thrombopoietin	DDIS (XRA)		BPH1, RWPE1, PC3	Coppe
TIMP-1	OIS (RAS), DDIS (XRA, BLEO)		HCA2, PrECs, BPH1, PC3, prostate fibroblasts (PSC27, PSC31, and PSC32)	Coppe, Bavik
tPA	DDIS (BLEO), REP		HCA2, IMR-90, BJ, JAS-3	West, Liu
uPA	DDIS (BLEO), REP		HCA2, IMR-90, BJ, JAS-3	West, Liu
uPAR	OIS (RAS), DDIS (XRA), REP		IMR-90, HCA2, WI-38, BJ, PrECs, BPH1, RWPE1, PC3	Rodier, Coppe
VEGF	OIS (RAS), DDIS (XRA)		PrECs, BPH1, RWPE1, PC3	Coppe

CHAPTER 1

p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype

"One of the symptoms of an approaching nervous breakdown is the belief that one's work is terribly important"

-Bertrand Russell

CHAPTER 1 ABSTRACT

Cellular senescence suppresses cancer by forcing potentially oncogenic cells into a permanent cell cycle arrest. Senescent cells secrete growth factors, proteases and inflammatory cytokines, termed the senescence-associated secretory phenotype (SASP). The SASP can be beneficial or deleterious, depending on the context. Although much is known about the pathways that regulate the senescence growth arrest, far less is known about pathways that regulate the SASP. We previously showed that DNA damage response (DDR) signaling is essential but not sufficient to establish and maintain the SASP. Additionally, p53, while required for the growth arrest, is not required for the SASP and in fact restrains the phenotype. Here, we delineate a crucial pathway for regulating the SASP and its relationship to the DDR and p53. We show, in normal human fibroblasts, that senescence-inducing stimuli such as ionizing radiation or oncogenic RAS activate p38MAPK with kinetics that parallel the development of the SASP. p38MAPK inhibition markedly reduced the secretion of most SASP factors, and constitutive activation of p38MAPK was sufficient to induce a robust SASP. Moreover, p53 restrained p38MAPK activation such that p38MAPK was more active in p53-deficient cells, and the amplified SASP caused by p53 deficiency was p38MAPK dependent. Further, p38MAPK activation was independent of the DDR and constitutive p38MAPK activation induced a SASP without inducing DDR signaling. Mechanistically, p38MAPK induced the SASP largely by increasing NF-κB transcriptional activity. These findings assign p38MAPK a novel role in SASP regulation – one that is necessary, sufficient, and independent of previously described pathways.

CHAPTER 1 INTRODUCTION

Cellular senescence halts the proliferation (used here interchangeably with growth) of cells that are at risk for malignant transformation. Many potentially oncogenic stimuli, ranging from direct DNA damage to the activation of certain oncogenes, can induce a senescence response (Campisi & d'Adda di Fagagna, 2007). Recent data showing that cellular senescence is a common response to oncogene activation *in vivo* (Prieur & Peeper, 2008) suggest that senescence may be as important as apoptosis for suppressing the development of cancer.

The senescent phenotype is multi-faceted. The chief hallmark of senescent cells – the senescence growth arrest -- is essentially irreversible in that it cannot be reversed by physiological stimuli. This arrest is established and maintained by two major tumor suppressor pathways governed by the p53 and p16INK4a/pRB proteins, respectively. Although many questions remain, we understand in broad strokes the interwoven and complementary mechanisms by which these pathways regulate the growth arrest (Campisi & d'Adda di Fagagna, 2007; Collins & Sedivy, 2003; Gil & Peters, 2006; Ohtani et al, 2004; Rodier et al, 2007). Senescent cells also develop an enlarged morphology, upregulate enzymes such as the senescence-associated β-galactosidase (SA-βgal), and show widespread changes in chromatin organization and gene expression (Campisi & d'Adda di Fagagna, 2007). Much less is known about the mechanisms that regulate these phenotypes. Of particular biological importance, senescent cells show a striking increase in the expression and secretion of numerous cytokines, chemokines, matrix metalloproteinases (MMPs), and other proteins that can alter local tissue environments. We termed this feature the senescence-associated secretory phenotype (SASP) (Coppe et al, 2008).

The SASP can be beneficial or deleterious, depending on the biological context. Among the benefits, some SASP factors -- for example, IL-6, IL-8, PAI-1, and IGFBP7 -- reinforce the senescence growth arrest in a cell autonomous manner, thereby suppressing tumorigenesis (Acosta et al, 2008; Kortlever et al, 2006; Kuilman et al, 2008; Wajapeyee et al, 2008). Other SASP factors may signal the immune system to clear senescent cells (Xue et al, 2007), and SASP MMPs can suppress the formation of fibrotic scars (Jun & Lau, 2010; Krizhanovsky et al, 2008). Among the deleterious effects, SASP MMPs can disrupt mammary alveolar and ductal morphogenesis in cell culture models (Parrinello et al, 2005). Perhaps more importantly, SASP factors can promote phenotypes associated with aggressive cancer (Krtolica et al, 2001), including cell proliferation (Bavik et al, 2006; Coppe et al, 2010b), angiogenesis (Coppe et al, 2006), epithelial-to-mesenchymal transitions and invasiveness (Coppe et al, 2008), and accelerated growth of xenografted tumors (Krtolica et al, 2001; Liu & Hornsby, 2007). Moreover, because the SASP includes pro-inflammatory cytokines, senescent cells, which increase with age *in vivo* (Dimri et al, 1995; Jeyapalan et al, 2007; Paradis et al, 2001; Zhou et al, 2008), may contribute to the low-level chronic inflammation that is a hallmark of aged mammalian tissues and most, if not all, major age-related diseases (Coppe et al, 2010a; Freund et al, 2010). Given the known and proposed importance of the SASP, it is crucial to understand the pathways that regulate this phenotype.

Although the senescence growth arrest and SASP are often coordinately induced, the pathways that regulate them do not completely overlap. For example, $p16^{INK4A}$ expression is sufficient to induce a senescence growth arrest, but does not induce or modify the SASP (Coppe et al, 2010a). Additionally, p53 is required for the growth arrest (Campisi & d'Adda di Fagagna, 2007; Courtois-Cox et al, 2008), but is not required for the SASP; quite the reverse – cells lacking functional p53 secrete markedly higher levels of most SASP components. Thus,

p53 actively restrains the SASP (Coppe et al, 2008), suggesting it may suppress tumorigenesis in part by limiting the development of a pro-inflammatory tissue environment created by the SASP. The pathway(s) by which p53 restrains the SASP is unknown.

The SASP is not an acute (rapid, transient) inflammatory response. It does not develop immediately after cells experience a senescence-inducing stimulus, and persists for long intervals, if not indefinitely (Coppe et al, 2010b; Coppe et al, 2008; Rodier et al, 2009). One partial regulator of the SASP is the DNA damage response (DDR). DNA damage is a common inducer of both the senescence growth arrest and the SASP, whether the damage is caused directly, for example by ionizing radiation, or indirectly, for example by hyperproliferative stimuli such as activated oncogenes (Campisi & d'Adda di Fagagna, 2007). DDR proteins such as ATM, CHK2, and NBS1 are essential for establishing and maintaining the expression of several SASP proteins, particularly inflammatory cytokines such as IL-6 and IL-8 (Rodier et al, 2009). However, the DDR is not sufficient for the SASP. A transient DDR, caused by a low level of ionizing radiation that does not induce senescence, does not induce the SASP (Rodier et al, 2009). Additionally, the SASP, like some other features of the senescent phenotype (e.g., cell enlargement and SA-βgal expression), takes several days to develop after the damaging event. Thus, there must be an additional, slower event that induces the SASP and is regulated independently of the DDR.

Here we show that this event is activation of the p38MAPK pathway. p38MAPK is a member of the mitogen-activated protein kinase (MAPK) family. MAPKs respond to a wide range of extracellular stimuli and mediate diverse cellular responses, all of which depend on the nature of the stimulus, cell type and physiological context. Like other MAPK members, p38MAPK is activated by phosphorylation, and this generally occurs rapidly (within minutes) and transiently (subsiding within a few hours) in response to acute cellular stress (Cuenda & Rousseau, 2007). p38MAPK is known to be important for the senescence growth arrest due to its ability to activate both the p53 and pRb/p16 growth arrest pathways (Kwong et al, 2009). p38MAPK inhibition can moderately delay the senescence arrest caused by dysfunctional telomeres, which resemble DNA double strand breaks (Iwasa et al, 2003), and the rapid senescence of cells from patients with Werner syndrome, a premature aging disorder (Davis & Kipling, 2009). Further, p38MAPK activity is required for the senescence arrest caused by oncogenic RAS, and constitutive p38MAPK activity can induce a senescence-like growth arrest in normal human cells (Deng et al, 2004; Wang et al, 2002). However, it is not known whether p38MAPK regulates the SASP. p38MAPK has been implicated in regulating specific cytokines such as IL-6, IL-8, and TNFα in other biological contexts (Ono & Han, 2000; Zhang et al, 2007), but these are generally acute responses, whereas the SASP is chronic and multi-faceted, comprising >40 cytokines, chemokines, growth factors, MMPs, shed receptors and ligands, etc. Recently, p38MAPK inhibition was shown to reduce the expression of IL-8 in MEK-induced senescence, leading us to ask whether it may play a more general role in SASP regulation (Acosta et al, 2008).

We show that p38MAPK activity is necessary and sufficient for development of a SASP in cells induced to senesce by either direct DNA damage or oncogenic RAS. We also show that p38MAPK is not activated with the usual acute kinetics, but rather activation occurs with delayed slow kinetics characteristic of the SASP. Further, p53 restrains the SASP by restraining p38MAPK activation and that activation occurs independently of the DDR. We found that p38MAPK regulates the SASP through NF-κB transcriptional activity, which we show is required for the expression of most SASP factors. These findings assign the p38MAPK

pathway a novel role in senescence – one that is necessary, sufficient and independent of previously described SASP-regulatory pathways.

CHAPTER 1 RESULTS

Unless indicated otherwise, error bars represent standard deviation.

p38MAPK is activated during the senescence response to genotoxic stress

p38MAPK is activated by tyrosine and threonine phosphorylation in response to a variety of stresses (Cuenda & Rousseau, 2007), including the response to oncogenic RAS (Ha-RAS^{V12}) (Wang et al, 2002), which indirectly causes DNA damage (Di Micco et al, 2006). To determine whether p38MAPK activation is a direct genotoxic stress response, we X-irradiated (XRA; 10Gy) presenescent (PRE) normal human fibroblasts (strain HCA2) to synchronously induce senescence (SEN(XRA)). After XRA, cells were cultured for 10 d, during which time they developed classic markers of senescence: growth arrest (no increase in cell number, low 5-bromodeoxyuridine (BrdU) labeling), an enlarged flattened morphology, and increased senescence-associated β-galactosidase (SA-βgal) activity (Dimri et al, 1995) (Figure 1-1A, 1-1B; not shown). To assess p38MAPK activation, we prepared whole cell lysates every 2 d after XRA until cells developed a complete senescent phenotype 8-10 d later. We analyzed levels of total and phosphorylated p38MAPK and its downstream target Hsp27 (Beyaert et al, 1996; Davis et al, 2005) by western blotting. Phosphorylated p38MAPK (p38-P) and phosphorylated Hsp27 (Hsp27-P) did not increase immediately after XRA (Figure 1-1C). Rather p38-P and Hsp27-P levels began to rise only 2-4 d after XRA, reaching maximal levels, which were sustained for weeks (not shown), 8-10 d later (Figure 1-1C). Thus, the p38MAPK response to senescence-inducing genotoxic stress differed markedly in kinetics from the rapid, transient activation that occurs after acute stresses (e.g., TNFα or LPS stimulation) (Cuenda & Rousseau, 2007) that do not induce senescence. Importantly, the kinetics of p38MAPK activation closely paralleled the kinetics with which the SASP develops (Coppe et al, 2008; Rodier et al, 2009).

p38MAPK activity is required for the SASP

The activation of p38MAPK during senescence was inhibited by the well-characterized small molecule SB203580 (SB). SB displaces ATP from the p38MAPKα and β ATP-binding pocket (Young et al, 1997), thereby preventing p38MAPK from phosphorylating its targets without preventing p38MAPK phosphorylation itself. As expected and determined by Hsp27-P levels, daily treatment with 10 μM SB, which was reported to have minimal off-target effects (Cuenda et al, 1995; Wilson et al, 1997), prevented p38MAPK activation after XRA (Figure 1-1C).

To determine the significance of the coincident rise in p38MAPK activity and the SASP, we added SB to SEN(XRA) cells for 48 h and then assessed IL-6, an indicator of SASP activity (Bhaumik et al, 2009; Coppe et al, 2010a; Coppe et al, 2008; Orjalo et al, 2009) by immunostaining (intracellular levels) and enzyme-linked immunoadsorbent assay (ELISA) of conditioned medium (CM) (secreted levels). Both assays showed that SB reduced IL-6 levels to near-PRE levels (Figures 1-1D, 1-1E); ELISA showed that SB also significantly reduced secretion of the SASP components IL-8 and GM-CSF (Figure 1-1E, $p<0.05$). Further, SB significantly reduced secreted IL-6 levels in CM from SEN(XRA) WI-38, an unrelated human

fibroblast strain (Figure 1-1F, $p<0.01$), and replicatively senescent (SEN(REP)) HCA2 and WI-38 cells (Figure 1-1G, $p<0.01$). Thus, the ability of the p38MAPK inhibitor to significantly reduce senescence-associated IL-6 secretion was not confined to XRA-induced senescence or a single cell strain. Taken together, these findings suggest that p38MAPK activation is necessary for the secretion of at least some components of the SASP.

Although SB is a well-characterized p38MAPK inhibitor, one report showed it can partially inhibit protein kinase B (PKB) at the concentration used here (10 μM) (Lali et al, 2000). To determine whether the effect of SB on SASP components was p38MAPK-specific, we generated an SB203580-insensitive p38MAPK mutant with three amino acid substitutions: T-106-M, H-107-P, L-108-F. These residues all reside in or around the ATP binding pocket, and the substitutions increase the size of the residue side chains, preventing SB from binding to the ATP pocket (Eyers et al, 1998; Gum et al, 1998). When this drug-resistant mutant (p38 DR) was expressed in senescent cells (via lentiviral infection), SB was no longer able to inhibit the phosphorylation of the downstream target of p38MAPK, Hsp27 (Figure 1-1H), demonstrating that expression of the mutant prevented SB from inhibiting p38MAPK signaling. We then asked whether SB was able to inhibit senescence-induced IL-6 in the presence of p38 DR. We found that, while expression of wild type p38 (p38 WT) did not prevent SB from inhibiting IL-6 in senescent cells, expression of p38 DR prevented IL-6 inhibition (Figure 1-1I). These data demonstrate that SB203580 acts via p38MAPK inhibition to inhibit the SASP.

Though the SASP-specific effects of SB were mediated by p38MAPK, SB inhibits both the α and β isoforms of p38MAPK (Enslen et al, 1998). To identify the isoform(s) that regulate the SASP, we depleted cells of p38MAPKα, the most abundantly expressed isoform, by RNA interference (RNAi). We expressed in SEN(XRA) cells either of two unrelated short hairpin (sh) RNAs that specifically target p38MAPKα (Figure 1-1J) using lentiviruses. We then assayed IL-6 levels in CM from cells expressing control (shGFP) or p38MAPKα-specific (shp38α) shRNAs. Both shp38α shRNAs significantly decreased secreted IL-6 levels in SEN(XRA) cells (Figure 1-1K, $p<0.01$). An shRNA against p38MAPKβ did not reduce secreted IL-6 (not shown). These data confirm that p38MAPK is essential for induction of the SASP and identify p38MAPKα as the major functional isoform.

The SASP is a complex network comprising >40 proteins (Coppe et al, 2010b; Coppe et al, 2008). To determine which SASP factors are regulated by p38MAPK, we analyzed CM from PRE and SEN(XRA) cells, with or without p38MAPK inhibition, using an array containing antibodies against 120 secreted proteins (see Materials and Methods). This analysis identified 37 proteins that were significantly upregulated in SEN(XRA), compared to PRE, cells (Figure 1-1L). The majority of these proteins (68%, 25/37) declined significantly ($p<0.05$) following p38MAPK inhibition (SEN(XRA)+SB) (Figures 1-1L asterisks; 1M), and the remaining SASP proteins exhibited non-significant ($p>0.05$) decreases (Figure 1-1L). The p38MAPK-regulated proteins included cytokines, chemokines, growth factors, shed ligands and, importantly, 9 of the 10 most robustly secreted SASP proteins. Hierarchical clustering (Eisen et al, 1998) of the array results showed that SEN(XRA) cells treated with SB had a SASP profile that more closely resembled PRE cells than untreated SEN(XRA) cells (Figure 1-1L). p38MAPK inhibition slightly increased the secreted levels of a few proteins, although the changes were not significant (Figure 1-1N, $p>0.05$ for all). The SASP also includes several matrix metalloproteases (MMPs), most prominently MMP1 and MMP3 (Coppe et al, 2010b). Using the same treatment regimen as above, p38MAPK inhibition had no effect on secreted MMP1 or MMP3 levels, and even when p38MAPK inhibition was started before XRA and continued until sample collection, only MMP3 was decreased; MMP1 was not affected (Figure 1-1O). Thus, p38MAPK is a less potent

regulator of SASP MMPs, but a strong positive regulator of many SASP chemokines, cytokines and growth factors.

p38MAPK inhibition mitigates a paracrine effect of senescent cells

Conditioned media from senescent cells stimulates the ability of cancer cells to invade a basement membrane (Coppe et al, 2008). To determine whether p38MAPK inhibition mitigates this cell non-autonomous effect of the SASP, we measured the ability of CM from PRE or SEN(XRA) cells expressing either a control shRNA (shGFP) or p38MAPKα (shp38α) shRNA to stimulate the invasiveness of MDA-MB-231 human breast cancer cells through a basement membrane. SEN(XRA) CM stimulated ~6-fold more invasion than PRE CM (Figure 1-1P, $p<0.001$), as expected (Coppe et al, 2008). p38MAPK depletion markedly reduced this stimulatory activity (Figure 1-1F, $p<0.001$), indicating that p38MAPK inhibition can mitigate an important biological consequence of the SASP.

p38MAPK inhibition mitigates the SASP induced by oncogenic RAS expression

Senescence can be induced by the activation of certain oncogenes, including the oncogenic form of H-RAS (RAS^{V12}) (Serrano et al, 1997). As reported (Di Micco et al, 2006), oncogenic H-RAS^{V12} expression stimulated hyperproliferation for several days, resulting in DNA damage and ultimately a senescence growth arrest 8-10 d later (SEN(RAS)) (not shown). Similar to SEN(XRA) cells, and as reported (Deng et al, 2004; Wang et al, 2002), (SEN(RAS) cells showed increased levels of phosphorylated p38MAPK (Figure 1-2A). Also as reported (Coppe et al, 2008), SEN(RAS) cells expressed an amplified SASP -- secretion of several proteins at significantly higher levels than those secreted by SEN(XRA) cells -- and secreted factors distinct from those that comprise the SEN(XRA) SASP. In HCA2 cells, the SEN(RAS) SASP included 83 proteins (Figure 1-2B). p38MAPK inhibition (SEN(RAS+SB) significantly reduced ($p<0.05$) the levels of 78% (65/83) of these proteins (Figure 1-2B, asterisks), including 9 of the 10 most robustly secreted SASP proteins. The remaining SASP proteins were all non-significantly ($p>0.05$) reduced by p38MAPK inhibition (Figure 1-2B). p38MAPK inhibition also significantly reduced MMP1 and MMP3 levels in SEN(RAS) cells, although to a lesser extent than it reduced most cytokines and chemokines (Figure 1-2C, $p<0.05$). The SASP proteins affected by p38MAPK inhibition in SEN(RAS) cells overlapped with many of those affected by p38MAPK inhibition in SEN(XRA) cells: of 23 factors upregulated in both SEN(XRA) and SEN(RAS), 78% (19/23) were significantly decreased by p38MAPK inhibition in both cases (Figure 1-2D). Thus, a majority of the SASP factors induced by both genotoxic stress (XRA) and oncogene activation (RAS) depends on p38MAPK activity.

p53 restrains the SASP by restraining p38MAPK activity

p53 is required for the senescence growth arrest, but not the SASP. Rather, p53 restrains the SASP – when cells lacking functional p53 are induced to senesce, the resulting SASP is markedly amplified compared to cells with wild type p53 (Coppe et al, 2008). The mechanism by which p53 restrains the SASP is not known. To determine the relationship between p53 and p38MAPK during development of the SASP, we inactivated p53 using retrovirally-delivered GSE22, a peptide that prevents p53 tetramerization, and thus p53 transcriptional activity (Ossovskaya et al, 1996). Because p53 monomers are not rapidly degraded, GSE22 activity can be monitored by the accumulation of p53 protein (Figure 1-2E, SEN(XRA)+GSE). We induced p53-deficient cells to senesce with XRA (SEN(XRA)+GSE), and compared phosphorylated p38MAPK levels with those in SEN(XRA) and SEN(RAS) cells.

Activated p38MAPK levels were highest in SEN(XRA)+GSE cells, followed by SEN(RAS) and then SEN(XRA) cells (Figure 1-2E). The relative levels of p38MAPK phosphorylation qualitatively matched the relative levels of IL-6 secretion (Figure 1-2E vs 1-2F), suggesting that p53 and RAS regulate the intensity of the SASP by regulating the level of p38MAPK activation. p53 also regulated the kinetics of SASP development by regulating the timing of p38MAPK activation. When p53 was inactivated by GSE22, p38MAPK phosphorylation occurred more rapidly after XRA compared to cells with wild type p53 (Figure 1-2G). To determine whether this increase in p38MAPK activity was responsible for the amplified SASP in p53-deficient cells, we inhibited p38MAPK with SB. The amplified levels of IL-6, IL-8, and GM-CSF were almost completely suppressed by p38MAPK inhibition (Figure 1-2H, $p<0.001$). We obtained similar results when we depleted cells of p53 by RNAi (Figures 1-2I, 1-2J, $p<0.001$). Importantly, inactivation of p53 did not simply sensitize the cell to any amount of DNA damage, suggesting that this relationship between p53, p38MAPK, and IL-6 is senescence-specific – whereas a senescence inducing dose of XRA (10 Gy) caused a marked increase IL-6 secretion after three days in GSE-expressing cells versus vector controls, a non-senescence inducing dose of XRA (0.5 Gy), which nevertheless induces 53BP1 foci in all nuclei and engages the DDR (Rodier et al, 2009) did not increase IL-6 in either GSE or vector expressing cells (Figure 1-2K). We conclude that p53 restrains p38MAPK activity after senescence induction, which in turn restrains the SASP and prevents development of an amplified SASP.

We investigated the mechanism by which p53 restrains p38MAPK activity. Reportedly, the expression of Wip1, a phosphatase that dephosphorylates both p38MAPK and CHK2, is p53 dependent (Oliva-Trastoy et al, 2007; Takekawa et al, 2000; Yu et al, 2007). Additionally, it is reported to be induced in response to DNA damage such as ionizing radiation (Fiscella et al, 1997). To determine whether this phosphatase was the mechanistic link between p53 and p38MAPK, we asked whether Wip1 overexpression was able to inhibit GSE-amplified IL-6 after XRA. Despite leading to high Wip1 levels (Figure 1-2L), Wip1 overexpression did not decrease IL-6 secretion in GSE-expressing, XRA treated cells (Figure 1-2M, $p>0.05$). Inversely, depletion of Wip1 by RNAi (Figure 2N) did not increase p38MAPK activation (Figure 1-2O) or IL-6 expression after XRA (Figure 1-2P, $p>0.05$). Taken together, these data demonstrate that Wip1 does not restrain p38MAPK or IL-6 after XRA and thus is not the mechanism by which p53 restrains p38MAPK activity at senescence.

p38MAPK activity is sufficient to induce a SASP

To determine whether p38MAPK activity is sufficient for development of a SASP, we infected PRE cells with a constitutively active mutant (MKK6EE) of MAP kinase kinase 6 (MKK6), which directly phosphorylates p38MAPK. As expected, MKK6EE expression caused constitutive phosphorylation of endogenous p38MAPK (Figure 1-3A). Moreover, MKK6EE expression induced SA-βgal activity in two different cell strains (Figure 1-3B). This growth arrest was accompanied by proliferative arrest (Figure 1-3C), a decrease in BrdU incorporation (Figure 1-3D), and senescent-like morphology (Figure 1-3E). These responses were prevented by the p38MAPK inhibitor SB203580, demonstrating that they depend on p38MAPK activity.

Using antibody arrays, we identified 19 factors that were significantly upregulated in MKK6EE-expressing cells relative to PRE controls (Figure 1-3F). p38MAPK inhibition (MKK6EE+SB) significantly ($p<0.05$) reduced the increased secreted levels of most of these proteins (84%, 16/19) (Figure 1-3C asterisks) and non-significantly ($p>0.05$) reduced the levels

of the remaining proteins (Figure 1-3F). Notably, 7 of the 10 most upregulated factors in SEN(XRA) cells, and 9 of the 10 most upregulated factors in SEN(RAS) cells, increased significantly upon MKK6EE expression (Figure 1-3G). We validated the array results by ELISA measurements of secreted IL-6, which was ~500-fold higher than in PRE cells (Figure 1-3H, $p<0.001$). Constitutive p38MAPK activity was also sufficient to induce MMP1 and MMP3, although to a lesser extent than it induced the cytokines and chemokines (Figure 1-3I, $p<0.01$). Lastly, we demonstrated that IL-6 was induced within one day of MKK6EE infection (Figure 1-3J, $p<0.01$) and increased over the next two days as MKK6EE levels increased (Figures 1-3J and 1-3K, $p<0.001$), suggesting that constitutive p38MAPK activation is the rate-limiting step for SASP induction. Together, these data show that constitutive p38MAPK activity is sufficient to induce a robust SASP that resembles the SEN(XRA) and SEN(RAS) SASPs, in addition to inducing a growth arrest, SA-βgal expression, and senescent-like morphology.

p38MAPK regulates the SASP independently of the DNA damage response

The DNA damage response (DDR) is required for expression of a subset of SASP proteins, including IL-6 and IL-8 (Rodier et al, 2009). To determine whether p38MAPK inhibition decreases the SASP by inhibiting the DDR, we induced senescence by XRA and measured the activities of several DDR proteins with or without p38MAPK inhibition (SB). p38MAPK inhibition had no effect on the rapid (within 2 h) phosphorylation of ATM, CHK2 or p53(Ser15), nor on the transient stabilization of p53 and expression of p21 after XRA (Figure 1-4A). p38MAPK inhibition also did not prevent the low level activation of these DDR proteins that persists after XRA (>2 d) (Rodier et al, 2009; Rodier et al, 2010) (Figure 1-4A). These data suggest that p38MAPK inhibition does not suppress the SASP by suppressing the DDR.

In the presence of existing DNA damage, p38MAPK can replenish short-lived DNA damage foci in a subset of cells via a ROS feedback loop (Passos et al, 2010). In agreement with those findings, p38MAPK inhibition had no effect on the formation or resolution of 53BP1 foci for the first 4 d after XRA, but slightly decreased foci number 6-8 d after XRA (Figure 1-4B, $p<0.01$), in SEN(REP) cells (Figure 1-4C, $p<0.01$), and in SEN(RAS) cells (Figure 1-4D, $p<0.05$). However, in contrast to XRA, REP, and RAS, constitutive p38MAPK activity (MKK6EE expression) did not substantially increase DNA damage, as measured by nuclear 53BP1 foci (Figure 1-4E). Thus, most SEN(RAS), SEN(XRA) and (SEN(REP) cells harbored ≥3 53BP1 foci per nucleus, but most cells induced to senesce by MKK6EE harbored <3 53BP1 foci and were not substantially different from PRE cells (Figure 1-4E). There was a slight, significant increase in the percentage of MKK6EE-induced senescent cells with ≥4 foci 53BP1 foci/nucleus, but these cells accounted for only ~6% of the total (Figure 1-4F, $p<0.05$). Further, constitutive p38MAPK activity did not induce ATM or CHK2 phosphorylation (Figure 1-4G), and depletion of ATM or CHK2 by RNAi (Figure 1-4H) had no effect on the IL-6, IL-8 or GM-CSF secretion induced by MKK6EE (Figure 1-4I, $p>0.05$), demonstrating that p38MAPK does not regulate the SASP via DDR modulation. Conversely, the DDR could regulate SASP factors by modulating p38MAPK activity. However, neither ATM nor CHK2 depletion suppressed p38MAPK phosphorylation in SEN(XRA) cells (Figure 1-4J). Together, these findings indicate that p38MAPK activity uniquely regulates the SASP independently of the DDR.

p53 restrains DDR signaling, but that is not the mechanism by which p53 restrains p38MAPK

Though p38MAPK activation at senescence in normal cells is not mediated by the canonical DDR, we were interested in whether the amplified p38MAPK activation seen in

senescent cells lacking functional p53 was DDR-dependent. p53 inactivation by GSE markedly increased the growth rate of PRE cells (Figure 1-4K) and led to a small but significant increase in the number of 53BP1 foci per nucleus (Figure 1-4L, $p<0.001$). Additionally, p53 inactivation increased DDR signaling in PRE cells, as measured by total and phosphorylated CHK2 (Figure 1-4M). However, despite this increase in DDR signaling, p53 inactivation did not increase (and in fact slightly decreased) both p38MAPK activation (Figure 1-4M) and IL-6 secretion (Figure 1-4N) in PRE cells, demonstrating that p38MAPK activation and the DDR are not coordinately regulated in p53-inactivated cells, and further supporting the conclusion that DDR signaling alone is not sufficient to induce the SASP.

After a senescence-inducing dose of DNA damage, p53 inactivation inhibits cell cycle arrest, potentially causing complex DNA damage by allowing cells to enter S-phase, leading to replication fork collapse when the DNA replication machinery encounters DNA double strand breaks (Coppe et al, 2008). Suggesting that p53 inactivation does, in fact, lead to additional DNA damage (or impaired resolution of existing DNA damage), p53 inactivation caused cells to more slowly resolve 53BP1 foci after XRA (Figure 1-4O, $p<0.05$ between 6 and 48 h after XRA). We hypothesized that, with this additional DNA damage, DDR signaling might be required for amplified p38MAPK activity. However, CHK2 depletion had no effect on amplified p38MAPK phosphorylation in post-XRA, GSE-expressing cells (Figure 1-4P). Combined with the data in the previous section, these observations demonstrate that p38MAPK is regulated independently of the canonical DDR in both normal senescence and in the p53-deficient background that leads to an amplified SASP.

ROS are not required for p38MAPK activation at senescence

Reactive oxygen species (ROS) are involved in the establishment and stabilization of the senescence growth arrest: elevated ROS levels are associated with replicative and oncogene-induced senescence (Lu & Finkel, 2008; Passos et al, 2007; Ramsey & Sharpless, 2006; Saretzki et al, 2003) and are a reported activator of p38MAPK (Jun & Lau, 2010; Passos et al, 2010). Additionally, p38MAPK activity can induce ROS production (Koli et al, 2008; Passos et al, 2010; Torres & Forman, 2003), generating a positive feedback loop. To determine if this feedback loops plays a role in p38MAPK regulation of the SASP, we examined whether ROS were required for p38MAPK activation at senescence, and whether constitutive p38MAPK activation induced ROS production.

We treated DNA damage-induced senescent cells with the ROS scavenger N-acetyl cysteine (NAC) to determine whether ROS signaling was necessary for sustained p38MAPK activation at senescence. NAC has been demonstrated to effectively blunt ROS signaling, allowing a subset of cells to escape the senescence growth arrest (Passos et al, 2010). Interestingly, 10 mM NAC treatment for 48 hours before CM collection led to a moderate decrease in IL-6 secretion (Figure 1-4Q, $p<0.05$). However, this was not accompanied by a decrease in p38MAPK activation (Figure 1-4R), suggesting either that ROS affects a different SASP-regulatory pathway, or that ROS act downstream of p38MAPK to regulate IL-6.

To distinguish between these two possibilities, we examined the role of ROS in cells with constitutively active p38MAPK. First, to measure of ROS in MKK6EE-expressing cells, we examined the level of H_2O_2 by DCFDA flow cytometry. Constitutive p38MAPK activation (by MKK6EE) led to a significant increase in H_2O_2 levels (Figure 1-4S, $p<0.01$); 10 mM NAC for 48 h before sample collection significantly reduced the H_2O_2 increase (Figure 1-4S, $p<0.01$). However, NAC treatment did not decrease MKK6EE-induced IL-6 secretion (Figure 1-4T,

p>0.05). These data suggest that, although ROS are partially required for senescence-induced IL-6 secretion, they regulate a pathway that is independent of p38MAPK.

Though ROS are not required for p38MAPK activation during normal XRA-induced senescence, inactivation of p53 might increase the production of ROS after XRA, leading to the amplified p38MAPK levels seen in these cells. To determine whether amplified p38MAPK activation or amplified IL-6 secretion was dependent on ROS signaling, we treated irradiated, GSE-expressing cells with 10 mM NAC for 48 h before sample collection. In this case, not only was there no effect on amplified p38MAPK phosphorylation (Figure 1-4U), but there was also no effect on amplified IL-6 secretion (Figure 1-4V, p>0.05). This later result suggests that the unknown, SASP-regulatory pathway that is ROS-dependent in normal senescence (refer to Figure 1-4Q) is either not ROS-dependent when p53 is inactivated, or the level of ROS is high enough in p53 inactivated cells that NAC is no longer effective.

As a whole, these data demonstrate that p38MAPK is not regulated by the DNA damage response or ROS signaling in normal or p53-deficient senescence. Additionally, p38MAPK does not regulate the SASP by activating the DDR. Though constitutive p38MAPK activity induced some ROS expression, this ROS expression did not play a role in the MKK6EE-induced SASP. Many of these pathways regulate p38MAPK in other cellular contexts; thus it is clear that the mechanisms of activation and integration of signaling pathways at senescence are distinct from the mechanisms of the more traditionally studied acute response. Consequently, neither the upstream nor downstream effectors of p38MAPK can necessarily be predicted from previous studies. With this in mind, we turned to investigate the downstream mechanisms by which p38MAPK induced SASP factors.

p38MAPK inhibition suppresses SASP component mRNA levels

Many SASP factors are upregulated at the level of mRNA abundance (Coppe et al, 2010b; Coppe et al, 2008). To understand the mechanism by which p38MAPK regulates the SASP, we used quantitative RT-PCR to determine mRNA levels of six SASP factors (GM-CSF, IL-6, IL-8, GROα, MCP-2, IL-1α) that declined significantly upon p38MAPK inhibition. For all six factors, p38MAPK inhibition (SB) markedly decreased mRNA abundance in SEN(XRA) cells (Figure 1-5A, p<0.05 for all genes). We obtained similar results using another cell strain (Figure 1-5B). For GM-CSF, IL-6 and IL-8, the magnitude of the decrease in mRNA abundance matched the magnitude of decrease in secreted protein level (Figure 1-5C; differences between mRNA and protein are not significant, p>0.05). Similar results were obtained for IL-6 in another cell strain (Figure 1-5D). In addition, constitutive p38MAPK activation was sufficient to induce the SASP mRNA levels as determined by IL-6 and IL-8 mRNA upon MKK6EE expression (Figure 1-5E, p<0.001). While these results do not rule out the possibility that p38MAPK stimulates the SASP by other regulatory mechanisms (e.g., translation, secretion), the data suggest that p38MAPK induces the SASP primarily by increasing mRNA abundance.

p38MAPK inhibition decreases the mRNA stability of some SASP factors

p38MAPK is reported to modulate mRNA stability in some cell contexts (Radtke et al, 2010; Wang et al, 1999). To understand how p38MAPK increases the mRNA levels of SASP genes, we examined the stability of mRNAs encoding five of the SASP proteins examined above. Two of these mRNAs (encoding GM-CSF and IL-6) showed increased stability at senescence; p38MAPK inhibition abolished this stability increase (Figure 1-5F, p<0.05). However, this was not the case for the three other mRNAs tested (encoding IL-8, GROα, and

MCP-2) (Figure 1-5F, $p>0.05$). Therefore, though p38MAPK seems to affect mRNA stability in a subset of SASP factors at senescence, we considered transcription as a more general mechanism by which p38MAPK might regulate SASP mRNA levels.

p38MAPK is necessary but not sufficient for IL-1α expression

IL-1α is multifunctional cytokine that initiates signal transduction cascades in multiple cell types (Apte et al, 2006). It is also a SASP component, though it is secreted at very low levels compared to major SASP factors like IL-6 and IL-8 (Figure 1-1). However, cell surface bound IL-1α is strongly expressed in senescent cells, and this expression is required for a positive feedback loop that induces the transcription of IL-6 and IL-8 (Orjalo et al, 2009). Because it is not highly secreted, measurement of mRNA level is a more accurate way to determine IL-1α expression levels than ELISA of CM. Because we saw that p38MAPK was required for expression of IL-1α mRNA at senescence (Figure 1-5A), we examined whether constitutive p38MAPK activity (by MKK6EE expression) was sufficient for IL-1α mRNA expression. As noted previously, MKK6EE induced the expression of IL-6 and IL-8 mRNA (Figure 1-5E); we found that MKK6EE also induced expression of IL-1β mRNA, but not IL-1α mRNA (Figure 1-5G). This unexpected result shows that, though p38MAPK activation and IL-1α are both required to induce IL-6 and IL-8 at senescence, and though IL-1α expression requires p38MAPK activity, the initial activation of IL-1α during the senescence program may be mediated by a p38MAPK-independent pathway. Consequently, while p38MAPK inhibition at senescence may dampen the senescence-induced IL-1α feedback loop (and consequently IL-6 and IL-8), activation of IL-1α is not the mechanism by which p38MAPK activity increases the transcription of SASP factors.

p38MAPK controls NF-κB activity in senescent cells

p38MAPK is known to regulate the activity of multiple transcription factors depending on context (Zarubin & Han, 2005); to identify the relevant factors that mediate the p38MAPK-induced SASP, we examined the promoters of the MKK6EE-induced factors (Figure 1-3F) for overrepresented transcription factor (TF) binding sites. We interrogated 200 bases upstream of each transcriptional start site using the 243 TF weight matrices in the TRANSFAC database. NF-κB binding motifs were most statistically overrepresented as compared to the promoters of all RefSeq genes (Figure 1-6A). Activated NF-κB was previously shown to be enriched at the IL-8 and GROγ promoters following MEK-induced senescence (Acosta et al, 2008). We therefore asked whether NF-κB activity increases during multiple types of senescence, and whether the increase is p38MAPK-dependent.

Inactive NF-κB dimers are sequestered in the cytoplasm by IκB inhibitors. NF-κB activating signals cause IκB degradation, allowing NF-κB complexes to translocate to the nucleus. Three NF-κB family members (RelA, RelB, C-Rel) have DNA binding and transactivation domains, but RelA is most strongly associated with increased inflammatory cytokine gene transcription (Karin, 2006; Perkins, 2007). By immunostaining, RelA was strongly cytoplasmic in PRE cells, but noticeably more nuclear in SEN(XRA) cells, despite a substantial fraction remained cytoplasmic (Figure 1-6B). However, the partial nuclear localization of RelA in SEN(XRA) cells was unaffected by p38MAPK inhibition (Figure 6B, SEN(XRA)+SB).

Nuclear localization alone is insufficient to activate NF-κB, which requires post-translational modifications for optimal DNA binding activity (Karin, 2006; Perkins, 2007). We therefore asked whether NF-κB DNA binding activity increases in multiple types of senescence.

NF-κB DNA binding activity increased ~5 fold in SEN(REP), SEN(RAS), and SEN(XRA) cells (two strains) (Figures 1-6C and 1-6D; $p<0.001$). Constitutive p38MAPK activity was sufficient to induce this activity, as cells expressing MKK6EE had NF-κB DNA binding levels similar to those in senescent cells (Figure 1-6C). However, despite constitutive p38MAPK activation inducing NF-κB DNA binding activity, the increase in NF-κB DNA binding activity in SEN(RAS) and SEN(XRA) cells was not inhibited by p38MAPK inhibition (Figure 1-6E, $p>0.05$). We verified that this assay can detect decreases in NF-κB DNA binding activity in senescent cells by depleting them of RelA by RNAi (Figure 1-6E, shRelA #2; see 1-6J for shRNA efficacy). This finding suggests that, though sufficient, p38MAPK is not necessary for increased NF-κB DNA binding.

Nonetheless, following XRA, NF-κB DNA binding activity increased slowly with kinetics that followed p38MAPK activation (Figure 1-6F). Thus, NF-κB DNA binding activity remained near PRE levels for 8 h after XRA, began to rise 24 h after XRA and reached maximal levels 8-10 d later (Figure 1-6F).

The activity of the NF-κB complex is also modulated by co-factors that influence transcriptional activity but not DNA binding activity (Saha et al, 2007; Vanden Berghe et al, 1999). To determine whether NF-κB transcriptional activity increases during senescence, we infected cells with a lentiviral-delivered reporter driven by an NF-κB-responsive promoter. This reporter showed that NF-κB transcriptional activity was >30-fold higher in SEN(XRA) compared to PRE cells (Figure 1-6G), and p38MAPK inhibition (SB) significantly decreased reporter activity ($p<0.001$). Together, these data support the idea that NF-κB transcriptional activity is positively regulated by one or more co-activators, which, in turn, are regulated by p38MAPK. Therefore, while NF-κB nuclear localization and DNA binding persist in the absence of p38MAPK activity, full transcriptional activity requires constitutive p38MAPK signaling. This observation fits with the model that NF-κB negatively regulates its own activity and thus requires a continuous signal to remain constitutively active (Perkins, 2007).

ATM, an important DDR component, is required for expression of a subset of SASP factors (Rodier et al, 2009). We therefore asked whether ATM is required for NF-κB transcriptional activity in senescent cells. In SEN(XRA) cells, shRNAs that efficiently depleted ATM (Figure 1-6H) decreased NF-κB transcriptional activity by 60-70% (Figure 1-6I, $p<0.001$). Thus, though the p38MAPK pathway and DDR act in parallel, they seem to converge on NF-κB signaling.

NF-κB is required for the SASP

Because p38MAPK regulates both the SASP and NF-κB activity, we asked whether NF-κB is required for the SASP. We expressed either of two unrelated shRNAs against RelA, both of which efficiently decreased RelA levels (Figures 1-6J and 1-6K) without substantially affecting RelB or C-Rel levels (Figure 1-6J). RelA depletion significantly decreased 73% (27/37) of the SASP proteins secreted by SEN(XRA) cells (Figure 1-6L, asterisks), including MMP1 and MMP3 (Figure 1-6M, $p<0.01$), and non-significantly ($p>0.05$) decreased all remaining SASP proteins (Figure 1-6L). Hierarchical clustering showed that RelA-depleted SEN(XRA) cells had a SASP profile that more closely resembled PRE cells than unmodified SEN(XRA) cells (Figure 1-6L), demonstrating that NF-κB regulates most of the SASP network. Not surprisingly, there was substantial overlap between the RelA-dependent and p38MAPK-dependent (Figure 1-1) SASP factors: 76% (19/25) of p38MAPK-dependent factors were also RelA-dependent (Figure 1-6N, Venn diagram). Of the ten most robustly secreted SASP proteins from Figure 1-1, eight were

both p38MAPK- and RelA-dependent (Figure 1-6N, table). Using IL-6, IL-8 and GM-CSF as SASP markers, we verified the RelA- dependence for SEN(RAS) cells (Figure 1-6O, $p<0.01$), and verified the dependence of IL-6 in two cell strains (HCA2 and WI-38, Figure 1-6P, $p<0.01$). We also found that amplified IL-6 induced by p53 inactivation (GSE) in post-XRA cells was RelA dependent (Figure 1-6Q, $p<0.01$). Lastly, we demonstrated that IL-6, IL-8, and GM-CSF secretion induced by MKK6EE were RelA-dependent (Figure 1-6R, $p<0.001$). Thus, NF-κB is required for SASP activity in all contexts tested, and we conclude that p38MAPK acts primarily through NF-κB to induce the SASP.

CHAPTER 1 DISCUSSION

Senescence is an important tumor suppressor mechanism because it prevents the growth of cells that are damaged or harbor activated oncogenes. Some SASP components reinforce the senescence growth arrest (Acosta et al, 2008; Kortlever et al, 2006; Kuilman et al, 2008; Wajapeyee et al, 2008), whereas others, particularly MMPs, can limit fibrosis during the repair of tissue damage (Jun & Lau, 2010; Krizhanovsky et al, 2008). However, the SASP can also create low-level chronic inflammation that can be deleterious long term, potentially leading to stem cell dysfunction, tissue degradation, and immunosenescence (Coppe et al, 2010a; Freund et al, 2010). This important anti-cancer defense, then, might be antagonistically pleiotropic and, ironically, promote cancer as senescent cells accumulate in tissues during aging (Dimri et al, 1995; Jeyapalan et al, 2007; Paradis et al, 2001; Zhou et al, 2008). Because the SASP is potentially causative in aging and many age-related diseases, it is important to understand how it is regulated. Our findings suggest that p38MAPK is crucial for the expression of many SASP cytokines and chemokines, which are largely pro-inflammatory and pro-carcinogenic (Coppe et al, 2010a; Davalos et al, 2010; Freund et al, 2010), but less important for the SASP MMPs, which may be beneficial, at least to the extent that they limit fibrosis during wound healing (Jun & Lau, 2010; Krizhanovsky et al, 2008).

The SASP develops when cells experience a stress severe enough to cause a senescence response. To date, these stresses are primarily genotoxic, leading to activation of a DDR. Persistent DDR signaling is necessary for the expression of several SASP factors (Rodier et al, 2009), and depletion of DDR proteins such as ATM, NBS1 or CHK2 suppresses the expression of SASP components, including IL-6, and IL-8. However, the DDR is activated immediately after damage, while the SASP takes days to develop, indicating that the DDR is not sufficient for SASP expression. Thus, there must be other, delayed molecular events that are required for development of the SASP and are regulated independently of the DDR. We show here that activation of the p38MAPK/NF-κB pathway is such an event.

p38MAPK was activated with slow kinetics after DNA damage in normal human cells, coinciding with expression of the SASP. p38MAPK inhibition effectively collapsed the senescence-associated cytokine network, preventing the pro-invasion paracine effects of senescent cells. Further, p38MAPK activity was sufficient to induce a SASP immediately upon expression, suggesting it is the limiting step for SASP development. Of particular importance, we found that p53 restrains the SASP by restraining p38MAPK activity. When p53 was inactivated and cells were then genotoxically stressed, p38MAPK was activated faster and to a higher level than in a wild-type p53 background. The increased activation correlated with the amplified SASP, and p38MAPK inhibition reduced the amplified SASP. Given that transformed and primary cells can differ in the kinetics with which the p38MAPK/NF-κB pathway is activated (Janssens & Tschopp, 2006), our data suggest that p53 represses the p38MAPK pathway

immediately after DNA damage in normal cells. This repression may allow time to repair the damage before cells commence signaling to the tissue microenvironment. Interestingly, the mechanism by which p53 restrains p38MAPK activity seems to be independent of Wip1, the p53-dependent, p38MAPK phosphatase (Fiscella et al, 1997; Oliva-Trastoy et al, 2007; Takekawa et al, 2000; Yu et al, 2007), as Wip1 overexpression did not reduce amplified IL-6 secretion in the p53-deficient, irradiated cells. Additionally, knockdown of Wip1 did not increase IL-6 secretion or p38MAPK phosphorylation in wild-type, irradiated cells. These data suggest that the mechanistic link between p53 and p38MAPK at senescence is distinct from their interaction in other cellular contexts.

The p38MAPK pathway acted in parallel to the DDR, rather than upstream or downstream. Inhibition of p38MAPK did not affect activation of important DDR factors such as ATM, CHK2, or p53. Additionally, though constitutive p38MAPK activation induced senescence growth arrest, it did not induce ATM or CHK2 activation, and depletion of ATM or CHK2 had no effect on the constitutive p38MAPK-induced SASP. These data suggest that p38MAPK does not regulate the SASP upstream of the DDR and demonstrates that senescence-associated pro-inflammatory cytokine secretion can occur in the absence of a DDR. Conversely, neither ATM nor CHK2 depletion altered p38MAPK phosphorylation at senescence. p53 inactivation led to amplified CHK2 signaling in presenescent cells, but this did not lead to increased IL-6 secretion (further demonstrating that the DDR is not sufficient for SASP activity) or p38MAPK phosphorylation. Additionally, p53 inactivation caused slower resolution of DNA damage in irradiated cells, as measured by the number of 53BP1 foci, but CHK2 depletion in this context also did not reduce p38MAPK phosphorylation. These observations demonstrate that p38MAPK does not act downstream of the canonical DDR in normal or p53-deficient cells.

p38MAPK inhibition caused a small decrease in the number of DNA damage foci in a SEN(XRA), SEN(REP), and SEN(RAS) cells, and constitutive p38MAPK activation was sufficient to induce these foci in a subset of cells. Constitutive p38MAPK activation also slightly increased ROS signaling, as measured by intracellular H2O2, supporting reports that p38MAPK can replenish and even induce short-lived DNA damage foci via generation of ROS (Passos et al, 2010). These ROS, in turn, can further activate p38MAPK, generating a positive feedback loop. We hypothesized that ROS signaling might play a role in p38MAPK's regulation of the SASP, either downstream by mediating p38MAPK signaling or upstream by activating p38MAPK. However, in cells with constitutively active p38MAPK, treatment with the antioxidant NAC, which reduced ROS levels, did not reduce IL-6 secretion, suggesting that ROS signaling is not a downstream mediator of p38MAPK with regards to SASP induction. Additionally, increased ROS signaling is not necessary for p38MAPK activation at senescence: NAC treatment decreased IL-6 secretion in normal senescent cells by about 50%, but did not reduce p38MAPK phosphorylation. NAC treatment did not reduce amplified p38MAPK phosphorylation in p53-deficient senescent cells, suggesting that, although increased ROS signaling may play a partial role in SASP regulation via an unknown pathway, it is not required for induction or maintenance of p38MAPK phosphorylation at senescence. Interestingly, this unknown pathway may be refractory to NAC inhibition in p53-deficient cells, as NAC treatment had no effect on amplified IL-6 secretion, possibly due to overwhelming ROS production. Alternatively, this pathway may be unnecessary for the SASP in p53-deficient cells, as other pathways such as p38MAPK become hyperactivated. Though the role of ROS at senescence and in SASP regulation remains incompletely understood, we conclude that the DDR-ROS feedback loop that maintains a subset of DNA damage foci (Passos et al, 2010) is not the mechanism by which p38MAPK regulates the SASP.

NF-κB was the crucial effector of p38MAPK signaling during senescence. We found that p38MAPK modulated SASP factor mRNA abundance, and NF-κB binding sites were the most statistically overrepresented transcription factor binding sites in the set of p38MAPK-induced proteins. NF-κB DNA binding activity was increased in DNA damage-induced senescence, RAS-induced senescence, replicative senescence, and in response to constitutive p38MAPK activation, and p38MAPK was required for senescence-induced NF-κB transcriptional activity. The SASP critically depended on NF-κB, whether senescence was induced by DNA damage, RAS, or constitutive p38MAPK activity. However, NF-κB activation is probably not the only means by which p38MAPK increases SASP gene expression – other senescence-associated transcription factors such as C/EBP are indirectly regulated by p38MAPK (Cortez et al, 2007) and, for at least some SASP genes, mRNA stability increased at senescence and decreased after p38MAPK inhibition. Nevertheless, NF-κB was crucial because virtually all SASP factors were decreased upon its depletion. The mechanism by which p38MAPK regulates NF-κB remains unclear: constitutive p38MAPK activity induced NF-κB nuclear localization, DNA binding and transcriptional activity, but only the transcriptional activity required p38MAPK. Additionally, though p38MAPK induced a SASP, it did not induce IL-1α expression, which is required for a positive feedback loop that reinforces NF-κB activity at senescence (Orjalo et al, 2009). These results suggest that p38MAPK does not regulate NF-κB activity indirectly (e.g. via cytokine feedback loops or posttranslational modifications of p65 in the cytoplasm), and we propose that p38MAPK may act directly on the assembled NF-κB transcriptional complex, possibly by affecting the activity of a co-activator(s) that is required for NF-κB transcriptional activity or altering the chromatin landscape around NF-κB binding sites (Saccani et al, 2002; Saha et al, 2007; Vanden Berghe et al, 1999).

Interestingly, DDR signaling was also required for NF-κB activity in senescent cells – ATM depletion decreased NF-κB transcriptional activity, suggesting that, though the DDR and p38MAPK pathways act independently of each other, they may converge to stimulate NF-κB transcriptional activity. It seems initially paradoxical that p38MAPK can be both sufficient for the NF-κB activity and independent of the DDR, if the DDR is required for NF-κB activity. However, though high levels of p38MAPK phosphorylation were sufficient to drive SASP activity in the absence of DDR signaling, lower levels of p38MAPK, such as those found in DNA damage-induced senescence, were evidently not sufficient, as the DNA damage-induced SASP also requires DDR signaling (Rodier et al, 2009). While the molecular mechanism behind this p38MAPK regulatory threshold remains unknown, it is possible that p38MAPK and/or downstream targets phosphorylate/acetylate several sites of the NF-κB transcriptional complex with varying degrees of affinity, as has been demonstrated in other contexts (Saha et al, 2007). Multiple post-translational modifications are necessary for NF-κB to have full transcriptional activity (Karin, 2006; Perkins, 2007). High p38MAPK activity may lead to phosphorylation/acetylation of all the necessary sites on NF-κB, even those for which the p38MAPK pathway has low affinity, leading to full SASP activation. Lower levels of p38MAPK may not be able to modify those low-affinity sites; consequently, DDR signaling, which presumably has higher affinity for those sites, is required. This model may also explain why a high level of p38MAPK activation is sufficient to induce NF-κB DNA binding activity, but increased NF-κB DNA binding activity in DNA damage- and RAS-induced senescence does not require p38MAPK signaling.

MMPs are an important feature of the SASP. These enzymes have matrix-degrading and fibrolytic activity, which may limit fibrosis during wound healing (Jun & Lau, 2010; Krizhanovsky et al, 2008). These activities are likely short-term beneficial effects of the SASP. The two most highly secreted MMPs are MMP1 and MMP3 (Coppe et al, 2010b). p38MAPK

activation induced these MMPs, but to a lesser extent than many cytokines and chemokines. Additionally, although prolonged p38MAPK inhibition reduced MMP levels in SEN(XRA) and SEN(RAS) cells, the effect was substantially smaller than the reduction in IL-6, IL-8, GM-CSF, and other SASP factors. On the other hand, NF-κB depletion effectively blunted both MMP1 and MMP3 secretion. Thus, p38MAPK-independent MMP-regulating pathways must be activated at senescence, but those pathways may also converge on NF-κB. More importantly, our data suggest it may be possible to reduce some SASP factors without affecting others, potentially mitigating the deleterious effects without strongly mitigating the benefits.

Although the role of senescence and the SASP in cancer, aging and age-related disease has yet to be fully established, senescent increase with age *in vivo*, and are found at sites of age-related pathology, including cancer, in humans. Our identification of the p38MAPK/NF-κB pathway as a necessary and sufficient, DNA damage-independent regulator of the SASP provides new insights into how senescent cells might be a source of the chronic inflammation that is a hallmark of aging and many age-related diseases.

CHAPTER 1 ACKNOWLEDGMENTS

We thank Drs. Eisuke Nishida (Kyoto University) for the pSRalpha-myc-MKK6-EE vector and Pierre Desprez for critically reading the manuscript. This work was supported by grants from the National Institutes of Health (AG09909, AG017242 & AG25901, JC), and a National Science Foundation Graduate Research Fellowship (AF).

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CHAPTER 1 FIGURE LEGENDS

Figure 1-1: p38MAPK is activated with slow kinetics during genotoxic stress-induced senescence and is required for the SASP

(A) Cell proliferation. Presenescent HCA2 cells (PRE) were mock irradiated or treated with 10 Gy X-radiation (XRA) and counted at the indicated intervals thereafter.

(B) SA-βgal staining. Cells were mock irradiated (PRE) or treated with 10 Gy X-ray and allowed to senesce (SEN(XRA)) for 10 d, then fixed and stained for SA-βgal.

(C) p38MAPK phosphorylation increases during DNA damage-induced senescence. Cells were irradiated and whole cell lysates collected at the indicated days thereafter. (+): p38MAPK was continuously inhibited by 10 μM SB203580 (SB) beginning 48 h before irradiation. Left: western blot analysis of the indicated proteins. Right: western blot quantitation, normalized to PRE levels. p38-P, phosphorylated p38MAPK; Hsp27-P, phosphorylated heat shock protein 27.

(D) p38MAPK inhibition decreases intracellular IL-6 in SEN(XRA) cells. PRE and SEN(XRA) cells were fixed and analyzed for IL-6 by immunofluorescence. +SB: p38MAPK was inhibited by SB023580 (SB) for 48 h prior to fixation.

(E) p38MAPK inhibition decreases IL-6, IL-8 and GM-CSF secreted by SEN(XRA) cells. Conditioned media (CM) were collected from PRE and SEN(XRA) cells and analyzed by ELISA. +SB: p38MAPK was inhibited by SB023580 (SB) for 48 h prior to CM collection.

(F) p38MAPK inhibition decreases secreted IL-6 in SEN(XRA) WI-38 cells. CM from PRE and SEN(XRA) WI-38 cells were collected and analyzed by ELISA. SB: p38MAPK was inhibited by SB023580 for 48 h prior to CM collection.

(G) p38MAPK inhibition decreases secreted IL-6 by SEN(REP) HCA2 and WI-38 cells. CM from PRE and replicatively senescent (SEN(REP)) cells were collected and analyzed by ELISA. SB: p38MAPK was inhibited by SB023580 for 48 h prior to CM collection.

(H) Efficacy of a drug (SB) resistant p38MAPK mutant. Cells were infected with a lentivirus expressing wild-type p38MAPK (p38 WT) or a drug resistant p38MAPK mutant (p38 DR) and selected. Cells were then irradiated and allowed to reach senescence. Whole cell lysates were collected and analyzed by western blotting for the indicated proteins. SB: SB203580 was added for 48 h prior to lysate collection.

(I) Expression of a drug resistant p38MAPK mutant prevents SB-mediated inhibition of IL-6 at senescence. Cells were infected as described in (H), irradiated, and allowed to reach senescence. CM were collected and analyzed by ELISA. SB: SB203580 was added for 48 h prior to CM collection.

(J) Efficacy of p38MAPKα shRNAs. Cells were infected with a lentivirus expressing either of two shRNAs against p38α (shp38α) or an shRNA against GFP (shGFP; control), and selected. Cells were irradiated and allowed to senesce (SEN(XRA)). Whole cell lysates were collected and analyzed by western blotting for the indicated p38MAPK isoforms and tubulin (control).

(K) p38MAPKα depletion decreases secreted IL-6. Cells were infected as in (J), irradiated, and allowed to reach senescence. CM were collected and analyzed ELISA.

(L) p38MAPK inhibition suppresses the SEN(XRA) SASP. CM from PRE and SEN(XRA) cells, with (SB) or without 48 h of p38MAPK inhibition, were analyzed by antibody arrays. Shown are factors for which the SEN(XRA) level was significantly increased ($p<0.05$) over PRE. For each detected protein, signals from all conditions were averaged to generate the baseline. Signals above baseline are yellow; signals below baseline are blue. The heat map key shows $\log_2$-fold changes from baseline. Asterisks indicate factors that were significantly decreased by p38MAPK inhibition ($p<0.05$). The relationship between samples is shown graphically by hierarchical clustering (left).

(M) p38MAPK inhibition suppresses the SEN(XRA) SASP. Shown are factors for which the change between SEN(XRA)+SB and SEN(XRA) was statistically significant ($p<0.05$) (marked by asterisks in (L)). Values indicate the $\log_2$-fold decrease from SEN(XRA) levels.

(N) p38MAPK inhibition slightly increases secreted levels of some proteins. Factors for which p38MAPK inhibition by SB023580 (SB) increased secreted levels, relative to untreated SEN(XRA) cells. Values indicate the $\log_2$-fold change between SEN(XRA)+SB and SEN(XRA). None of the factors increased by SB were components of the SEN(XRA) SASP.

(O) p38MAPK inhibition decreases MMP3 but not MMP1 secreted by SEN(XRA) cells. CM from PRE and SEN(XRA) cells were collected and analyzed by ELISA. SB: p38MAPK was inhibited by SB023580 for 48 h prior to CM collection (2d), or p38MAPK was inhibited by SB203580 starting 48 h prior to irradiation and continuing until CM collection (cont).

(P) p38MAPK depletion decreases the ability of senescent cells to stimulate cancer cell invasiveness. CM from cells described in (J) were analyzed for the ability to stimulate invasiveness of MDA-MB-231 breast cancer cells in a Boyden chamber invasion assay.

Figure 1-2: p38MAPK drives the amplified SASPs induced by RAS or p53 inactivation

(A) p38MAPK is phosphorylated during RAS^{V12}-induced senescence. PRE cells were infected with a lentivirus expressing oncogenic RAS^{V12}, selected, and allowed to senesce (SEN(RAS)) for 10 d. Whole cell lysates were then collected and analyzed by western blotting for the indicated proteins. Presenescent controls (PRE) were infected with an insertless vector.

(B) p38MAPK inhibition suppresses the SEN(RAS) SASP. Cells were infected as described in (A). CM from PRE and SEN(RAS) cells were analyzed by antibody arrays as described in Figure 1-1L. +SB: p38MAPK was inhibited by SB203580 for 48 h prior to CM collection. Shown are the 83 factors for which the SEN(RAS) level was significantly increased over PRE. PRE and SEN(RAS) values were averaged to generate the baseline. Signals above baseline are yellow; signals below baseline are blue. The heat map key show $\log_2$-fold changes from baseline. The relationship between samples is indicated graphically by hierarchical clustering (top). Asterisks indicate the factors significantly decreased by p38MAPK inhibition.

(C) p38MAPK inhibition decreases MMP1 and MMP3 secreted by SEN(RAS) cells. PRE Cells were infected as described in (A). CM from PRE and SEN(RAS) cells were collected and analyzed by ELISA. SB: p38MAPK was inhibited by SB023580 for 48 h prior to CM collection (2d), or p38MAPK was inhibited by SB203580 starting 48 h prior to irradiation and continuing until CM collection (cont).

(D) The SEN(XRA) and SEN(RAS) SASPs are similarly dependent on p38MAPK. The table lists SASP factors that are significantly increased over PRE control in both SEN(XRA) and SEN(RAS) cells. • indicates factors significantly decreased by p38MAPK inhibition in both SEN(XRA) and SEN(RAS) cells.

(E) Amplified p38MAPK phosphorylation in SEN(RAS) cells and SEN(XRA) cells lacking functional p53 (SEN(XRA)+GSE). Cells were infected with a lentivirus expressing GSE22 (GSE) or an insertless vector, selected, then irradiated (XRA) or infected with a lentivirus expressing oncogenic RAS^{V12} (RAS) and allowed to senesce. Whole cell lysates were collected and analyzed by western blotting for the indicated proteins.

(F) SEN(RAS) and SEN(XRA) cells lacking functional p53 secrete amplified IL-6 levels. Cells were treated as in (E), then CM were collected and analyzed by ELISA.

(G) p53 inactivation accelerates p38MAPK phosphorylation after XRA. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE), selected, and irradiated. Whole cell lysates were collected at the specified timepoints and analyzed by western blotting for the indicated proteins.
(H) GSE-amplified levels of IL-6, IL-8 and GM-CSF are p38MAPK dependent. Cells were infected as described in (G) and irradiated. CM were collected 3 d later and analyzed by ELISA. SB: p38MAPK was inhibited by SB203580 for 48 h prior to CM collection.
(I) Efficacy of p53 shRNAs. Cells were infected with a lentivirus expressing an shRNA against p53 (shp53) or GFP (shGFP; control) and selected. Whole cell lysates were collected and analyzed by western blotting for the indicated proteins.
(J) Amplified levels of IL-6, IL-8 and GM-CSF induced by p53 depletion are p38MAPK dependent. Cells were infected with a lentivirus expressing an shRNA against p53 (shp53) or GFP (shGFP; control) and selected. Cells were then irradiated and CM were collected 3 d later and analyzed by ELISA. SB: p38MAPK was inhibited by SB203580 for 48 h prior to CM collection.
(K) Amplified levels of IL-6 are only induced by p53 inactivation upon senescence inducing-DNA damage, not in response to low level DNA damage. Cells were infected with a lentivirus lacking an insert (vector) or expressing GSE22 (GSE), selected, and then mock irradiated (PRE) or irradiated with either 0.5 Gy or 10 Gy. CM were collected 3 d later and analyzed by ELISA.
(L) Efficacy of Wip1 overexpression construct. Cells were infected with a lentivirus lacking an insert (vector) or expressing wild type Wip1 (Wip1) and selected. Whole cell lysates were collected and analyzed by western blotting.
(M) Wip1 overexpression does not prevent GSE-amplified IL-6 secretion after XRA. Cells were infected as described in (L) and selected. Cells were then irradiated (10 Gy) and CM were collected 3 d later and analyzed by ELISA.
(N) Efficacy of Wip1 shRNAs. Cells were infected with a pool of 3 lentiviruses expressing independent shRNAs against Wip1 (shWip1), or an equivalent titer of a scrambled control shRNA (shScramble) and selected. Total RNA was extracted and Wip1 (PPM1D) mRNA levels were analyzed by quantitative RT-PCR.
(O) Wip1 depletion does not increase p38MAPK phosphorylation after XRA. Cells were infected as in (N), selected, and irradiated (XRA). Whole cell lysates were collected 3 d later and analyzed by western blotting.
(P) Wip1 depletion does not increase IL-6 secretion after XRA. Cells were infected as in (N), selected, and irradiated. CM were collected 3 d later and analyzed by ELISA.

Figure 1-3: Constitutive p38MAPK activation is sufficient to induce a SASP

(A) PRE cells were infected with a lentivirus expressing a constitutively active MAP kinase kinase 6 mutant (MKK6EE) and selected. Whole cell lysates were collected 8 d after infection and analyzed by western blotting for the indicated proteins. Presenescent controls (PRE) were infected with an insertless vector.
(B) MKK6EE induces SA-βgal expression. HCA2 and WI-38 cells were infected as described in (A), fixed 8 d after infection, and stained for SA-βgal.
(C) MKK6EE induces a p38MAPK-dependent growth arrest. Cells were infected as described in (A) and counted at the indicated intervals thereafter. +SB: p38MAPK was continuously inhibited by SB203580 beginning 48 h before infection.
(D) MKK6EE suppresses BrdU incorporation. Cells were infected as described in (A), and 7 d later cultured with BrdU for 24 h, fixed, and immunostained for incorporated BrdU.

BrdU-positive cells were quantified by CellProfiler. SB: p38MAPK was continuously inhibited with SB023580 beginning 48 h before infection.

(E) MKK6EE induces a p38MAPK-dependent senescence morphology. Cells were infected as described in (A) and photographed through a phase contrast microscope 8 d after infection. Representative images are shown. +SB: p38MAPK was continuously inhibited with SB023580 beginning 48 h before infection.

(F) MKK6EE induces a SASP. Cells were infected as described in (A), and CM were collected 8 d after infection. +SB: p38MAPK was inhibited by SB203580 for 48 h prior to CM collection. Proteins secreted by the indicated cells were analyzed using antibody arrays. Shown are proteins for which the MKK6EE level was significantly increased ($p<0.05$) over PRE. For each protein, PRE and MKK6EE values were averaged to generate the baseline. Signals above baseline are yellow; signals below baseline are blue. The heat map key show $\log_2$-fold changes from baseline. The relationship between samples is shown graphically by hierarchical clustering (left). Asterisks indicate factors significantly decreased by p38MAPK inhibition ($p<0.05$).

(G) The MKK6EE SASP resembles the SEN(XRA) and SEN(RAS) SASPs. Shown are the 10 most upregulated factors in the SEN(XRA) and SEN(RAS) SASPs. + indicates factors that are significantly increased by MKK6EE expression.

(H) MKK6EE increases secreted IL-6. Cells were infected as described in (A). CM were collected 8 d after infection and analyzed by ELISA. SB: p38MAPK was continuously inhibited with SB023580 beginning 48 h before infection.

(I) MKK6EE increases secreted MMP1 and MMP3 levels. Cells were infected as described in (A). CM were collected 8 d after infection and analyzed by ELISA.

(J) MKK6EE increases secreted IL-6 within one day after infection. Cells were infected as described in (A). CM were collected at the designated timepoints and analyzed by ELISA.

(K) MKK6EE levels after infection. Cells were infected as described in (A). Whole cell lysates were collected at the designated timepoints and analyzed by western blotting for the indicated proteins.

Figure 1-4: p38MAPK induces the SASP independently of the DNA damage response

(A) p38MAPK inhibition does not prevent the DDR. Whole cell lysates were collected at the specified intervals after irradiation and analyzed by western blotting for the indicated proteins. Where indicated, p38MAPK was continuously inhibited by SB203580 (+) beginning 48 h before irradiation. ATM-P, Ser 1981 phosphorylated ATM; CHK2-P, Thr 68 phosphorylated CHK2.

(B) Effect of p38MAPK inhibition on 53BP1 foci formation and resolution. Cells were fixed at the indicated intervals after irradiation and analyzed by immunostaining for 53BP1. Foci were quantitated using CellProfiler. Cells with ≥3 53BP1 foci/nucleus were scored. Error bars indicate the margin of error at 95% confidence. +SB: p38MAPK was continuously inhibited by SB203580 beginning 48 h before irradiation.

(C) Effect of p38MAPK inhibition on 53BP1 foci in replicatively senescent cells. SEN(REP) cells were fixed and analyzed by immunostaining for 53BP1. Foci were quantitated using CellProfiler. +SB: p38MAPK was inhibited by SB023580 for 6 d prior to fixation.

(D) Effect of p38MAPK inhibition on 53BP1 foci in Ras-induced senescent cells. SEN(RAS) cells were then fixed and analyzed by immunostaining for 53BP1. Foci were quantitated using CellProfiler. +SB cont: p38MAPK was continuously inhibited by SB203580 beginning 48 h before infection.

(E) Constitutive p38MAPK activation does not induce 53BP1 foci. Cells were fixed 8 d after MKK6EE expression (MKK6EE), 10 days after RAS expression (SEN(RAS)), 8 d after

irradiation (SEN(XRA)), and after replicative senescence (69 population doublings) (SEN(REP)) and immunostained for 53BP1. Foci were quantified using CellProfiler to score cells with ≥3 53BP1 foci per nucleus. Error bars indicate the margin of error at 95% confidence.

(F) Constitutive p38MAPK activation does not induce 53BP1 foci. The same data depicted in (E), displayed here as a histogram.

(G) Constitutive p38MAPK activation does not induce a DDR. Whole cell lysate was collected 8 d after MKK6EE infection and analyzed by western blotting for the indicated proteins. Presenescent controls (PRE) were infected with an insertless vector. ATM-P, Ser 1981 phosphorylated ATM; CHK2-P, Thr 68 phosphorylated CHK2.

(H) Efficacy of ATM and CHK2 depletion by RNAi. Cells were simultaneously infected with a lentivirus expressing MKK6EE and a lentivirus expressing an shRNA against ATM (shATM #12), CHK2 (shCHK2 #2, shCHK2 #12), or GFP (shGFP; control) and selected. Whole cell lysates were collected 8 d after infection and analyzed by western blotting for the indicated proteins. Presenescent controls (PRE) were infected with an insertless vector.

(I) ATM or CHK2 depletion does not prevent the SASP induced by constitutive p38MAPK activation. Cells were infected as in (H) and selected. CM were collected 8 d after infection and analyzed by ELISA.

(J) ATM or CHK2 depletion does not prevent p38MAPK phosphorylation at senescence. Cells were irradiated; 6 d later cells were infected with a lentivirus expressing shRNAs against ATM (shATM #12), CHK2 (shCHK2 #2, shCHK2 #12) or GFP (shGFP; control) and selected. 10 d after irradiation, whole cell lysates were collected and analyzed by western blotting for the indicated proteins.

(K) p53 inactivation increases the proliferation rate of PRE cells. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE) and counted at the indicated intervals thereafter.

(L) p53 inactivation increases the number of 53BP1 foci per nucleus in PRE cells. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE) and selected. 6 d after infection, cells were fixed and analyzed by immunostaining for 53BP1. Foci were quantitated using CellProfiler.

(M) p53 inactivation increases DDR signaling but not p38MAPK phosphorylation in PRE cells. Cells were infected as in (L). Whole cell lysate was collected 6 d after infection and analyzed by western blotting for the indicated proteins.

(N) p53 inactivation does not increase IL-6 secretion in PRE cells. Cells were infected as in (L). CM were collected 6 d after infection and analyzed by western blotting for the indicated proteins.

(O) p53 inactivation increases average number of 53BP1 foci per nucleus after XRA. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE) and selected. Cells were irradiated, fixed at the indicated intervals thereafter, and analyzed by immunostaining for 53BP1. Foci were quantitated using CellProfiler. Average foci per nucleus were scored.

(P) DDR signaling is not required for the amplified p38MAPK phosphorylation in irradiated, GSE-expressing cells. Cells were simultaneously infected with a lentivirus expressing GSE22 (GSE) and a lentivirus expressing an shRNA against CHK2 (shCHK2 #2, shCHK2 #12), or GFP (shGFP; control), selected, then irradiated. Whole cell lysate was collected 3 d later and analyzed by western blotting for the indicated proteins. NS, nonspecific band.

(Q) NAC partially inhibits senescence-induced IL-6 secretion. PRE or SEN(XRA) cells were treated with 10 mM N-acetyl cysteine (NAC) for 48 h. CM were then collected and analyzed by ELISA.
(R) NAC does not inhibit senescence-induced p38MAPK phosphorylation. SEN(XRA) cells were treated with 10 mM NAC for 48 hr, then whole cell lysates were collected and analyzed by western blotting for the indicated proteins.
(S) Constitutive p38MAPK activation increases ROS signaling. Cells were infected with a lentivirus lacking an insert (PRE) or expressing MKK6EE (MKK6EE) and selected. 8 d after infection, cells were collected and H_2O_2 levels were measured by flow cytometry. NAC: ROS signaling was inhibited with 10 mM NAC for 48 h before collection.
(T) NAC does not inhibit MKK6EE-induced IL-6. Cells were infected and treated with NAC as described in (S). CM were collected 8 d after infection and analyzed by ELISA.
(U) NAC does not inhibit amplified p38MAPK phosphorylation in irradiated, GSE-expressing cells. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE), selected, then irradiated. Whole cell lysates were collected 3 d later and analyzed by western blotting for the indicated proteins. NAC: ROS signaling was inhibited by 10 mM NAC for 48 h before collection.
(V) NAC does not inhibit amplified IL-6 secretion in irradiated, GSE-expressing cells. Cells were infected and irradiated as described in (U). CM were collected 3 d later and analyzed by ELISA. NAC: ROS signaling was inhibited by 10 mM NAC for 48 h before collection.

Figure 1-5: p38MAPK mediates SASP mRNA levels

(A) p38MAPK inhibition decreases SASP mRNA levels in HCA2 cells. Total RNA was extracted from PRE and SEN(XRA) HCA2 cells and mRNA levels for the indicated genes were analyzed by quantitative RT-PCR. +SB: p38MAPK was inhibited with SB203580 for 48 h prior to sample collection. For each gene, the four signals were averaged to generate the baseline. Signals above baseline are red; signals below baseline are green. The heat map key shows $\log_2$-fold changes from baseline. p38MAPK inhibition significantly decreased ($p<0.05$) mRNA levels for all genes assayed.
(B) p38MAPK inhibition decreases SASP mRNA levels in WI-38 cells. Total RNA was extracted from PRE and SEN(XRA) WI-38 cells and mRNA levels for the indicated genes were analyzed by quantitative RT-PCR. +SB: p38MAPK was inhibited with SB203580 for 48 h prior to sample collection. For each gene, the four signals were averaged to generate the baseline. Signals above baseline are red; signals below baseline are green. The heat map key shows $\log_2$-fold changes from baseline. p38MAPK inhibition significantly decreased ($p<0.05$) mRNA levels for all genes assayed except GM-CSF.
(C) Decreased mRNA levels closely match decreased secreted protein levels in HCA2 cells. CM and RNA were collected from SEN(XRA) HCA2 cells. mRNA levels for IL-6, IL-8 and GM-CSF were analyzed by quantitative RT-PCR; secreted protein levels were analyzed by ELISA. SEN(XRA) mRNA and protein levels were set to 1 for each factor. SB: p38MAPK was inhibited with SB203580 for 48 h prior to sample collection. For all factors, the fold decrease in mRNA level after p38MAPK inhibition was not significantly different from the fold decrease in protein level ($p>0.05$).
(D) Decreased IL-6 mRNA levels closely match decreased secreted IL-6 protein levels in WI-38. CM and RNA were collected from SEN(XRA) WI-38 cells. mRNA levels for IL-6 were analyzed by quantitative RT-PCR; secreted protein levels were analyzed by ELISA. SEN(XRA) mRNA and protein levels were set to 1. SB: p38MAPK was inhibited with SB203580 for 48 h prior to sample collection. The fold decrease in mRNA level after

p38MAPK inhibition was not significantly different from the fold decrease in protein level ($p>0.05$).

(E) Constitutive p38MAPK activity increases mRNA levels of IL-6 and IL-8. Cells were infected with a lentivirus lacking an insert (PRE) or expressing MKK6EE (MKK6EE) and selected. Total RNA was extracted 8 d after infection and mRNA levels for the indicated genes were analyzed by quantitative RT-PCR.

(F) mRNA stability of SASP genes. At time=0, actinomycin D (10 ug/mL) was added to SEN(XRA) or PRE cells to halt transcription. RNA was isolated at the indicated intervals thereafter, and levels of the indicated transcripts were measured via quantitative RT-PCR. Transcript levels were set to 100% at time=0. +SB: p38MAPK was inhibited with SB023580 for 48 h before the addition of actinomycin D.

(G) Constitutive p38MAPK activity increases the mRNA level of IL-1β but not IL-1α. Cells were treated as described in (E) and mRNA levels for the indicated genes were analyzed by quantitative RT-PCR.

Figure 1-6: p38MAPK regulates the SASP via NF-κB transcriptional activity

(A) Transcription factor (TF) binding sites (BS) in MKK6EE-induced genes. Genes encoding SASP proteins upregulated by MKK6EE expression (Figure 1-3F) were analyzed for statistically overrepresented TFBS in the 200 bp upstream of the transcriptional start site. "% of sequences" indicates the percentage of sequences with at least 1 binding site for each indicated weight matrix. TFBS are sorted by p value.

(B) RelA partially localizes to the nucleus during damage-induced senescence. PRE and SEN(XRA) cells were fixed and immunostained for RelA. Representative images are shown. +SB: p38MAPK was inhibited with SB203580 for 48 h prio to fixation.

(C) NF-κB DNA binding activity increases in multiple types of senescence. Cells were induced to senesce (SEN) by the indicated stimuli, and whole cell lysates were collected and assayed for NF-κB DNA binding activity.

(D) NF-κB DNA binding increases in senescent WI-38 cells. PRE and SEN(XRA) WI-38 whole cell lysates were collected and assayed for NF-κB DNA binding activity.

(E) p38MAPK inhibition does not decrease senescence-induced NF-κB DNA binding activity. SEN(RAS) and SEN(XRA) whole cell lysates were collected and assayed for NF-κB DNA binding activity. SB: p38MAPK was inhibited for 48 h prior to collection. shRelA #2: RelA was depleted via lentiviral infection of an shRNA against RelA before senescence induction. PRE controls were mock irradiated or infected with a lentivirus expressing an shRNA against GFP, as appropriate.

(F) NF-κB DNA binding activity kinetics after DNA damage. Cells were irradiated, whole cell lysates were collected at the indicated intervals thereafter and assayed for NF-κB DNA binding activity.

(G) p38MAPK inhibition reduces senescence-induced NF-κB transcriptional activity. Cells were infected with a lentivirus expressing an NF-κB luciferase reporter construct, irradiated, and allowed to senesce (SEN(XRA)). Cells were lysed, and luciferase activity was measured. SB: p38MAPK was inhibited with SB203580 for 48 h prior to lysis.

(H) Efficacy of ATM shRNAs. Cells were infected with a lentivirus expressing either of two shRNAs against ATM (shATM #11, #12) or GFP (shGFP; control) and selected. Whole cell lysates were collected and analyzed by western blotting for the indicated proteins.

(I) ATM depletion reduces senescence-induced NF-κB transcriptional activity. Cells were infected with a lentivirus expressing an NF-κB luciferase reporter construct and selected. Cells were then infected with a lentivirus expressing either of two shRNAs against ATM (shATM #11, #12) or GFP (shGFP; control), selected, irradiated, and

allowed to senesce (SEN(XRA)). Cells were then lysed, and luciferase activity was measured.

(J) Efficacy of RelA shRNAs. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA #1, #2) or GFP (shGFP; control) and selected. Whole cell lysates were collected and analyzed by western blotting for the indicated NF-κB family members and tubulin (control).

(K) Validation of RelA shRNAs by immunofluorescence. Cells were infected as in (J), then mock irradiated (PRE) or irradiated and allowed to senescence (SEN(XRA)). Cells were then fixed, and immunostained for RelA. Representative images are shown.

(L) RelA depletion suppresses the SASP of SEN(XRA) cells. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control), selected, then mock irradiated (PRE) or irradiated and allowed to senesce (SEN(XRA)). Secreted proteins were detected by antibody arrays as described for Figure 1-1. Shown are factors for which the SEN(XRA) level was significantly increased ($p<0.05$) over PRE. For each protein, the six signals were averaged to generate the baseline. Signals above baseline are yellow; signals below baseline are blue. The heat map key show $\log_2$-fold changes from baseline. The relationship between samples is indicated graphically by hierarchical clustering (left). Asterisks indicate factors that are significantly decreased by both RelA shRNAs ($p<0.05$).

(M) Cells were infected and irradiated as described in (L). CM were collected and analyzed by ELISA.

(N) Most p38MAPK-dependent SASP proteins are RelA dependent. Left: proportional Venn diagram displaying the overlap between p38MAPK-dependent factors (red), RelA-dependent factors (blue), and the SEN(XRA) SASP (yellow). 76% of p38MAPK-dependent factors are also RelA-dependent (dashed area). Right: shown are the 10 most upregulated SEN(XRA) SASP factors from Figure 1-1. + indicates proteins dependent on RelA or p38MAPK.

(O) SEN(RAS)-induced IL-6, IL-8, and GM-CSF are RelA dependent. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then infected with a lentivirus lacking an insert (PRE) or expressing RAS^{V12} and allowed to senesce (SEN(RAS)). CM were collected and analyzed by ELISA.

(P) RelA depletion decreases secreted IL-6 levels in senescent HCA2 and WI-38 cells. HCA2 (top) or WI-38 (bottom) cells were infected and irradiated as described in (L). CM were collected and analyzed by ELISA.

(Q) GSE-amplified levels of IL-6 are RelA dependent. Cells were infected with a lentivirus lacking an insert (vector control) or expressing GSE22 (GSE) and selected. Cells were then infected with a lentivirus expressing either an shRNA against RelA (shRelA) or GFP (shGFP; control), selected, and irradiated. CM were collected 3 d later and analyzed by ELISA.

(R) MKK6EE-induced IL-6, IL-8 and GM-CSF are RelA dependent. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then infected with a lentivirus lacking an insert (PRE) or expressing MKK6EE (MKK6EE). CM were collected 8 d after infection and analyzed by ELISA.

Figure 1-1
(1 of 2)

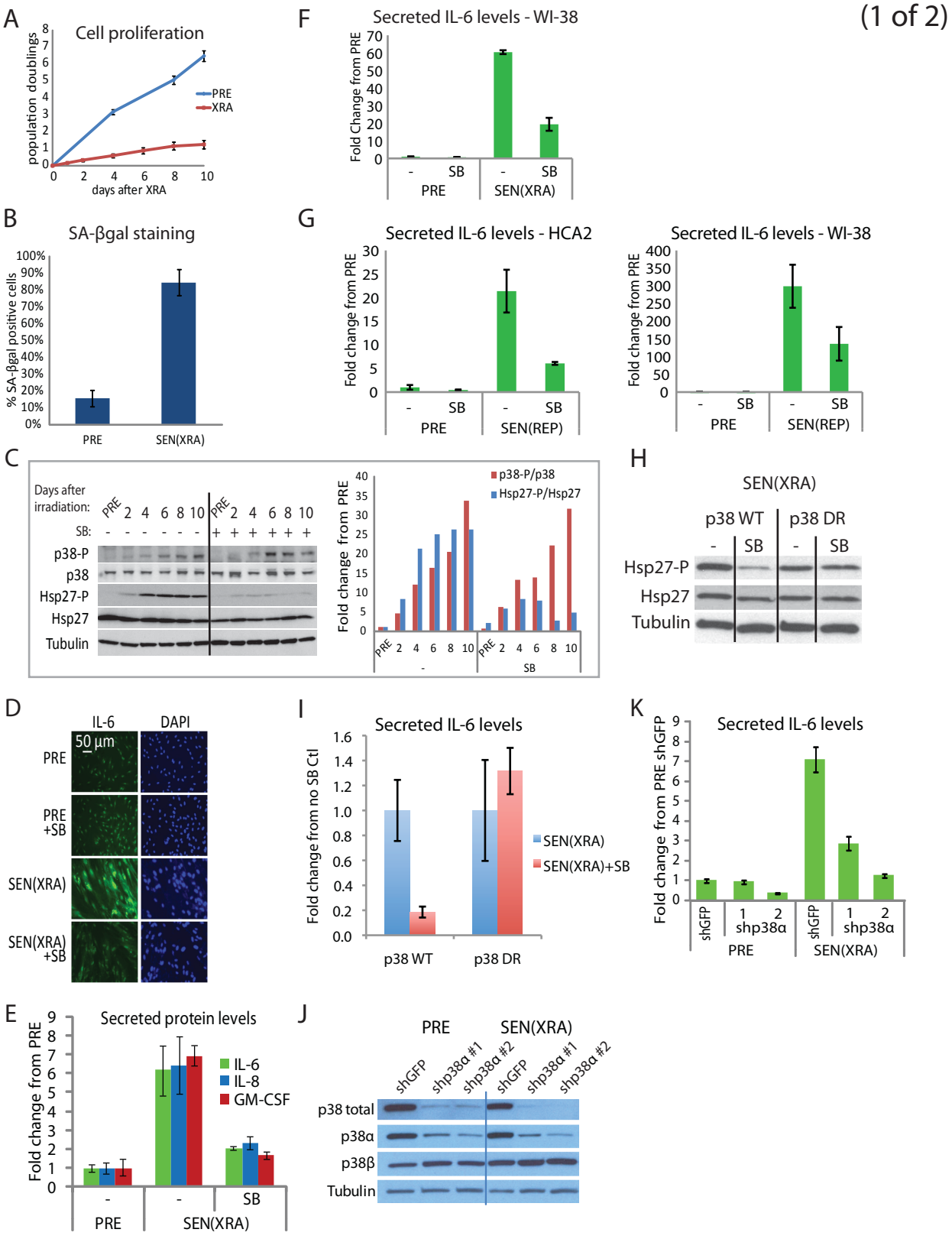
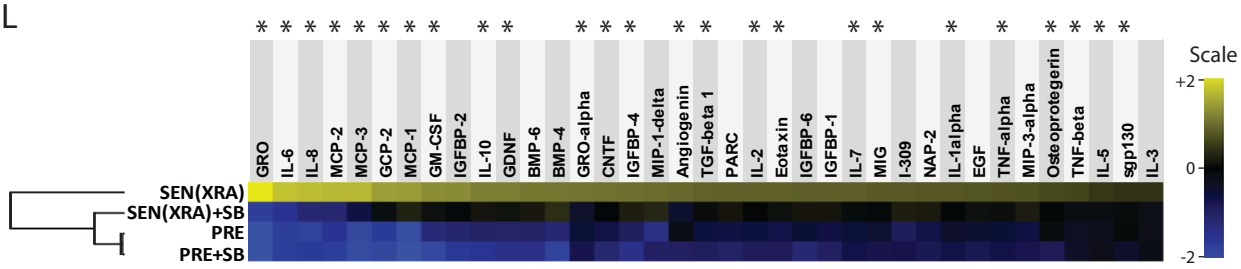


Figure 1-1
(2 of 2)

L



M

SASP proteins significantly reduced by p38 inhibition

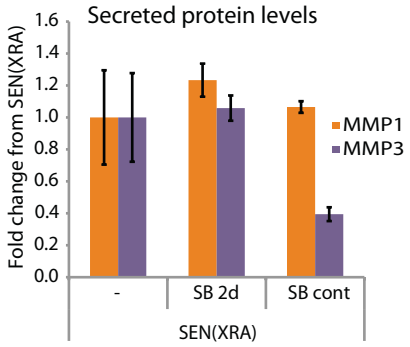
Factor	Log2 change from SEN(XRA)
GRO	-3.31
IL-6	-2.84
IL-8	-2.47
MCP-2	-2.43
MCP-3	-1.99
GCP-2	-1.35
GRO-alpha	-1.26
Angiogenin	-1.22
GM-CSF	-1.04
MCP-1	-0.99
CNTF	-0.91
GDNF	-0.90
IL-10	-0.87
IL-7	-0.80
MIG	-0.74
IL-2	-0.73
TGF-beta 1	-0.72
IL-1alpha	-0.71
Eotaxin	-0.68
IGFBP-4	-0.67
TNF-beta	-0.64
Osteoprotegerin	-0.59
TNF-alpha	-0.58
IL-5	-0.53
sgp130	-0.46

N

Proteins increased by p38 inhibition

Factor	Log2 change from SEN(XRA)	p-value
VEGF-D	0.01	0.99
MIP-1beta	0.03	0.91
GITR	0.05	0.87
FGF-9	0.09	0.67
b-NGF	0.09	0.82
HGF	0.10	0.78
sTNF RII	0.15	0.69
Oncostatin M	0.15	0.48
Axl	0.18	0.51
IL-2 Rapha	0.21	0.37
bFGF	0.21	0.26
Angiopoietin-2	0.22	0.32
AgRP	0.31	0.40
BTC	0.34	0.55
VEGF	0.39	0.57
Amphiregulin	0.41	0.12
HCC-4	1.04	0.16

O



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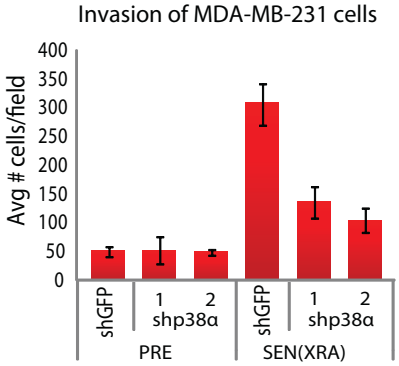
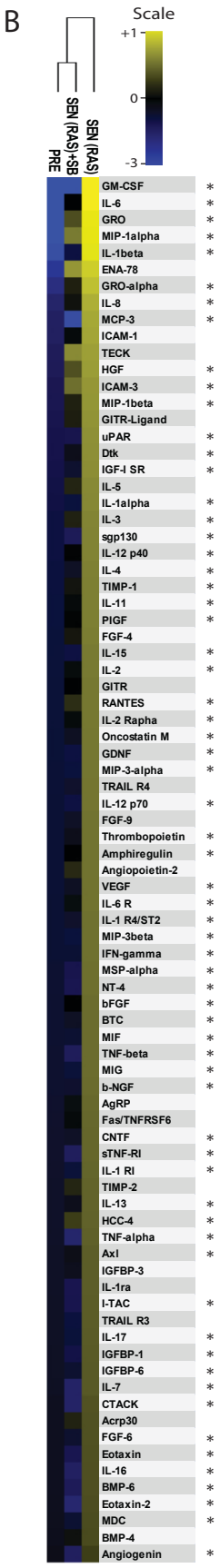


Figure 1-2
(1 of 2)



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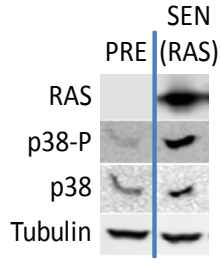

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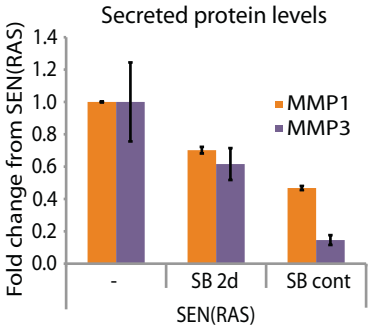
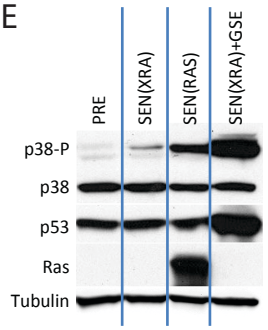


D

Factor	p38MAPK-dependent in XRA and RAS
GM-CSF	•
IL-6	•
GRO	•
GRO-alpha	•
MCP-3	•
IL-8	•
IL-5	
IL-1alpha	•
IL-3	
sgp130	•
IL-2	•
GDNF	•
TNF-beta	•
MIG	•
CNTF	•
TNF-alpha	•
IGFBP-1	
IGFBP-6	•
IL-7	•
Eotaxin	•
BMP-6	
BMP-4	
Angiogenin	•

E



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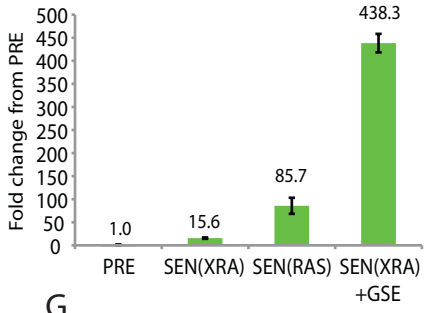

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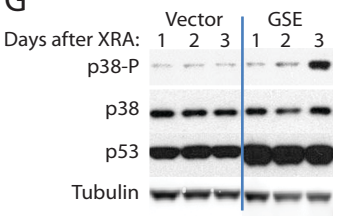

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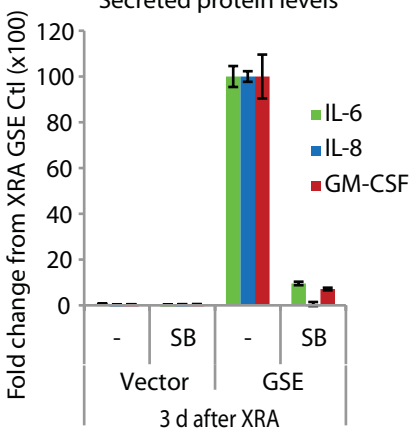

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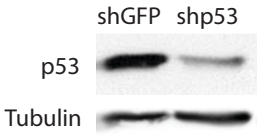

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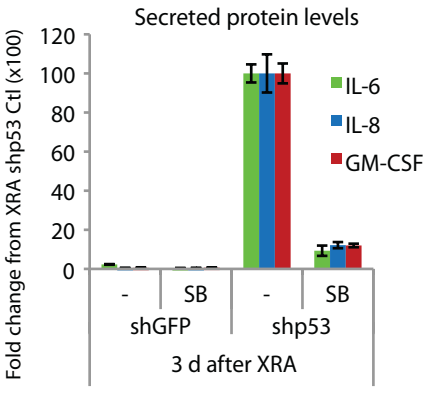
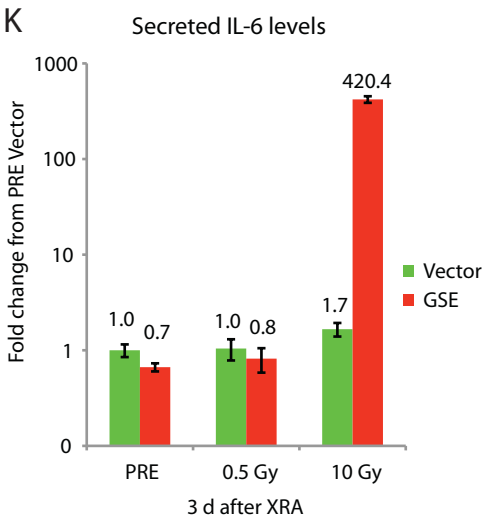
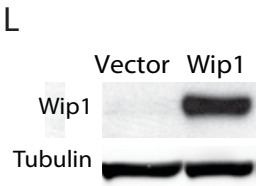
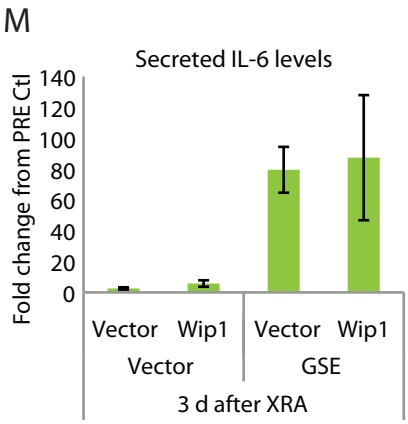
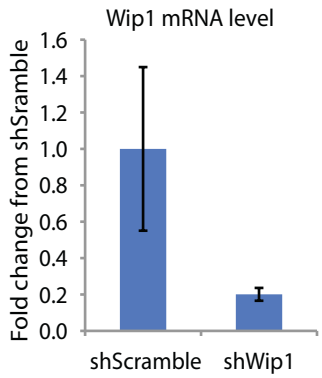


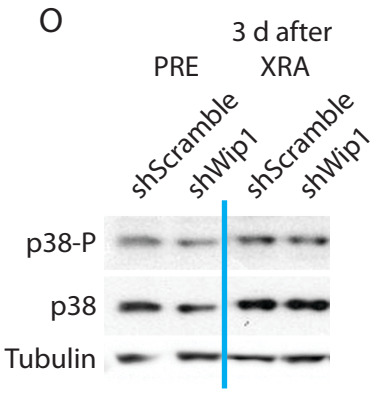
Figure 1-2
(2 of 2)











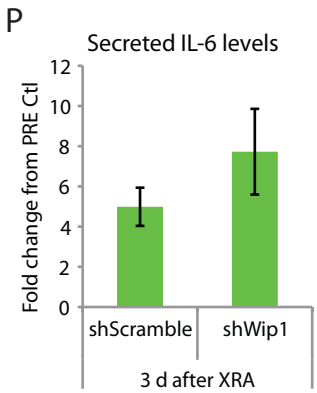


Figure 1-3
(1 of 1)

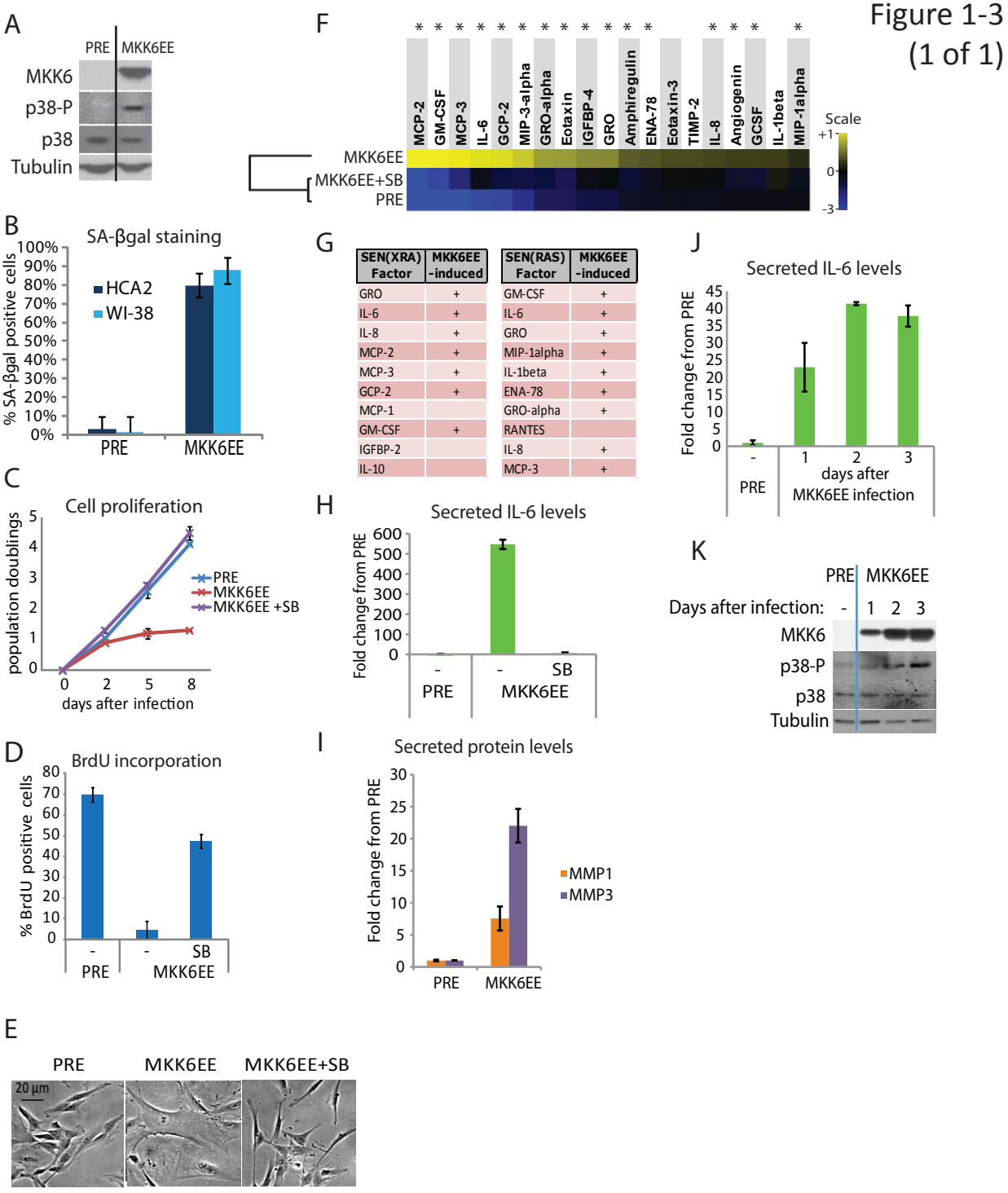


Figure 1-4
(1 of 2)

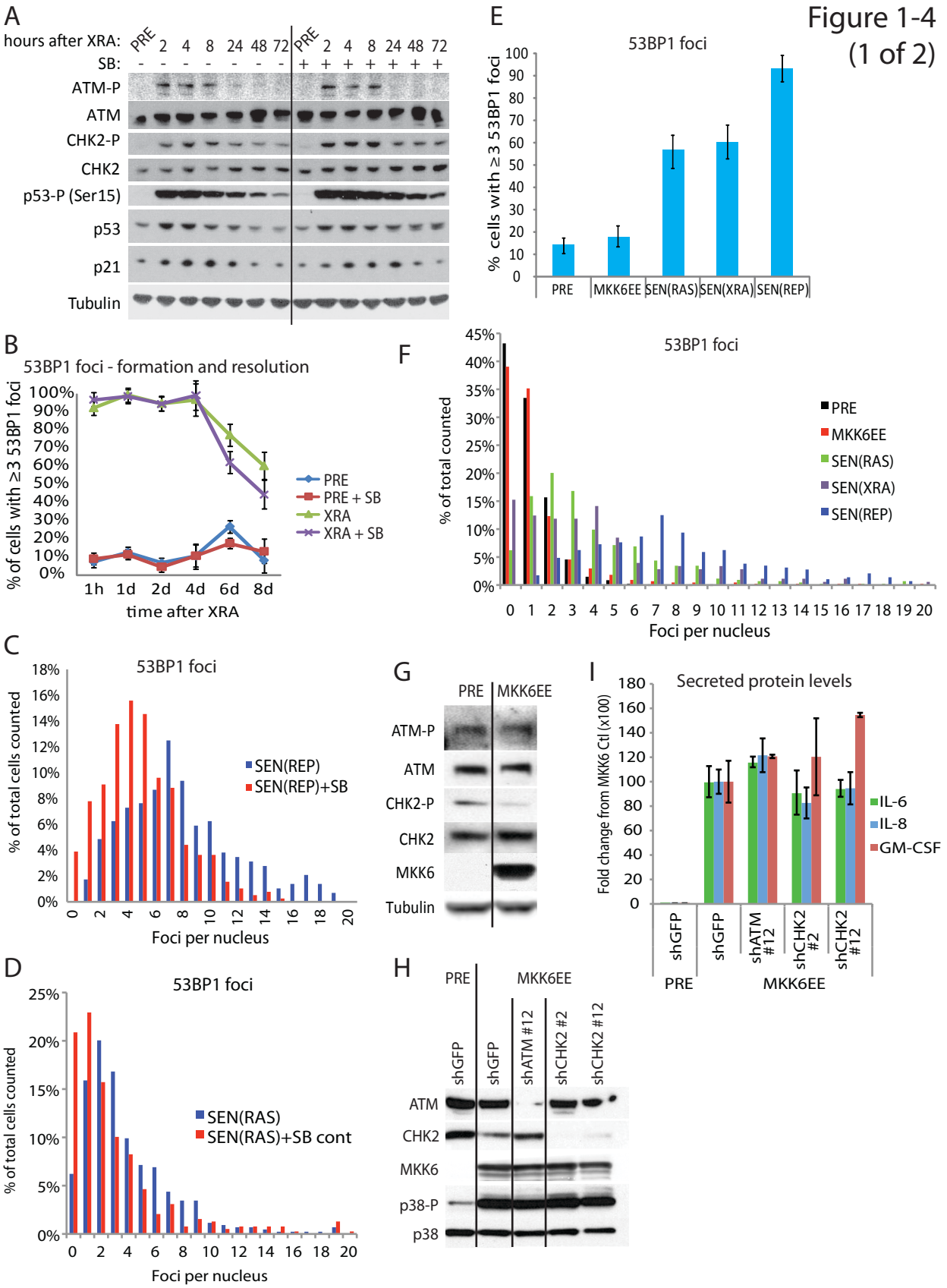
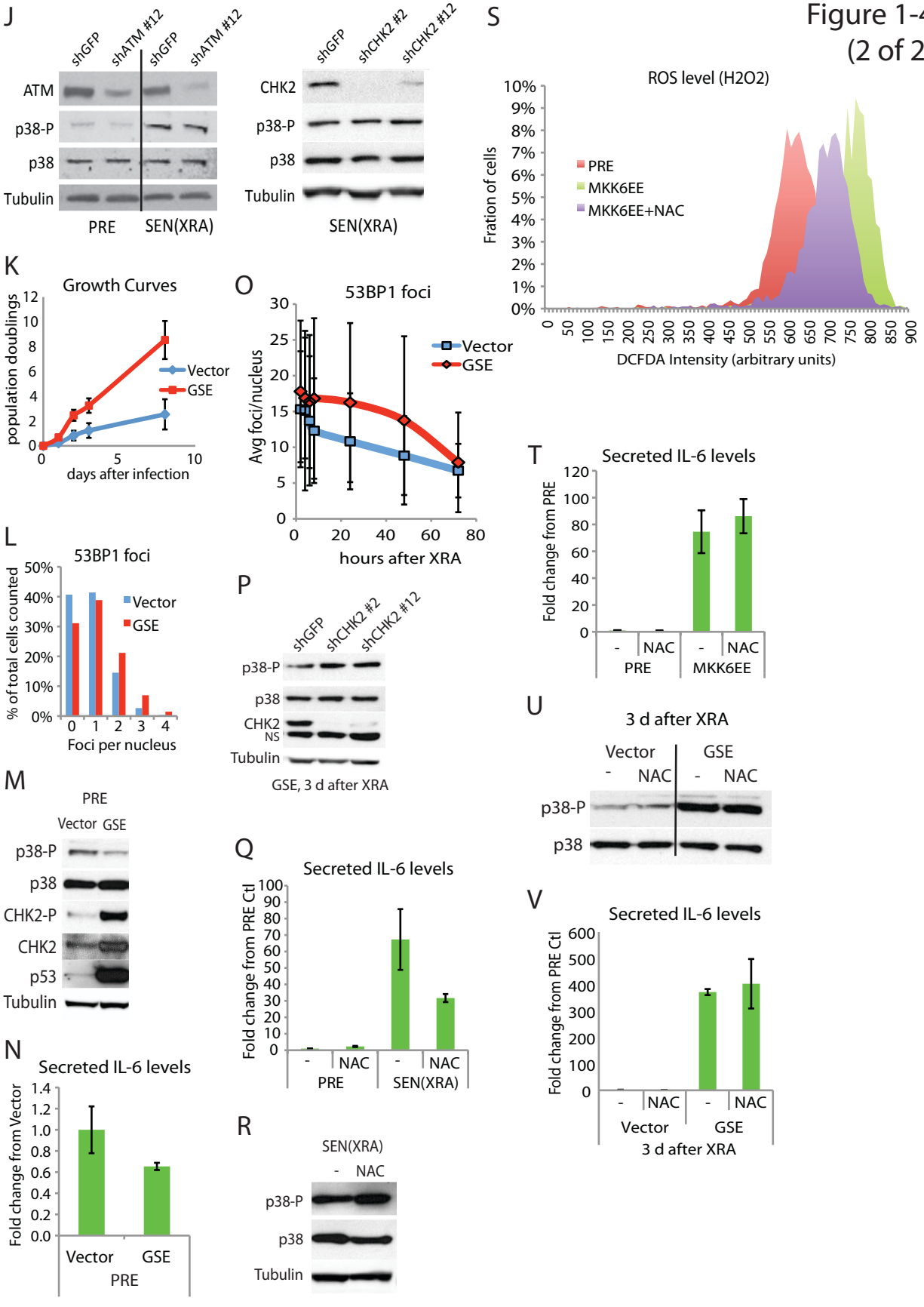
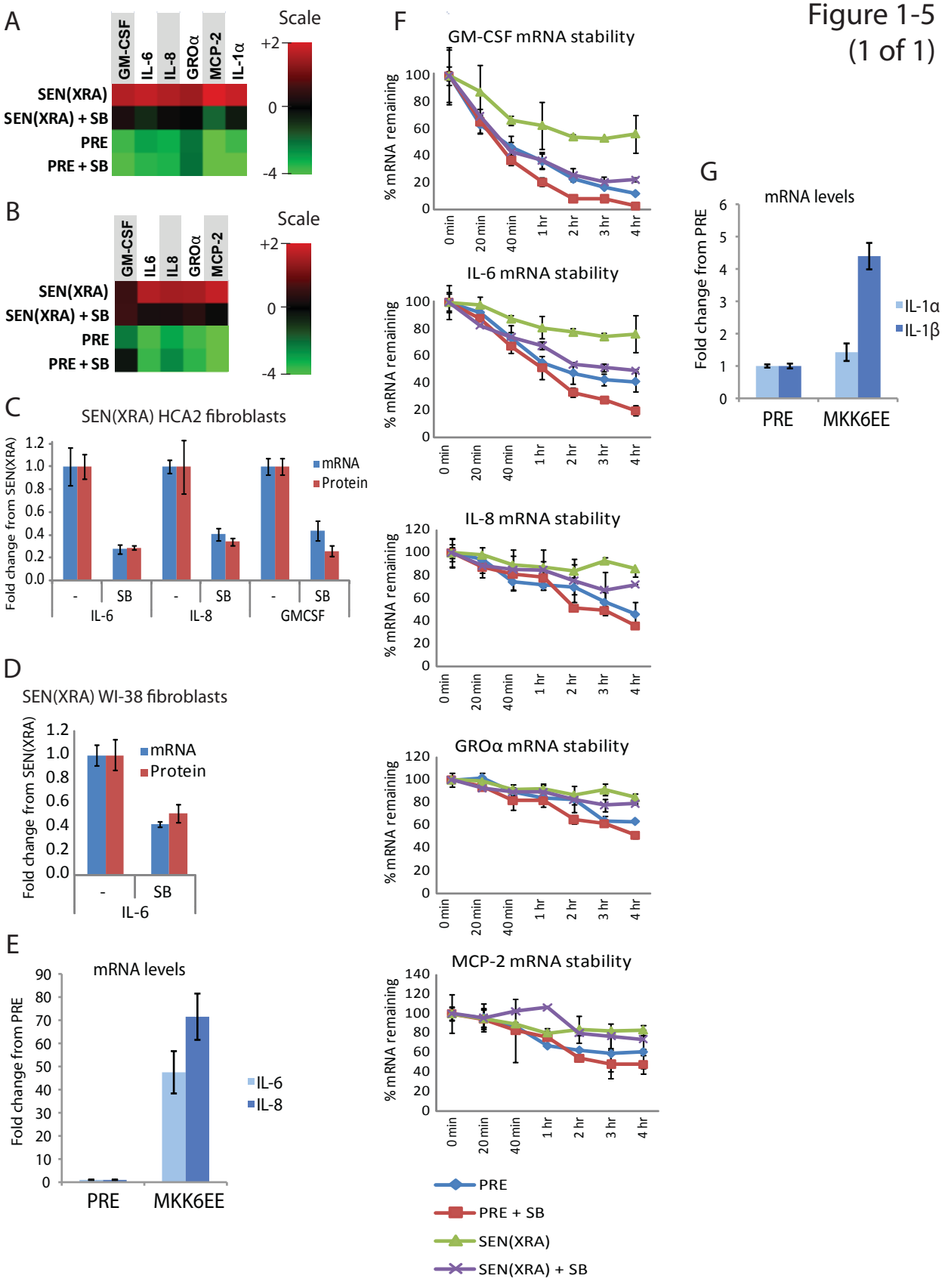


Figure 1-4
(2 of 2)



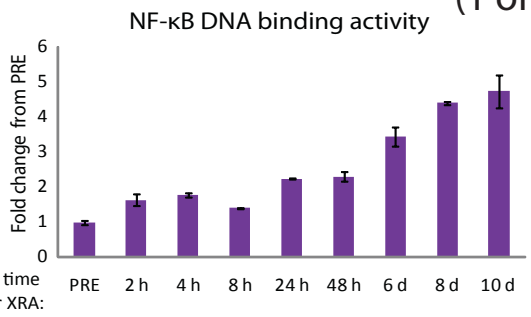

A
GM-CSF
IL-6
IL-8
GROα
MCP-2
IL-1α
SEN(XRA)
SEN(XRA) + SB
PRE
PRE + SB
Scale
+2
0
-4
B
GM-CSF
IL6
IL8
GROα
MCP-2
SEN(XRA)
SEN(XRA) + SB
PRE
PRE + SB
Scale
+2
0
-4
C
SEN(XRA) HCA2 fibroblasts
Fold change from SEN(XRA)
mRNA
Protein
-
SB
IL-6
IL-8
GMCSF
D
SEN(XRA) WI-38 fibroblasts
Fold change from SEN(XRA)
mRNA
Protein
IL-6
E
mRNA levels
Fold change from PRE
IL-6
IL-8
PRE
MKK6EE
F
GM-CSF mRNA stability
IL-6 mRNA stability
IL-8 mRNA stability
GROα mRNA stability
MCP-2 mRNA stability
% mRNA remaining
0 min
20 min
40 min
1 hr
2 hr
3 hr
4 hr
PRE
PRE + SB
SEN(XRA)
SEN(XRA) + SB
G
mRNA levels
Fold change from PRE
IL-1α
IL-1β
PRE
MKK6EE

A

Overrepresented TFBS in the set of MKK6EE-induced genes

Rank	TRANSFAC Matrix ID	Transcription Factor	% of Sequences	P-Value
1	V$NFKB_Q6	NF-kappaB	44%	2.21E-10
2	V$CEBPA_01	C/EBPalpha	44%	1.50E-09
3	V$CEBPB_01	C/EBPbeta	50%	3.26E-09
4	V$NFKAPPAB65_01	NF-kappaB (p65)	44%	6.37E-09
5	V$YY1_01	YY1	50%	7.91E-09
6	V$CEBP_Q2	C/EBP	50%	2.94E-08
7	V$PBX1_01	Pbx-1	50%	8.87E-08
8	V$CEBP_01	C/EBP	56%	1.30E-07
9	V$OCT1_03	1-Oct	56%	1.39E-07
10	V$NFKB_C	NF-kappaB	39%	1.41E-07

F



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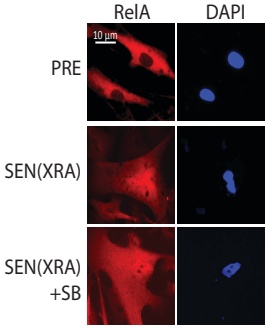

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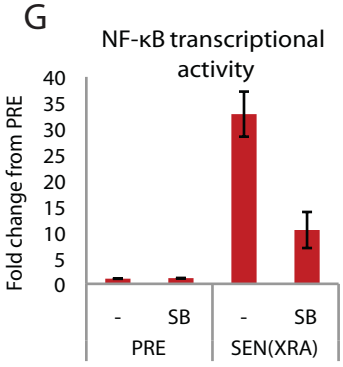

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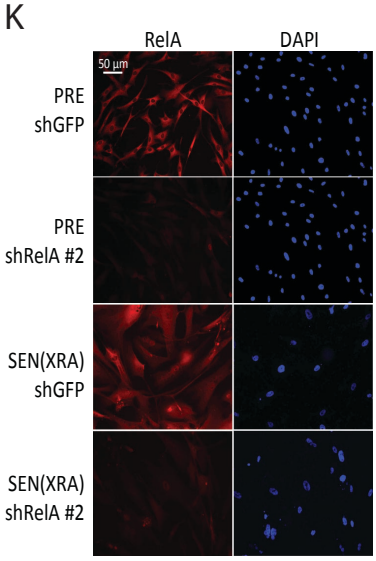

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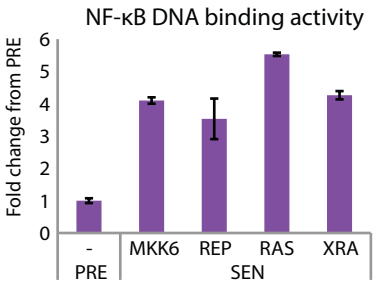

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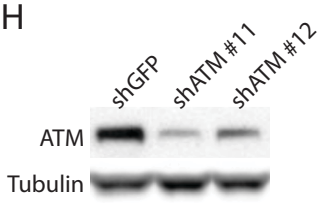

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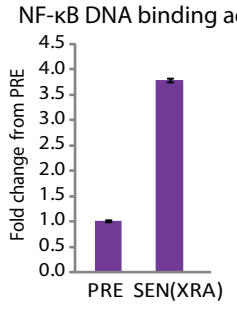

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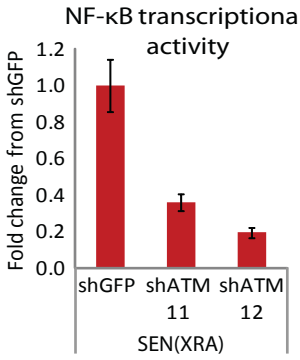

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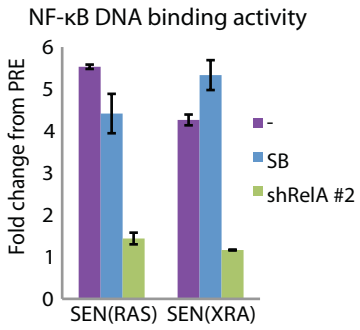

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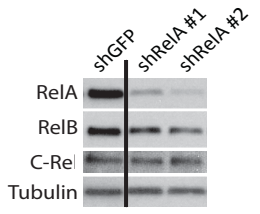
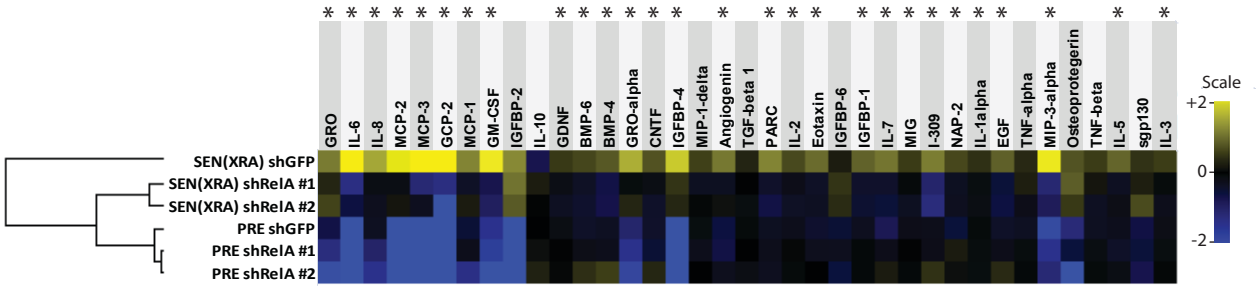


Figure 1-6
(2 of 2)

L



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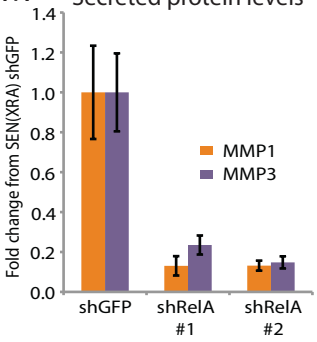

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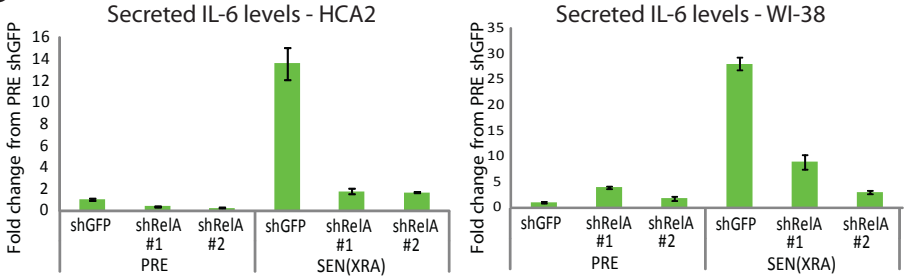

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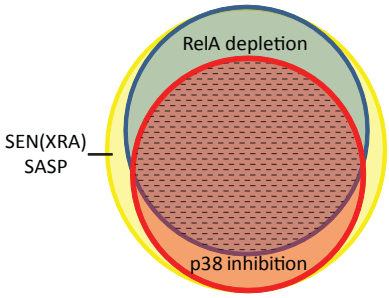
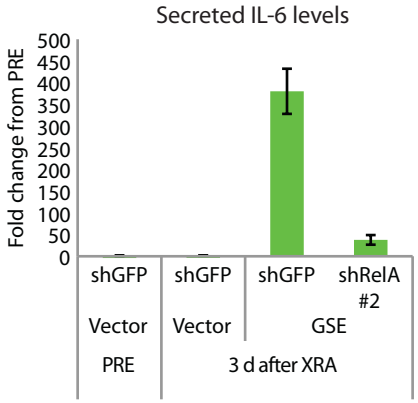


Factor	RelA	p38MAPK
GRO	+	+
IL-6	+	+
IL-8	+	+
MCP-2	+	+
MCP-3	+	+
GCP-2	+	+
MCP-1	+	+
GM-CSF	+	+
IGFBP-2		
IL-10		+

Q



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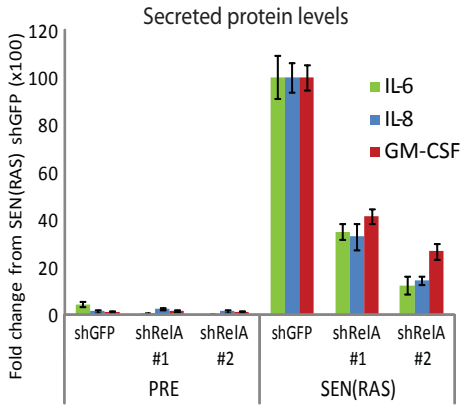

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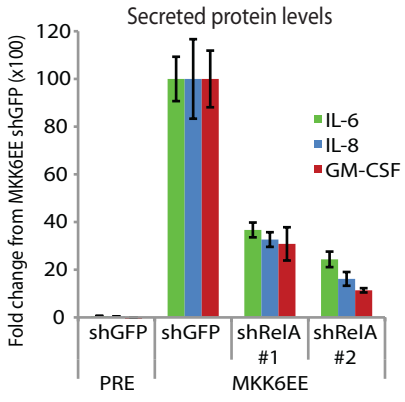

CHAPTER 2

p38MAPK regulates the senescence growth arrest and the senescence-associated secretory phenotype through distinct pathways

"If you are out to describe truth, leave elegance to the tailor"
-Albert Einstein

CHAPTER 2 ABSTRACT

p38MAPK signaling through NF-κB is necessary and sufficient for the senescence associated secretory phenotype (SASP) in both DNA damage-induced and oncogene-induced senescence driven by RAS. We investigated whether the p38MAPK/NF-κB pathway was involved in the senescence growth arrest. Although p38MAPK was required for efficient RAS-induced growth arrest, it was not required for DNA damage-induced growth arrest. Interestingly, DNA damage induced by X-radiation was unable to induce immediate growth arrest in proliferating RAS cells unless $p16^{INK4A}$ levels were high. NF-κB was not required for growth arrest induced by DNA damage or RAS, nor was NF-κB required for growth arrest induced by constitutively active p38MAPK. These data lead to two conclusions: First, RAS-induced growth arrest is dependent on p38MAPK because RAS signaling counteracts DDR/p53 growth arrest signals, rendering them insufficient for growth arrest; this demonstrates that RAS-induced growth arrest requires stronger growth arrest signals than DNA damage-induced growth arrest and thus is mechanistically distinct from DNA damage-induced growth arrest. Second, p38MAPK but not NF-κB can regulate senescence growth arrest, demonstrating a bifurcation in the growth arrest/SASP pathways downstream of p38MAPK. These findings indicate that the SASP and growth arrest can be independently regulated, suggesting possibilities for mitigating the deleterious effects of the SASP without adversely affecting the tumor suppressive growth arrest.

CHAPTER 2 INTRODUCTION

The senescence phenotype is complex – it involves widespread chromatin reorganization and gene expression changes, of which the senescence-associated secretory phenotype is only one part. Depending on the mode of senescence induction, the gene expression profile of senescent cells can differ substantially, as can the myriad of senescence pseudo-markers (Campisi & d'Adda di Fagagna, 2007; Collado & Serrano, 2010). As we have learned more about the phenotype, it has become apparent that there may be no features that are sufficient for a cell to be labeled "senescent". However, there is at least one feature that is necessary: proliferative arrest. Irrespective of all the other markers of the phenotype, if a cell is not arrested, it is not senescent (Kuilman et al, 2010).

There are three broad categories of senescence growth arrest, identified by senescence-inducing stimulus: replicative senescence, chromatin/DNA damage-induced senescence, and oncogene-induced senescence (Campisi & d'Adda di Fagagna, 2007). This third category is by far the most diverse, as virtually any gene whose activation can induce irreversible growth arrest is considered part of it; for example Ras, Raf, MEK, Akt, STAT5A, E2F1/3, Cyclin E, mos, or cdc6 (Courtois-Cox et al, 2008; Mallette et al, 2007; Prieur & Peeper, 2008). With rare exceptions that require further mechanistic investigation (Michaloglou et al, 2005; Olsen et al, 2002), the senescence growth arrest in all three cases is mediated by the p53 pathway, the $p16^{INK4A}$ pathway, or both; the differences between the categories are the upstream signals that activate these two major growth arrest pathways (Campisi & d'Adda di Fagagna, 2007).

However, though the field has partitioned senescence by the inducing stimulus, a common model is that all three categories of senescence growth arrest are actually the same category: DNA damage-induced senescence (Hemann & Narita, 2007; Zglinicki, 2005). Replicative senescence is caused by excessive cell division, which leads to critically short telomeres, which activates DNA damage response (DDR) factors such as ATM, CHK2, NBS1, and γ-H2AX (Deng et al, 2008) and eventually results in p53 activation. Inhibition of this DDR can induce cell cycle reentry, at least in some cell types (Herbig et al, 2004). The cells that fail to reenter the cell cycle upon DDR inactivation are halted because of $p16^{INK4A}$ expression, though $p16^{INK4A}$ expression may be a consequence of culture stress rather than of telomere shortening (Beausejour et al, 2003; Itahana et al, 2003; Zhang, 2004).

Many oncogenes induce DNA damage by overly rapid replication that leads to defects in DNA replication fork progression (Di Micco et al, 2006) and/or by production of reactive oxygen species (ROS) as signaling molecules, which then damage DNA (Lee et al, 1999). Supporting these respective mechanisms, some oncogenes can only induce senescence if the cells attempt to proceed through S-phase (Bartkova et al, 2006; Di Micco et al, 2006), and growing cells in low ambient oxygen can inhibit some types of oncogene-induced growth arrest (Lee et al, 1999; Lu & Finkel, 2008). Lastly, inactivation of DDR factors can prevent efficient oncogene-induced growth arrest in some contexts (Bartkova et al, 2006; Di Micco et al, 2006; Mallette et al, 2007).

However, evidence suggests that the reductionist theory that all senescence growth arrest is mediated by DNA damage is not completely correct. Though DNA damage clearly can induce growth arrest, some oncogenes such as E1A induce senescence without increasing DNA damage foci (Mallette et al, 2007), and not all oncogenes, for example Raf, Myc, and MEK, increase ROS (Dolado et al, 2007). Consequently, we hypothesized that there may be differences in the growth arrest regulatory pathways between DNA damage-induced senescence and oncogene-induced senescence.

To determine whether these pathway differences exist, we compared the role of the p38MAPK/NF-κB pathway in DNA damage-induced and RAS-induced growth arrest. p38MAPK is a known regulator of the senescence growth arrest in some contexts: p38MAPK inhibition can delay the replicative senescence arrest caused by dysfunctional telomeres (Iwasa et al, 2003) or the premature senescence of cells from patients with the premature aging Werner syndrome (Davis & Kipling, 2008). p38MAPK can mediate the expression of $p16^{INK4A}$ and the phosphorylation of p53, and both pathways are involved in RAS-induced growth arrest (Kwong et al, 2009; Sun et al, 2007). Finally, as we demonstrated in Chapter 1, p38MAPK activity is sufficient to induce a senescence-like growth arrest in normal human cells (Deng et al, 2004; Wang et al, 2002).

Here, we show that p38MAPK is required for oncogenic RAS-induced senescence growth arrest, but not DNA damage-induced growth arrest, demonstrating that the two inducers of senescence have different growth arrest requirements. We found that X-radiation, despite immediately arresting normal cells, could not induce immediate growth arrest in proliferating RAS cells, demonstrating that RAS signaling counteracts the p53 growth arrest signal initiated by the DDR, (likely because of the pro-mitogenic signaling that drive RAS-induced hyperproliferation), leading to a "weaker" net growth arrest signal. The concept of a net growth arrest signal that is tuned by mitogenic signaling as well as the p53/$p16^{INK4A}$ pathways explains why p38MAPK is required for RAS- but not DNA damage-induced growth arrest.

Interestingly, despite being downstream of p38MAPK, NF-κB was not required for either type of growth arrest. We also found that p38MAPK activation is sufficient to induce senescence growth arrest independent of a DDR or ROS production. Combined with others' results demonstrating that $p16^{INK4A}$ and p53, which are downstream of p38MAPK and required for growth arrest (Kwong et al, 2009), are not required for the SASP (Coppe et al, 2010a; Coppe et al, 2008), these data suggest that p38MAPK is a divergence point for regulation of the SASP versus the senescence growth arrest. This pathway bifurcation creates potential targets, such as NF-κB, that may inhibit the SASP without sacrificing the tumor-suppressing growth arrest.

CHAPTER 2 RESULTS

Unless indicated otherwise, error bars represent standard deviation.

Many studies of senescence are performed on cells cultured in ambient oxygen (20% O_2), an environmental situation that induces artifactual oxidative stress and can confound studies. 20% O_2 induces mouse embryonic fibroblasts to senescence via oxidative damage, whereas the same cells grown at a more physiological 3% O_2 can grown indefinitely, due to their endogenous expression of telomerase (Parrinello et al, 2003). Additionally, human fibroblasts can achieve up to 50% more population doublings when grown at 3% O_2 as compared to 20% O_2 (Chen et al, 1995), and some oncogenes only induce senescence at 20% O_2 (Lee et al, 1999). Additionally, growth at 20% O_2 alters other senescence-related phenotypes, such as the SASP (Coppe et al, 2008; Parrinello et al, 2003). Consequently, studies performed on inducers of senescence at ambient oxygen are really studying the combination of two senescence-inducers: the experimental inducer and oxidative damage. This can create situations in which DNA damage or ROS are implicated in ways that may not be physiologically relevant. To avoid such artifacts, we cultured all of our cells at 3% O_2.

p38MAPK is required for RAS-induced senescence

Though others have demonstrated that p38MAPK is required for RAS-induced senescence (Deng et al, 2004; Kwong et al, 2009; Sun et al, 2007; Wang et al, 2002), we asked what role p38MAPK plays in RAS-induced senescence without the increased oxidative damage and ROS signaling from 20% O_2 culture conditions. To model oncogene-induced senescence, we overexpressed an oncogenic form of H-RAS (RAS^{V12}), which induces growth arrest after 7-10 d (Serrano et al, 1997). To inhibit p38MAPK, we used the small molecule inhibitor SB203580 (SB) (Cuenda et al, 1995; Wilson et al, 1997; Young et al, 1997). We treated RAS cells with either of two modes of p38MAPK inhibition by SB (Figure 2-1A). In one case, we added SB for 48 h before sample collection, starting 8 d post-RAS infection (RAS+SB post). In the other case, we treated cells with SB for a total of 12 d, starting 2 days pre-RAS infection and continuing until sample collection (RAS+SB pre). These two regimens allowed us to distinguish between phenotypes that were preventable by p38MAPK, and phenotypes that were reversible. In both cases, SB was replaced daily.

We infected presenescent (PRE) normal human fibroblasts (strain HCA2) with a lentivirus expressing oncogenic RAS^{V12} (RAS). As reported (Di Micco et al, 2006), expression of RAS initially stimulated hyperproliferation for several days and ultimately induced a senescence growth arrest after 10 d (Figure 2-1B). Although others have reported that culturing cells at 3% O_2 prevents RAS-induced growth arrest by inhibiting ROS signaling (Lee et al, 1999), we observed efficient growth arrest by 10 d, a discrepancy that may be due to differences in RAS levels (Deng et al, 2004). As reported (Deng et al, 2004; Wang et al, 2002), cells induced to senesce by RAS (SEN(RAS)) showed increased levels of phosphorylated p38MAPK and $p16^{INK4A}$ (Figure 2-1C). Also as reported (Deng et al, 2004; Kwong et al, 2009; Wang et al, 2002), p38MAPK was required for induction of RAS-induced growth arrest: when we inhibited p38MAPK with SB starting before RAS infection and continuing for the length of the experiment, we found that growth arrest but not hyperproliferation was prevented (Figure 2-1B, RAS+SB pre). However, p38MAPK was unable to reverse the RAS-induced growth arrest: when p38MAPK was inhibited with SB for 48 hours after RAS-induced senescence had already been established (8 d after infection), the cells did not divide (Figure 2-1B, RAS+SB post), despite this being enough time for effective p38MAPK inhibition and SASP reduction (Chapter 1).

Because 48 h may not have been long enough for division to occur, we examined whether p38MAPK inhibition induced arrested cells to enter S-phase, as measured by BrdU incorporation. Though continuous p38MAPK inhibition prevented the RAS-induced decrease in BrdU incorporation (Figure 2-1D, RAS+SB pre), p38MAPK inhibition after RAS-induced growth arrest had been established did not cause cells to reenter S-phase (Figure 2-1D, RAS+SB post). We also found that p38MAPK inhibition was able to prevent, but not reverse, RAS-induced morphological changes (Figure 2-1E) and SA-βgal staining (Figure 2-1F). These data demonstrate that RAS-induced growth senescence, once established, does not require p38MAPK signaling.

We (Chapter 1) and others have seen that RAS signaling generates DNA damage, as measured by DNA damage foci formation (Di Micco et al, 2006; Dolado et al, 2007; Mallette et al, 2007; Moiseeva et al, 2009). There are two primary theories, which are not mutually exclusive, to explain how that DNA damage is generated. First, RAS stimulates hyperproliferation (Figure 2-1B), which causes replication fork collapse as cells attempt to proceed through S-phase; this replication fork collapse causes DNA damage and a subsequent DDR (Di Micco et al, 2006). Second, RAS signaling induces the production of ROS through a mechanism that involves NADPH oxidases and mitochondrial dysfunction (Dolado et al, 2007; Moiseeva et al, 2009). ROS then induce DNA damage, which activates a DDR. Others have implicated p38MAPK in a positive feedback loop that is required for sustained ROS production at senescence (Passos et al, 2010), and as we have shown, p38MAPK activation is sufficient to

induce some ROS signaling (Chapter 1). If the second hypothesis is correct, i.e. if DNA damage in RAS-induced senescence is driven primarily by ROS, and ROS production requires a p38MAPK-mediated feedback loop, then p38MAPK inhibition in RAS-expressing cells should decrease ROS and consequently the level of DNA damage. To address this hypothesis, we examined DNA damage foci in SEN(RAS) cells, with and without p38MAPK inhibition, by immunostaining for the DDR adapter protein 53BP1. Whereas PRE controls had, on average, <1 53BP1 focus per nucleus, RAS-expressing cells had >3 53PB1 foci per nucleus (Figure 2-1G). p38MAPK inhibition for 48 h before collection had no effect on 53BP1 foci number (SB post). Continuous p38MAPK inhibition starting before RAS expression decreased the average number of foci slightly (SB pre, $p<0.05$), but the levels were still significantly higher than in presenescent controls ($p<0.05$). Notably, these remaining foci persisted despite what appeared to be a substantial decrease in ROS: the cytoplasmic vacuoles seen in RAS cells (Figure 2-1E), which are formed by accumulation of H_2O_2 (Moiseeva et al, 2009), were completely absent in RAS-expressing cells exposed to continuous p38MAPK inhibition (Figure 2-1E).

p38MAPK is not required for DNA damage-induced senescence

As discussed earlier, a prevailing theory is that multiple inducers of senescence, including RAS, mediate growth arrest via the DNA damage response and thus, in effect, are a subcategory of DNA damage-induced senescence (Di Micco et al, 2006; Hemann & Narita, 2007; Zglinicki, 2005). To explore this theory further, we asked whether p38MAPK inhibition was required for direct DNA damage-induced growth arrest. To model DNA damage-induced senescence, we treated presenescent cells with 10 Gy X-radiation (XRA), which induces widespread DNA double strand breaks that activate a DDR (Costes et al, 2010; Morgan et al, 1996). Proliferative arrest occurs within 24 h (see Figure 2-2G), followed by the induction of other senescence markers over 7-10 days (Chapter 1 and Figure 2-2). In this case, we treated irradiated cells with only one mode of p38MAPK inhibition by SB (Figure 2-2A): a total of 12 d with SB, starting 2 days before irradiation and continuing until sample collection (XRA+SB). The same treatment regimen was applied to presenescent cells as a control, which were mock irradiated on day 0. SB was replaced daily.

Although p38MAPK has been reported to mediate DNA damage-induced growth arrest in other contexts and cell types (Hong et al, 2009; Passos et al, 2010), continuous p38MAPK inhibition had no effect on XRA-induced growth arrest in our system (Figure 2-2B). p38MAPK inhibition also had no effect on the XRA-induced decrease in BrdU incorporation (Figure 2-2C), morphological changes (Figure 2-2D), or increase in SA-βgal staining (Figure 2-2E). This lack of effect was not due to inefficient p38MAPK inhibition – using phosphorylation of Hsp27, a downstream target of p38MAPK, as a marker of p38MAPK activity, we determined that p38MAPK signaling was increased in SEN(XRA) cells, and that signaling was reduced to presenescent levels by SB treatment (Figure 2-2F). However, this p38MAPK inhibition had no effect on $p16^{INK4A}$ expression (Figure 2-2F), demonstrating that XRA cells activate p38MAPK-independent pathways to induce $p16^{INK4A}$ and subsequent growth arrest. This is in contrast to RAS cells, which reportedly require p38MAPK for $p16^{INK4A}$ expression (Kwong et al, 2009). Consequently, unlike RAS-induced growth arrest, XRA-induced growth arrest cannot be prevented (or reversed) by p38MAPK inhibition.

We reasoned that, despite having no long-term effect on XRA-induced growth arrest phenotypes, p38MAPK inhibition might have an effect on more subtle phenotypes, such as cell cycle checkpoint activation immediately after XRA, affecting entry into S-phase. To test this, we examined BrdU incorporation (in 24 h pulses started at the indicated times) during the 24 hours after XRA in exponentially growing cells. We found that p38MAPK inhibition had no effect on the rapid decrease in BrdU incorporation during this time (Figure 2-2G, $p>0.05$ for all post-XRA

timepoints). Interestingly, the only case for which SB treatment caused a significant increase in BrdU incorporation over untreated controls was in presenescent cells, represented by timepoint 0 h (Figure 2-2G). Whereas ~80% of untreated presenescent cells incorporated BrdU during a 24 h pulse, ~90% of SB-treated presenescent cells did ($p<0.05$). This effect was subtle, but reproducible, leading to SB-treated cells accumulating slightly more population doublings over a 10 d period (Figure 2-2B, $p<0.05$). The mechanism behind this increase in growth rate is unknown, but may involve p38MAPK inhibition preventing stress-induced (which is $p16^{INK4A}$-mediated) senescence in a subset of cells in the otherwise-presenescent population.

Similar to the effect on RAS-induced 53BP1 foci, continuous p38MAPK inhibition slightly reduced the average number of 53BP1 foci in SEN(XRA) cells (Figure 2-2H, $p<0.05$). p38MAPK inhibition also decreased the average number of 53BP1 foci in replicatively senescent (SEN(REP)) cells (Figure 2-2I, $p<0.05$), though like XRA-induced senescence, this was not accompanied by increased DNA synthesis or replication, as measured by BrdU incorporation (Figure 2-2I).

As both untreated and SB-treated cells began with the same number of DNA damage foci immediately after XRA (or in SEN(REP)) (Chapter 1), the effect of p38MAPK inhibition on final 53BP1 foci number must either be a result of increased foci resolution or less additional foci formation. We have shown that p38MAPK inhibition does not affect the activation of DDR proteins, which are responsible for sensing and repairing DNA damage, so the more likely explanation is the latter – p38MAPK inhibition reduces the formation of additional foci. This may be a consequence of inhibiting a ROS feedback loop, which can generate short-lived DNA damage foci that are required for efficient growth arrest in some contexts (Passos et al, 2010). However, as we have described, DNA damage foci reduction by p38MAPK inhibition was not correlated with prevention of XRA-induced or replicative growth arrest in our system. Nevertheless, we asked whether ROS signaling was required for XRA-induced growth arrest. We found that treatment with NAC did not prevent the XRA-induced growth arrest (Figure 2-2J), irrespective of whether NAC was given for the first 24 hours after XRA (NAC first 24 h) to block any initial surge in ROS, for the last 48 hours before sample collection (NAC post) to disrupt any existing ROS feedback loop, or continuously, starting before XRA (NAC pre). These data suggest that ROS signaling is not required for XRA-induced growth arrest in our system.

Initial DNA damage signaling is not sufficient to drive RAS-induced growth arrest

p38MAPK was required for RAS-induced growth arrest but not XRA-induced growth arrest, and this difference is likely due to XRA activating $p16^{INK4A}$ via p38MAPK-independent pathways. This is surprising, as many reports suggest that RAS-induced senescence is mediated by DNA damage caused by hyperproliferation or ROS (Courtois-Cox et al, 2008; Di Micco et al, 2006; Mallette et al, 2007); thus, one would expect both inducers of senescence to activate similar pathways. To investigate this further, we asked whether DNA damage was actually sufficient to drive RAS-induced growth arrest. To answer this question, we induced widespread DNA damage in proliferating, RAS-expressing cells via XRA. As previously demonstrated, XRA causes normal cells to arrest almost immediately (Figure 2-2). If RAS-induced senescence is driven only by accumulation of DNA damage, this intense burst of DNA damage (sufficient to arrest normal cells) should immediately drive RAS cells out of their hyperproliferative phase and into growth arrest. If instead, RAS cells require additional signals, the cells may continue to divide even after being irradiated.

To determine the correct timepoint after RAS infection for irradiation, we examined RAS expression in the three days after RAS^{V12} lentiviral infection (Figure 2-3A). We found that RAS expression was detectable within 1 day after infection, but continued to increase over the next 2

days. This increase was paralleled by an increase in p38MAPK phosphorylation and $p16^{INK4A}$ expression. Interestingly, RAS-expressing cells continued to proliferate during this time and after (Figure 2-1B), demonstrating that the increase in $p16^{INK4A}$ was not sufficient to drive growth arrest. However, to avoid the effects of this increase in $p16^{INK4A}$ expression, we chose to irradiate RAS-expressing cells 1 day after infection (Figure 2-3B), when they were already proliferating measurably faster than PRE cells (Figure 2-1). Surprisingly, we found that, while control cells (Figure 2-3B, PRE) virtually ceased cell proliferation immediately after XRA (XRA), RAS-expressing cells continued to proliferate after XRA (RAS+XRA), though they grew slower than mock irradiated RAS-expressing cells (RAS) and eventually arrested 7 days after XRA. Importantly, this eventual growth arrest was due to XRA rather than RAS, as p38MAPK inhibition, which prevented RAS-induced growth arrest (RAS+SB), did not prevent growth arrest in irradiated RAS cells (RAS+SB+XRA), though it did delay it slightly. We next asked whether XRA had a differential effect on cells that had been expressing RAS for 3 days, instead of just 1 day, reasoning that the increase in $p16^{INK4A}$ levels after 3 days of RAS expression might prime the cell for growth arrest. In this case, we found that irradiation stopped RAS-expressing cells with the same kinetics as normal cells, though mock irradiated RAS cells continued to proliferate (Figure 2-3C).

These results demonstrate that XRA-induced DNA damage is not sufficient to drive growth arrest in RAS-expressing cells, though it is sufficient in normal cells. Therefore, RAS signaling counteracts growth arrest signals, and these pro-mitogenic signals are almost certainly the same signals that lead to RAS-induced hyperproliferation. XRA treatment did slow and eventually stop the proliferation of RAS cells, even with p38MAPK inhibited, demonstrating that XRA activates p38MAPK-independent pathways that are not activated by RAS; this fits with our observation that XRA-induced $p16^{INK4A}$ expression is p38MAPK independent (Figure 2-2). However, XRA-induced $p16^{INK4A}$ takes several days to develop (Campisi, unpublished data), explaining why several days were required for RAS cells to stop after XRA, as long as they were irradiated when RAS-induced $p16^{INK4A}$ levels were low (i.e. 1 day after RAS expression). If RAS-induced $p16^{INK4A}$ levels were high, on the other hand (i.e. 3 days after RAS expression), XRA arrested RAS cells immediately. We suggest that the RAS-induced increase in $p16^{INK4A}$, though insufficient to prevent proliferation on its own, cooperates with the XRA-induced signals to induce growth arrest. Supporting this conclusion, p38MAPK inhibition, which prevents RAS-induced $p16^{INK4A}$ expression (Kwong et al, 2009), allowed irradiated, RAS-expressing cells to proliferate slightly longer than the same cells without p38MAPK inhibition (Figure 2-3B, compare RAS+XRA to RAS+SB+XRA).

NF-κB does not enforce senescence growth arrest

Certain SASP factors are reported to reinforce the senescence growth arrest (Acosta et al, 2008; Kuilman et al, 2008; Wajapeyee et al, 2008; Yang et al, 2006), and the p38MAPK/NF-κB pathway is required for expression of those factors at senescence (Chapter 1). Consequently, we hypothesized that SASP inhibition might be one way that p38MAPK inhibition prevents growth arrest. To address this further, we examined whether NF-κB, which mediates the SASP downstream of p38MAPK (Chapter 1), was required for RAS-induced or XRA-induced growth arrest. We expressed, via lentiviral vector, either of two unrelated shRNAs against RelA, the SASP-mediating subunit of NF-κB. Both shRNAs efficiently decreased RelA levels (Figure 2-4A). Subsequently, we either expressed oncogenic RAS (RAS) or irradiated the cells (XRA). As discussed earlier, RAS expression stimulated hyperproliferation for several days, followed by growth arrest; however, unlike p38MAPK inhibition, RelA depletion had no effect on RAS-induced growth arrest (Figure 2-4B). RelA depletion also had no effect on XRA-induced growth arrest (Figure 2-4C). Supporting these data, RelA depletion had no effect on the decrease in BrdU incorporation (Figure 2-4D), the morphological changes (Figure 2-4E), or the increase in

SA-βgal staining (Figure 2-4F) induced by RAS or XRA. These data show that, despite being critical for SASP expression (Chapter 1), NF-κB is not required for senescence growth arrest, suggesting that SASP inhibition is not the primary mechanism by which p38MAPK inhibition prevents growth arrest.

Additionally, though NF-κB and p38MAPK inhibition have qualitatively equal effects on SASP factor expression, they do not have equal effects on DNA damage foci. Whereas p38MAPK inhibition slightly decreases the average number of 53BP1 foci per nucleus in SEN(XRA) and SEN(RAS) cells (Figures 2-1, 2-2), NF-κB depletion had no significant effect on the average number of 53BP1 foci per nucleus in either type of senescence (Figure 2-4G, $p>0.05$), though the effect, if existent, trended towards a decrease. RelA depletion also had no effect on DDR activation after XRA, as measured by p53 phosphorylation on Ser15 (Figure 2-4H), which is a downstream marker of DDR signaling (Siliciano et al, 1997). The data suggest that NF-κB is not the conduit by which p38MAPK affects DNA damage foci.

p38MAPK induces senescence growth arrest independently of DNA damage or ROS production

To study possible mechanisms by which p38MAPK activation prevents cell proliferation, we took advantage of a constitutively active mutant (MKK6EE) of the upstream kinase of p38MAPK, MAP kinase kinase 6 (MKK6), which directly phosphorylates p38MAPK (Enslen et al, 1998; Raingeaud et al, 1996). To verify that the effects of MKK6EE were mediated by p38MAPK, we inhibited p38MAPK with SB. We used two treatment regimens for p38MAPK inhibition (Figure 2-5A): in one case, we treated cells with SB for 48 h before sample collection, starting 5 d post-MKK6EE infection (MKK6EE+SB post). In the other case, we treated cells with SB for a total of 9 d, starting 2 days pre-MKK6EE infection and continuing until sample collection (MKK6EE+SB pre). In both cases, SB was replaced daily.

We infected cells with a lentivirus expressing MKK6EE, which led to constitutive phosphorylation of endogenous p38MAPK (Figure 2-5B). When cells were cultured at 3% O_2, MKK6EE expression induced $p16^{INK4A}$ expression (Figure 2-5B), proliferative arrest (Figure 2-5C), a halt to DNA replication as measured by BrdU incorporation (Figure 2-5D), and an enlarged senescent morphology (Figure 2-5E), as reported for 20% O_2 (Iwasa et al, 2003; Wang et al, 2002). When we inhibited p38MAPK with SB beginning pre-MKK6EE expression and continuing for the length of the experiment, we found that all of these responses were prevented (Figure 2-5B-E, MKK6EE+SB pre), demonstrating that MKK6EE-induced senescence is completely mediated by p38MAPK. However, when we inhibited p38MAPK with SB for 48 hours after MKK6EE-induced senescence had already been established (5 d after infection), none of these responses were reversed (Figure 2-5B-E, MKK6EE+SB post), demonstrating that the p38MAPK-induced senescence phenotype, once established, is self perpetuating and does not require continued p38MAPK signaling.

We next examined the role of DNA damage in MKK6EE-induced senescence. MKK6EE expression induced a small but significant increase in the average number of 53BP1 foci per nucleus (Figure 2-5F, $p<0.05$). However, as we discussed in Chapter 1, this average increase comes from a shift towards more foci in ~6% of cells – those with ≥4 foci (Figure 2-5G). The reason this shift only occurs in a small subset of cells is unclear – it may be that existing DNA damage sensitizes a cell to acquiring or responding to new damage, e.g. from increased ROS. Supporting the idea that only a small subset of cells accumulates DNA damage signaling, MKK6EE expression induced no detectable increase in average DDR signaling, as measured by ATM and CHK2 phosphorylation (Figure 2-5H). Additionally, ATM and CHK2 were not required for MKK6EE-induced growth arrest. Stable depletion of ATM or CHK2 by RNAi starting

before MKK6EE expression (Figure 2-5I) did not lead to any increase in cumulative population doublings (PDs) versus control during the 7 days after MKK6EE infection (Figure 2-5J).

As we demonstrated in Chapter 1, and as others have shown (Koli et al, 2008; Passos et al, 2010; Torres & Forman, 2003), MKK6EE expression induced an increase in ROS, as measured by intracellular H_2O_2 (Figure 2-5K, $p<0.05$). However, ROS scavenging by N-acetyl cysteine (NAC), which decreased H_2O_2 levels by about half when added to cells for 48 h before collection (Figure 2-5K, $p<0.05$), did not affect MKK6EE-induced growth arrest. NAC treatment led to no increase in cumulative PDs versus the untreated MKK6EE control during the 7 days after MKK6EE infection, regardless of whether NAC was added for the 48 h before collection (NAC post) or was given continuously, starting before MKK6EE infection (NAC pre) (Figure 2-5L). These data suggest that ROS signaling is not required for p38MAPK-induced growth arrest.

Conversely, p53 was essential for MKK6EE-induced growth arrest. We inactivated p53 using retrovirally-delivered GSE22, a peptide that prevents p53 tetramerization and thus p53 transcriptional activity (Ossovskaya et al, 1996). Because p53 monomers are not rapidly degraded, GSE22 activity can be monitored by the accumulation of p53 protein (Figure 2-5M). We infected GSE-expressing cells with a lentivirus expressing MKK6EE. Seven days after MKK6EE-infection, cells with inactive p53 had the same level of MKK6 expression and p38MAPK phosphorylation as cells with wild-type p53 (Figure 2-5M). However, whereas MKK6EE-expressing cells with wild-type p53 were growth arrested and had divided less than once since MKK6EE infection, MKK6EE-expressing cells with inactive p53 were still proliferating and had accumulated the same PDs as normal presenescent cells (Figure 2-5N). Taken together, these data suggest that constitutive p38MAPK (by MKK6EE expression) induces growth arrest that is dependent on p53 activity but is independent of DNA damage, DDR signaling, or ROS.

CHAPTER 2 DISCUSSION

A required feature of senescence – in fact, the only required feature of senescence – is proliferative arrest, though it is important to recognize that not every arrested cell is a senescent cell (Kuilman et al, 2010). Senescence proliferative arrest is an important *in vivo* tumor suppressor mechanism, particularly in the case of oncogene-induced senescence (Braig et al, 2005; Chen et al, 2005; Collado et al, 2005; Collado & Serrano, 2010; Kuilman et al, 2010; Michaloglou et al, 2005). However, the secretory phenotype of senescent cells may have deleterious effects on the tissue environment, including the promotion of malignant phenotypes (Campisi & d'Adda di Fagagna, 2007; Freund et al, 2010). Paradoxically, then, senescence – an important line of cancer defense – might promote cancer or other disease over the long term as senescent cells accumulate in tissues (Campisi, 2005; Dimri et al, 1995; Jeyapalan et al, 2007; Paradis et al, 2001; Zhou et al, 2008). Thus, understanding how the SASP and the growth arrest can be uncoupled could lead to therapies that mitigate the deleterious effects of the SASP without sacrificing tumor suppression.

In Chapter 1, we demonstrated that the p38MAPK/NF-κB pathway is necessary and sufficient for the SASP. However, p38MAPK is also a known regulator of the senescence growth arrest in some contexts (Davis et al, 2005; Iwasa et al, 2003; Kwong et al, 2009; Passos et al, 2010; Sun et al, 2007), and others have demonstrated that the SASP is important to reinforce the growth arrest (Acosta et al, 2008; Kuilman et al, 2008), suggesting that these two sides of the senescence phenotype – the SASP and the growth arrest – might be inseparable,

at least with regard to the p38MAPK/NF-κB pathway. To explore this further, we examined the role of p38MAPK and NF-κB in senescence growth arrest.

Whereas p38MAPK was required for the SASP regardless of how senescence was induced (Chapter 1), its role in the senescence growth arrest depended on the mode of senescence induction. Inhibition of p38MAPK prevented RAS-induced growth arrest but had no effect on the growth arrest following DNA damage by X-radiation (XRA). This was not due to differences in p38MAPK-mediated inhibition of DDR signaling: p38MAPK inhibition had a qualitatively similar effect on DNA damage foci in RAS and XRA cells, which was a small reduction in the average foci per nucleus. Instead, we determined that XRA induces $p16^{INK4A}$ via an unknown, p38MAPK-independent mechanism, whereas RAS-induced $p16^{INK4A}$ requires p38MAPK (Kwong et al, 2009).

The presence of distinct growth arrest pathways in XRA versus RAS cells was surprising, as RAS-induced growth arrest is widely considered to be a result of DNA damage, generated either by hyperproliferation or ROS (Courtois-Cox et al, 2008; Di Micco et al, 2006; Mallette et al, 2007). However, we found that XRA-induced DNA damage, which was sufficient to immediately arrest normal cells, did not immediately arrest proliferating RAS cells unless those cells had been expressing RAS for several days and thus had high levels of $p16^{INK4A}$. The primary conclusion of this result is that induction of p53 by DNA damage signaling is not sufficient to drive growth arrest in a RAS-expressing background, and consequently RAS-induced growth arrest cannot exclusively be a DNA damage response, though DNA damage may contribute to the eventual RAS-induced growth arrest. 10 Gy of X-radiation induces widespread DNA damage that immediately drives normal cells into G_0 via p53 activation, but RAS expression prevented that immediate growth arrest unless $p16^{INK4A}$ levels were high (3 days after RAS infection). We suggest that increased $p16^{INK4A}$ expression, in combination with p53-mediated growth arrest signals from DNA damage, but neither alone, is sufficient to counteract the mitogenic signaling from RAS. Consequently, in an unirradiated RAS cell, despite early induction of $p16^{INK4A}$, growth arrest takes several days, during which time the cell accumulates DNA damage (from hyperproliferation or ROS – see discussion below), which then cooperates with $p16^{INK4A}$ signaling. Supporting this conclusion, others have demonstrated that both p53 and $p16^{INK4A}$ are required for RAS-induced senescence (Kwong et al, 2009). Conversely, in XRA cells, which have no hyper-mitogenic signaling, the initial p53 response to DNA damage is sufficient to induce immediate growth arrest, which is then "locked in" via a later increase in $p16^{INK4A}$.

A more general interpretation of these results has implications for our understanding of growth arrest in the context of tumorigenesis. The broad concept arising from the above discussion is that activation of growth arrest pathways does not raise an impenetrable barrier to proliferation, but rather, a cell's proliferative state is determined by the net signal from growth arrest signals versus mitogenic signals. This is in contrast to current understanding of senescence growth arrest; whereas current explanations of tumor growth assume that a malfunction or suppression of growth arrest pathways is a necessary part of tumorigenesis (Campisi & d'Adda di Fagagna, 2007; Courtois-Cox et al, 2008; Di Micco et al, 2007), these results demonstrate that oncogenic signaling can force a cell to proliferate, perhaps indefinitely, if just one of the two major growth arrest pathways (i.e. p53 and $p16^{INK4A}$) is activated. Of note, we saw that RAS cells expressed high amounts of $p16^{INK4A}$ 3 days after infection, but still proliferated faster than PRE controls. Consequently, when examining cells *in vivo*, particularly in and around malignant and pre-malignant tumors, the use of $p16^{INK4A}$ as a biomarker of senescence may lead to incorrect conclusions about proliferative status (irrespective of mutations to downstream targets of $p16^{INK4A}$ such as pRb), though evidence suggests that the final transition into full-blown carcinoma may indeed require inactivation of growth arrest

pathways in some cases (Collado & Serrano, 2010). Additionally, the theory that additive growth arrest signals are required for growth arrest in an oncogenic background may explain why p53 activation has no effect on early tumor formation, but can drive regression of late-stage, high-grade tumors with high MAPK activity (Feldser et al, 2010; Junttila et al, 2010).

There are multiple mechanisms by which p38MAPK might regulate RAS-induced growth arrest in addition to $p16^{INK4A}$ expression. p38MAPK can phosphorylate p53, both directly and through mediators such as PRAK (Kwong et al, 2009; Sun et al, 2007). p38MAPK may also phosphorylate DDR proteins such as Chk2 (Wang et al, 2000) or generate a ROS feedback loop that generates DNA damage, which signals to p53 (Passos et al, 2010). Additionally, certain SASP factors reinforce growth arrest in some types of senescence (Acosta et al, 2008; Kuilman et al, 2008; Wajapeyee et al, 2008; Yang et al, 2006), and we have demonstrated that those factors depend on p38MAPK activity (Chapter 1). It is likely that all of these regulatory methods are involved in p38MAPK-mediated growth arrest, in a manner that depends on cell type and conditions such as mitogenic signaling (as discussed above). However, our data allow for predictions about the general relevance of these different p38MAPK-mediated mechanisms:

First, although continuous p38MAPK inhibition reduces SASP factors to the same qualitative extent in DNA damage- and RAS-induced senescence (Chapter 1), it only inhibits growth arrest in RAS-induced senescence. Additionally, NF-κB depletion, which eliminates both the DNA damage and RAS-induced SASPs equally or better than p38MAPK inhibition (Chapter 1), had no effect on growth arrest in either context (discussed further below). Consequently, we conclude that neither XRA- nor RAS-induced growth arrest is primarily mediated by an autocrine, SASP-factor loop.

Second, the data suggest that p38MAPK activation does not induce growth arrest via engagement of the DDR or by generating ROS that induce DNA damage. We demonstrate here that constitutive p38MAPK activation (by MKK6EE) leads to complete growth arrest without activation of DDR proteins ATM and CHK2. Previous results demonstrating that MKK6EE expression leads to Chk2 activation (Wang et al, 2000) may have been confounded by culture at ambient (20%) oxygen, which significantly increases ROS levels, alters senescent phenotypes, and may prime cells for DDR activation (Coppe et al, 2010b; Lee et al, 1999; Parrinello et al, 2003). p38MAPK activation did slightly increase DNA damage foci in a subset (~6%) of cells, which may be been caused by the detected increase in H_2O_2 levels. However, this increase in ROS and slight increase in DNA damage signaling did not mediate p38MAPK-induced growth arrest, as neither depletion of DDR proteins nor NAC treatment, which reduced ROS levels, prevented the growth arrest. p38MAPK inhibition slightly (but significantly) decreased DNA damage foci in RAS-induced senescence, which correlated with the prevention of growth arrest; however, even with continuous p38MAPK inhibition, RAS-expressing cells contained significantly more DNA damage foci than PRE cells, demonstrating that p38MAPK prevents growth arrest without eliminating DDR signaling. These data suggest that, although genotoxic stress and DDR signaling can induce growth arrest, such as after X-radiation (Kuilman et al, 2010), p38MAPK does not mediate growth arrest via DDR signaling or DNA damage propagation.

Much has been published on the generation of ROS by RAS expression, which seems to involve NADPH oxidases and widespread mitochondrial dysfunction, as well as p38MAPK activation (Dolado et al, 2007; Lu & Finkel, 2008; Moiseeva et al, 2009). High levels of ROS have been implicated in DNA damage foci generation (Jun & Lau, 2010; Passos et al, 2010; Rai, 2010). Interestingly, however, our data suggest that ROS may not be the primary physiological source of DNA damage in RAS-expressing cells. We found that RAS-expressing cells accumulated significant levels of DNA damage foci even when grown at 3% O_2 and when

p38MAPK was continuously inhibited, both interventions that are reported to decrease ROS levels (Lee et al, 1999; Passos et al, 2010). While more direct measurements are required to demonstrate that these interventions reduced ROS in our system, continuous p38MAPK inhibition prevented RAS-induced generation of ROS-filled vacuoles, strongly suggesting that p38MAPK inhibition mitigated ROS production. Thus, while a subset of 53BP1 foci in RAS-induced senescent cells may be created and/or maintained by a p38MAPK-mediated ROS feedback loop, it appears that the majority of DNA damage foci induced by RAS signaling are p38MAPK- and ROS-independent. As the primary alternate source of RAS-induced DNA damage – hyperproliferation – is p38MAPK- and ROS- independent, it is likely that the more direct and physiologically relevant source of DNA damage in RAS-expressing cells comes from hyperproliferation and replication fork collapse, rather than ROS production. Studies demonstrating that ROS signaling generates widespread DNA damage in RAS-induced senescence (Moiseeva et al, 2009; Rai et al, 2010) may have been confounded by culture at ambient (20%) oxygen which, as discussed earlier, significantly increases ROS levels and may generate unphysiologically high levels of DNA damage.

Many questions remain about the role of ROS in senescence and its relationship with p38MAPK activation. For one, it is unclear whether ROS act primarily downstream or primarily upstream of p38MAPK. While most studies suggest that ROS activate p38MAPK, which then mediates growth arrest (Dolado et al, 2007; Han & Sun, 2007; Kuilman et al, 2010), others have demonstrated that the increase in ROS levels in RAS-expressing cells requires intact p53 and Rb pathways, the activation of which requires p38MAPK (Kwong et al, 2009; Moiseeva et al, 2009). Additionally, we have demonstrated that constitutive p38MAPK activation increases ROS levels, albeit only moderately, suggesting that some sources of ROS induction are downstream of p38MAPK activation. Irrespective of which comes first, we consider it very likely that ROS and p38MAPK reinforce each other in a positive feedback loop, as others have argued (Passos et al, 2010). In addition to the source of ROS being unclear, the consequence of increased ROS at senescence also requires further investigation. Many have demonstrated that ROS are required for efficient RAS-induced growth arrest, and this is often assumed to be a result of increased DNA damage and genotoxic stress. However, it seems that the increased ROS signaling in RAS cells may not generate much DNA damage; rather, per the feedback loop discussed above, we hypothesize that ROS primarily reinforces p38MAPK activation. Supporting this conclusion, we found that, in XRA-induced senescence, a situation where p38MAPK was not required for growth arrest, NAC was not required for growth arrest either, demonstrating that ROS signaling may not be required in all types of senescence.

Though p38MAPK does not seem to primarily mediate growth arrest by SASP, DDR, or ROS activation, p38MAPK does mediate growth arrest via p53 – we found that p53 inactivation completely prevented p38MAPK-induced growth arrest. In agreement with these findings, others have demonstrated that p38MAPK can phosphorylate p53 and induce $p16^{INK4A}$ expression, and that both of these responses are required for RAS-induced growth arrest (Kwong et al, 2009). Thus, we suggest that direct activation of the two major growth arrest pathways, rather than indirect activation of these pathways via SASP feedback loops or DNA damage, is the primary mechanism by which p38MAPK mediates senescence growth arrest. We also found that, once p38MAPK has activated these pathways, blocking p38MAPK signaling cannot cause cells to reenter the cell cycle, supporting the work of others who have demonstrated that some types of senescence, particularly those involving extended $p16^{INK4A}$ expression, are genetically irreversible, even when all upstream signaling is absent (Dai & Enders, 2000).

In contrast to p38MAPK inhibition, NF-κB depletion had no discernible effect on the senescence growth arrest, morphology, or SA-βgal expression, regardless of the inducing

stimulus. Because cytokine/chemokine signals reinforce the growth arrest in some types of senescence (Acosta et al, 2008; Kuilman et al, 2008; Wajapeyee et al, 2008; Yang et al, 2006), one might expect NF-κB depletion to at least partially mitigate the growth arrest. However, a recent report showed that, while constitutively active IκBa (which inhibits NF-κB) cannot immortalize normal fibroblasts damaged by repeated exposure to low levels of H_2O_2, it can immortalize 50% of identically treated fibroblasts that express hTERT (Wang et al, 2009). Thus, NF-κB depletion might mitigate a senescence growth arrest in cells with a permissive background (e.g., TERT expression), that experienced only weak growth arrest signals (e.g., low levels of H_2O_2), or that have increased mitogenic signaling, leading to "weak" growth arrest (though RAS-induced senescence is apparently not weak enough to require NF-κB).

To summarize, our data suggest that p38MAPK regulates the SASP via NF-κB, but establishes senescence growth arrest by mediating the expression of $p16^{INK4A}$ and both directly and indirectly phosphorylating p53 (Kwong et al, 2009; Sun et al, 2007). However, neither $p16^{INK4A}$ nor p53 are required for the SASP (Coppe et al, 2010a; Coppe et al, 2008). Additionally, others have demonstrated that PRAK, a kinase directly downstream of p38MAPK that is required for p53 activation, is not required for cytokine expression in mice (Shi et al, 2003; Sun et al, 2007). These findings lead us to hypothesize that p38MAPK is the final common node between the pathways that regulate the SASP and the pathways that regulate the growth arrest. Additionally, our data demonstrate that, while both RAS and XRA-induced senescence have similar SASP regulation, their growth arrest regulation differs. We propose a model (Figure 2-6) in which both inducers of senescence activate the DDR and the p38MAPK pathway. However, the relative contribution of each pathway differs: XRA generates widespread DNA damage, inducing a DDR that activates p53 (and later $p16^{INK4A}$ via a p38MAPK-independent mechanism). p38MAPK also likely drives some p53/$p16^{INK4A}$ activity, but that signaling is dispensable because of the strong DDR signaling. Activation of the p53 growth arrest pathways is sufficient to cause immediate growth arrest because there are no hyper-mitogenic signals to drive proliferation. Conversely, RAS induces strong mitogenic signals, preventing immediate growth arrest despite an increase in p38MAPK/$p16^{INK4A}$ by day 3 that is higher than that seen in fully senescent XRA cells. These mitogenic signals lead to a period of hyperproliferation that generates some DNA damage, which, while insufficient to drive growth arrest on its own, cooperates with the amplified p38MAPK/$p16^{INK4A}$ signaling to induce growth arrest. Consequently, RAS-induced growth arrest requires p38MAPK but XRA-induced growth arrest does not.

Chronic inflammation, which may be partly driven by cellular senescence (Campisi & d'Adda di Fagagna, 2007), is associated with multiple age-related pathologies, including cancer (Karin, 2006; Vasto et al, 2009). Both NF-κB and p38MAPK have been suggested as potential targets for therapeutic suppression of chronic inflammation (Coulthard et al, 2009; Pasparakis, 2009). However, p38MAPK inhibition might compromise the ability of cells to senesce in response to oncogenic mutations, trading chronic inflammation for increased cancer risk; therefore, targeting NF-κB may be a safer strategy. Of course, NF-κB is an important regulator of the immune system and cannot be globally inhibited without dangerous repercussions (Pasparakis, 2009). However, targeted NF-κB inhibition -- in specific tissues or for short durations -- may be efficacious in ameliorating age-related pathologies to which senescent cells contribute, without interfering with tumor suppression.

CHAPTER 2 ACKNOWLEDGMENTS

We thank Drs. Eisuke Nishida (Tokyo University) for the pSRalpha-myc-MKK6-EE vector. This work was supported by grants from the National Institutes of Health (research

grants AG09909 & AG017242 to JC; center grant AG025708 to the Buck Institute) and a National Science Foundation Graduate Research Fellowship (AF).

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CHAPTER 2 FIGURE LEGENDS

Figure 2-1: p38MAPK is required for RAS-induced senescence

(A) Schematic of RAS infection and p38MAPK inhibition conditions. Cells were infected with a lentivirus expressing oncogenic RAS^{V12} (RAS). The p38MAPK inhibitor, SB203580 (10 uM) was either not added to cultures (-), added to cultures for 48 h before collection, after RAS was expressed for 8 days (+SB post), or added to cultures pre-RAS infection and continued until sample collection (+SB pre). In all cases, presenescent controls (PRE) were infected with an insertless vector.

(B) p38MAPK inhibition prevents but cannot reverse RAS-induced cell cycle arrest. Cells were treated as in (A) and counted at the indicated intervals thereafter.

(C) RAS expression induces p38MAPK phosphorylation and $p16^{INK4A}$ expression. Cells were infected as in (A) and whole cell lysates were collected 10 d later and analyzed by western blotting for the indicated proteins.

(D) p38MAPK inhibition prevents but cannot reverse RAS-induced reduction of DNA synthesis, as measured by BrdU incorporation. Cells were treated as in (A). 9 d after infection, BrdU was added to the media. 24 h later cells were fixed and immunostained for nuclear BrdU.

(E) p38MAPK inhibition prevents but cannot reverse RAS-induced morphological changes. Cells were treated as in (A) and photographed through a phase contrast microscope 10 d after infection. Representative images are shown.

(F) p38MAPK inhibition prevents but cannot reverse RAS-induced SA-βgal expression. Cells were treated as in (A) and stained for SA-βgal 10 d after infection.

(G) p38MAPK inhibition partially prevents but does not reverse RAS-induced DNA damage foci, as measured by 53BP1. Cells were treated as in (A), fixed 10 d later and immunostained for 53BP1. Foci were quantitated using CellProfiler. Error bars represent the standard error of the mean.

Figure 2-2: p38MAPK is not required for DNA damage-induced senescence

(A) Schematic of irradiation and p38MAPK inhibition conditions. Cells were treated with 10 Gy X-radiation (XRA). SB was either not added to cultures (-), or added to cultures pre-XRA and continued until sample collection (+SB). In all cases, presenescent controls (PRE) were mock irradiated.

(B) p38MAPK inhibition does not prevent XRA-induced cell cycle arrest. Cells were treated as in (A) and counted at the indicated intervals thereafter.

(C) p38MAPK inhibition does not prevent XRA-induced reduction of DNA synthesis, as measured by BrdU incorporation. Cells were treated as in (A). 9 d after XRA, BrdU was added to the media. 24 h later cells were fixed and immunostained for nuclear BrdU.

(D) p38MAPK inhibition does not prevent XRA-induced morphological changes. Cells were treated as in (A) and photographed through a phase contrast microscope 10 d after XRA. Representative images are shown.

(E) p38MAPK inhibition does not prevent XRA-induced SA-βgal expression. Cells were treated as in (A) and stained for SA-βgal 10 d after XRA.

(F) XRA induces p38MAPK signaling that is inhibited by SB203580 treatment. Cells were treated as in (A) and whole cell lysates were collected 10 d after XRA and analyzed by western blotting for the indicated proteins.

(G) p38MAPK inhibition does not alter the kinetics of BrdU decrease after XRA. Cells were pretreated for 48 h with SB (+SB) or not and then irradiated (XRA). At the

indicated timepoints, BrdU was added to the media. 24 h later cells were fixed and immunostained for nuclear BrdU.

(H) p38MAPK inhibition partially reduces DNA damage foci after XRA, as measured by 53BP1. Cells were treated as in (A), fixed 10 d later and immunostained for 53BP1. Foci were quantitated using CellProfiler. Error bars represent the standard error of the mean.

(I) p38MAPK inhibition partially reduces DNA damage foci in replicatively senescent cells (SEN(REP)), as measured by 53BP1, but does not reverse replicative growth arrest. Cells were cultured to replicative senescence, and then p38MAPK was inhibited (or not) for 6 d with SB (replaced daily). Cells were cultured with BrdU for the last 24 h. Cells were then fixed and immunostained for 53BP1 and BrdU. Foci were quantitated using CellProfiler. Error bars represent the standard error of the mean.

(J) ROS inhibition by NAC does not prevent XRA-induced growth arrest. Cells were irradiated with 10 Gy (XRA). NAC (10 mM) was either not added to cultures (-), added to cultures for the 24 h immediately after XRA (NAC first 24 h), added to cultures 48 h before collection, starting 8 days after XRA (+NAC post), or added to cultures pre-XRA and continued until sample collection (+NAC pre). Cells were counted 10 d after XRA. In all cases, NAC was replaced daily.

Figure 2-3: Initial DNA damage signaling is not sufficient to drive RAS-induced growth arrest

(A) Infection with a lentivirus expressing RAS induces RAS, p38-P, and $p16^{INK4A}$ over a 3-day period. Cells were infected with a lentivirus lacking an insert (PRE) or expressing oncogenic RAS^{V12} (RAS) on day zero. Whole cell lysates were collected at the indicated intervals thereafter and analyzed by western blotting for the indicated proteins.

(B) Irradiation of day 1 RAS-expressing cells does not induce immediate growth arrest. Cells were infected as in (A). On day 1 after infection, cells were mock irradiated or irradiated with 10 Gy (XRA). Cells were counted at the indicated intervals after infection. Where indicated, SB was added to cultures pre-RAS infection and continued until sample collection.

(C) Irradiation of day 3 RAS-expressing cells induces immediate growth arrest. Cells were infected as in (A). On day 3 after infection, cells were mock irradiated or irradiated with 10 Gy (XRA). Cells were counted at the indicated intervals after infection.

Figure 2-4: NF-κB does not enforce senescence growth arrest
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(A) Efficacy of RelA shRNAs. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA #1, #2) or GFP (shGFP; control) and selected. Whole cell lysates were collected and analyzed by western blotting for the indicated proteins.

(B) RelA depletion does not prevent RAS-induced growth arrest. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then infected with a lentivirus expressing RAS^{V12} (RAS) and counted at the indicated intervals thereafter. In all cases presenescent controls (PRE) were infected with an insertless vector instead of RAS.

(C) RelA depletion does not prevent XRA-induced growth arrest. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then irradiated (XRA) and counted at the indicated intervals thereafter. In all cases presenescent controls (PRE) were mock irradiated.

(D) RelA depletion does not prevent the RAS- or XRA-induced decrease in BrdU incorporation. Cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then irradiated (SEN(XRA)) or infected with a lentivirus expressing RAS^{V12} (SEN(RAS)), cultured for 9 d, then cultured for 24 h with BrdU, fixed, and immunostained for nuclear BrdU. Controls were either mock irradiated (PRE) or infected with an insertless vector (PRE (Vector)).
(E) RelA depletion does not prevent RAS- or XRA-induced morphological changes. Cells were treated as in (D) and imaged by phase contrast microscope 10 d after infection/XRA.
(F) RelA depletion does not significantly reduce RAS- or XRA-induced DNA damage foci, as measured by 53BP1. Cells were treated as in (D), fixed 10 d after infection/XRA, and immunostained for 53BP1. Foci were quantitated using CellProfiler. Error bars represent the standard error of the mean.

Figure 2-5: p38MAPK induces senescence growth arrest independently of DNA damage or ROS production

(A) Schematic of MKK6EE infection and p38MAPK inhibition conditions. Cells were infected with a lentivirus expressing a constitutively active mutant (MKK6EE) of MAP kinase kinase 6 (MKK6), which directly phosphorylates p38MAPK. SB was either not added to cultures (-), added to cultures for 48 h before collection, after MKK6EE was expressed for 5 days (+SB post), or added to cultures pre-MKK6EE infection and continued until sample collection (+SB pre). In all cases, presenescent controls (PRE) were infected with an insertless vector.
(B) MKK6EE expression activates endogenous p38MAPK and $p16^{INK4A}$. Cells were treated as described in (A) and whole cell lysates were collected 7 days after infection and analyzed by western blotting for the indicated proteins.
(C) p38MAPK inhibition prevents but cannot reverse MKK6EE-induced cell cycle arrest. Cells were treated as in (A) and counted at the indicated intervals thereafter.
(D) p38MAPK inhibition prevents but cannot reverse MKK6EE-induced reduction of DNA synthesis, as measured by BrdU incorporation. Cells were treated as in (A). 6 d after infection, 5-bromo-2-deoxyuridine (BrdU), was added to the media. 24 h later cells were fixed and immunostained for nuclear BrdU.
(E) p38MAPK inhibition prevents but cannot reverse MKK6EE-induced, senescent-like morphological changes. Cells were treated as in (A) and photographed through a phase contrast microscope 7 d after infection. Representative images are shown.
(F) MKK6EE slightly increases the average number of DNA damage foci per nucleus, as measured by 53BP1. Cells were infected as in (A), fixed 7 d later and immunostained for 53BP1. Foci were quantitated using CellProfiler. Error bars represent the standard error of the mean.
(G) MKK6EE expression primarily increases the small percentage of cells with ≥4 53BP1 foci per nucleus. Cells were infected as in (A), fixed 7 d later and immunostained for 53BP1. Foci were quantitated using CellProfiler. The data are identical to (F), displayed as a histogram.
(H) MKK6EE expression does not induce a DDR. Cells were infected as in (A). Whole cell lysate was collected 8 d after MKK6EE infection and analyzed by western blotting for the indicated proteins. ATM-P, Ser 1981 phosphorylated ATM; CHK2-P, Thr 68 phosphorylated CHK2.
(I) Efficacy of ATM and Chk2 depletion by RNAi. Cells were simultaneously infected with a lentivirus expressing MKK6EE and a lentivirus expressing an shRNA against ATM (shATM #12), CHK2 (shChk2 #2, shChk2 #12), or GFP (shGFP; control) and

selected. Whole cell lysates were collected 8 d after infection and analyzed by western blotting for the indicated proteins.

(J) ATM or CHK2 depletion does not prevent MKK6EE-induced growth arrest. Cells were infected as in (I) and counted 8 d later.

(K) MKK6EE increases ROS signaling. Cells were infected as in (A). 8 d after infection, cells were collected and H_2O_2 levels were measured by flow cytometry. NAC: ROS signaling was inhibited with 10 mM NAC for 48 h before collection.

(L) ROS inhibition by NAC does not prevent MKK6EE-induced growth arrest. Cells were infected as in (A) and treated according to the schematic in (A), except that NAC was used instead of SB. Cells were counted 7 d after infection.

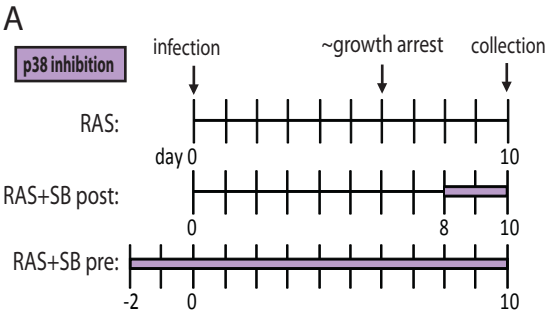
(M) Efficacy of GSE22. Cells were infected with a lentivirus expressing GSE22 (GSE) to inactive p53 and selected. Cells were then infected with MKK6EE and whole cells lysates were collected 7 d later and analyzed by western blotting for the indicated proteins. Accumulation of p53 in GSE-expressing cells represents inactive monomers.

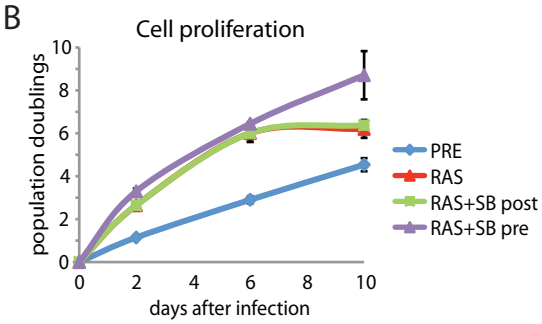
(N) p53 is required for MKK6EE-induced growth arrest. Cells were treated as in (M) and counted 7 d after MKK6EE infection.

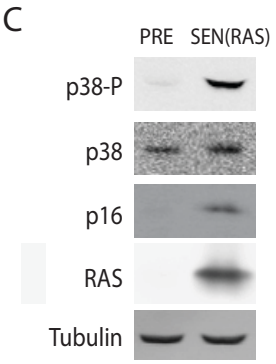
Figure 2-6: The role of p38MAPK in the SASP and growth arrest in both RAS- and XRA-induced senescence

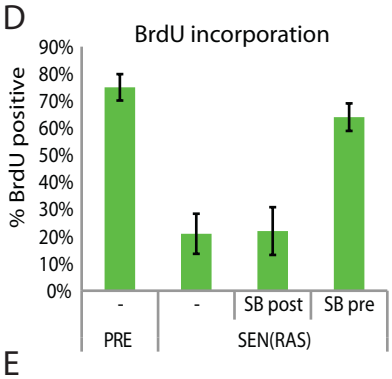
p38MAPK acts as a bifurcation between the pathways that regulate the SASP and the pathways that regulate the growth arrest. p38MAPK regulates the SASP via NF-κB in both RAS- and XRA-induced senescence. In both cases, neither $p16^{INK4A}$ nor p53 are required for the SASP. However, the role of p38MAPK in growth arrest regulation depends on the senescence inducer. XRA generates high levels of DNA damage, leading to a strong DNA damage response (DDR) that is sufficient to fully activate p53 (and later $p16^{INK4A}$) and induce growth arrest. p38MAPK is activated later, and though it also activates p53/$p16^{INK4A}$, this signaling is dispensable because of the strong DDR. The initial p53 activation is sufficient to induce growth arrest because there is no hyper-mitogenic signaling in these cells. Conversely, RAS strongly activates p38MAPK almost immediately, which then activates $p16^{INK4A}$ and p53, as well as the amplified SASP. However, RAS also induces strong mitogenic signaling that prevents immediate growth arrest, leading to a period of hyperproliferation that causes DNA damage and a subsequent DDR. Cells arrest after this period, once both the $p16^{INK4A}$ and p53 pathways are fully activated via a combination of p38MAPK signaling and DNA damage. Consequently, RAS cells require p38MAPK for growth arrest, whereas XRA cells do not. Closed arrows signify large contributions; open arrows signify relatively smaller contributions.

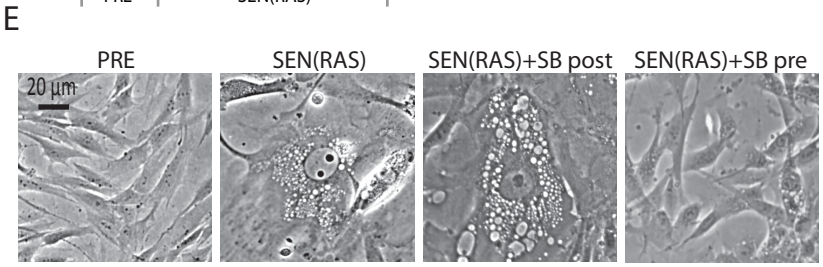
Figure 2-1
(1 of 1)

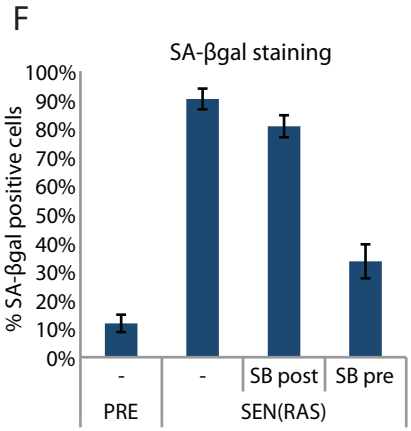


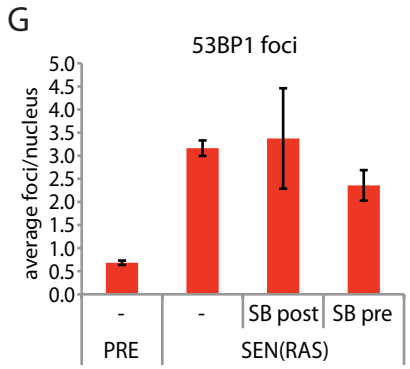












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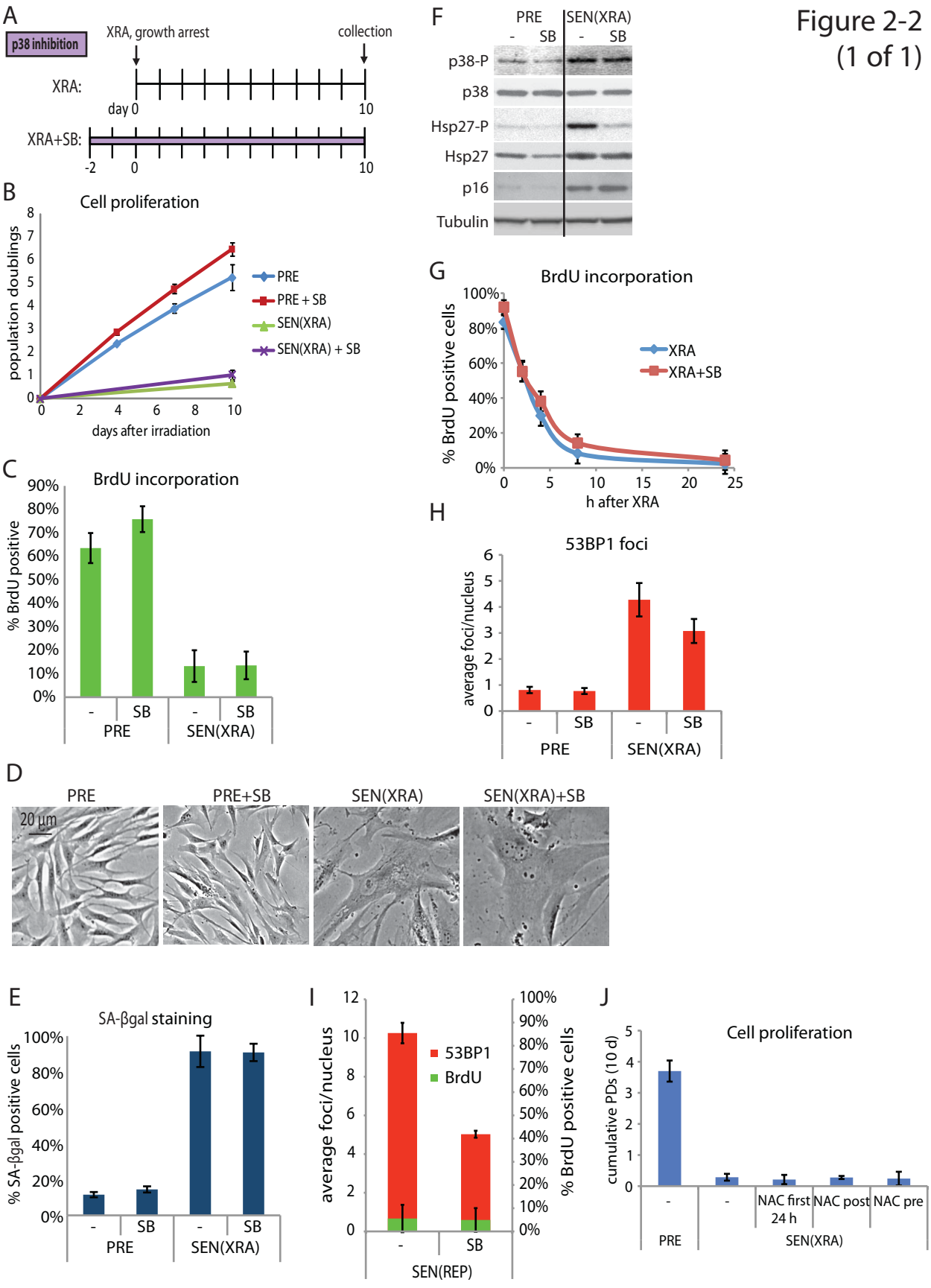

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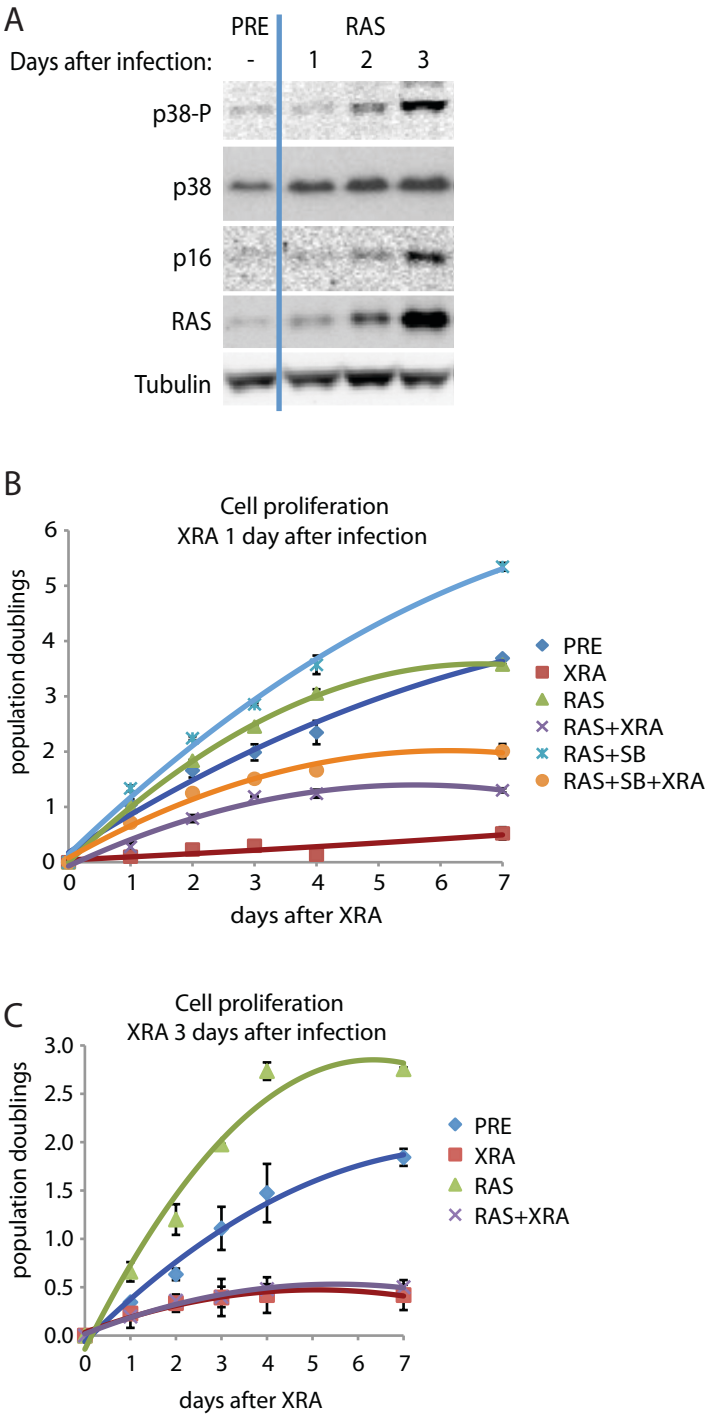

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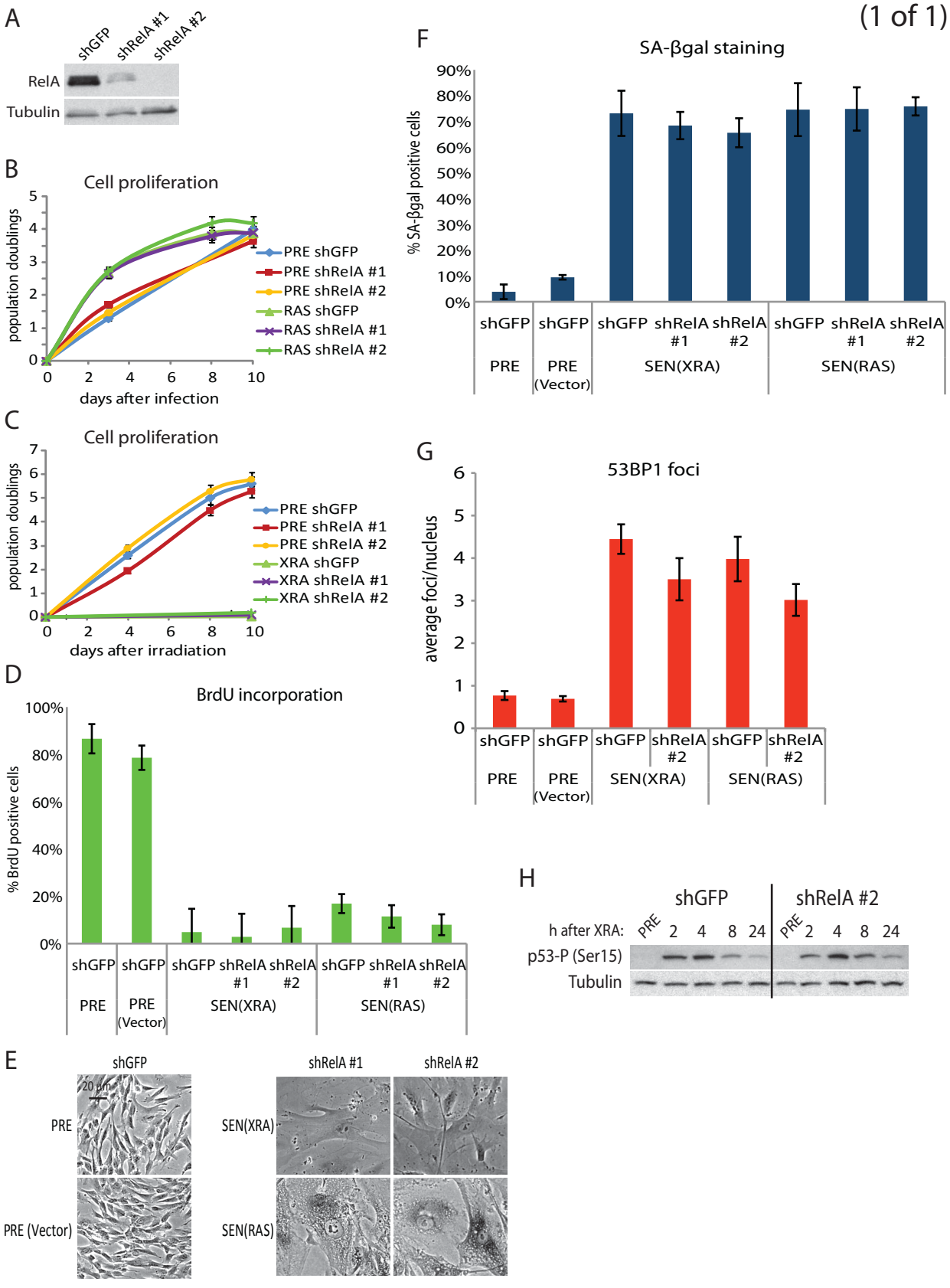

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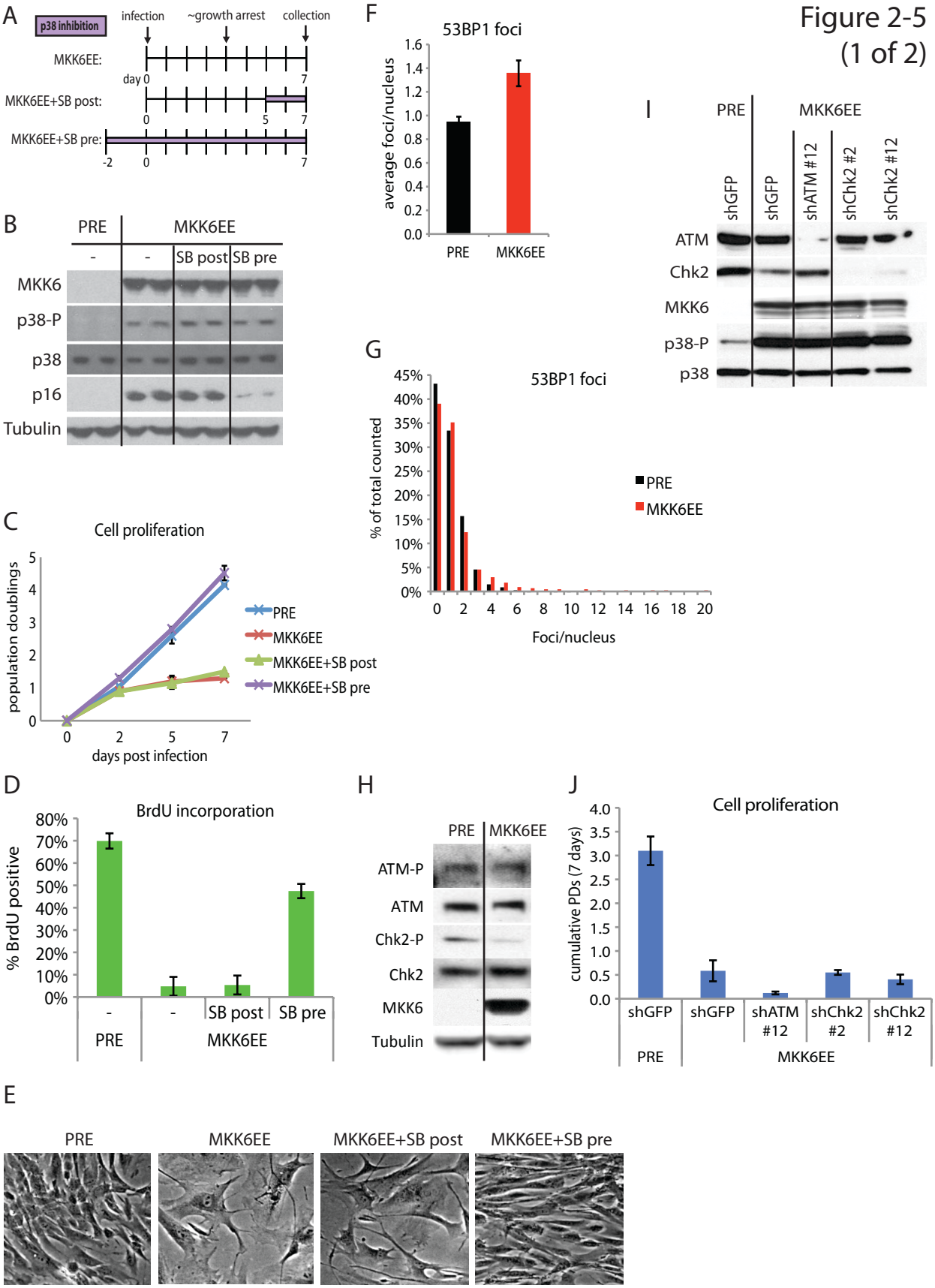

Figure 2-5
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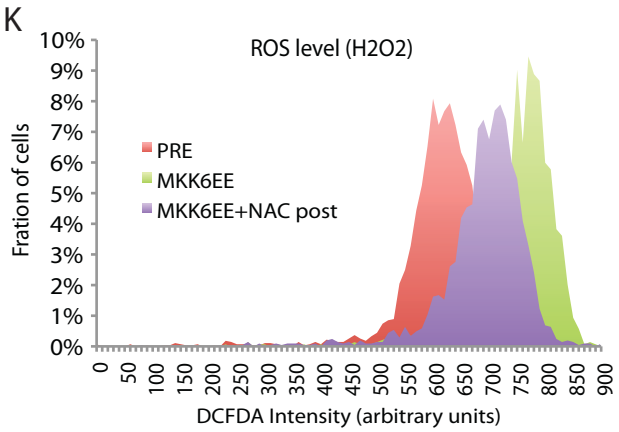

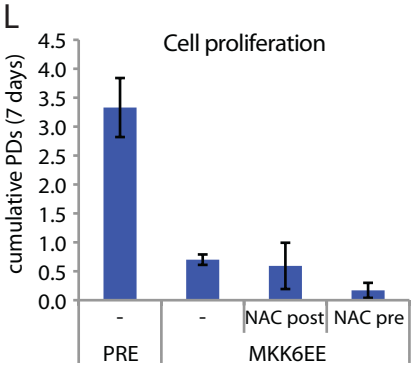

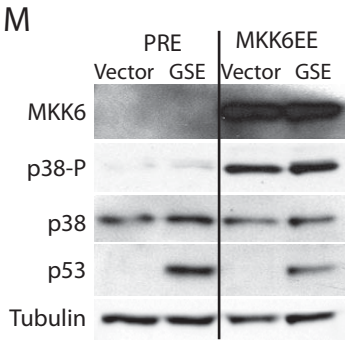

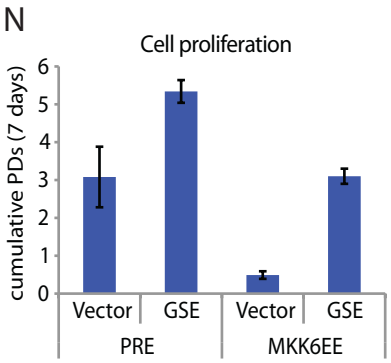

Figure 2-6
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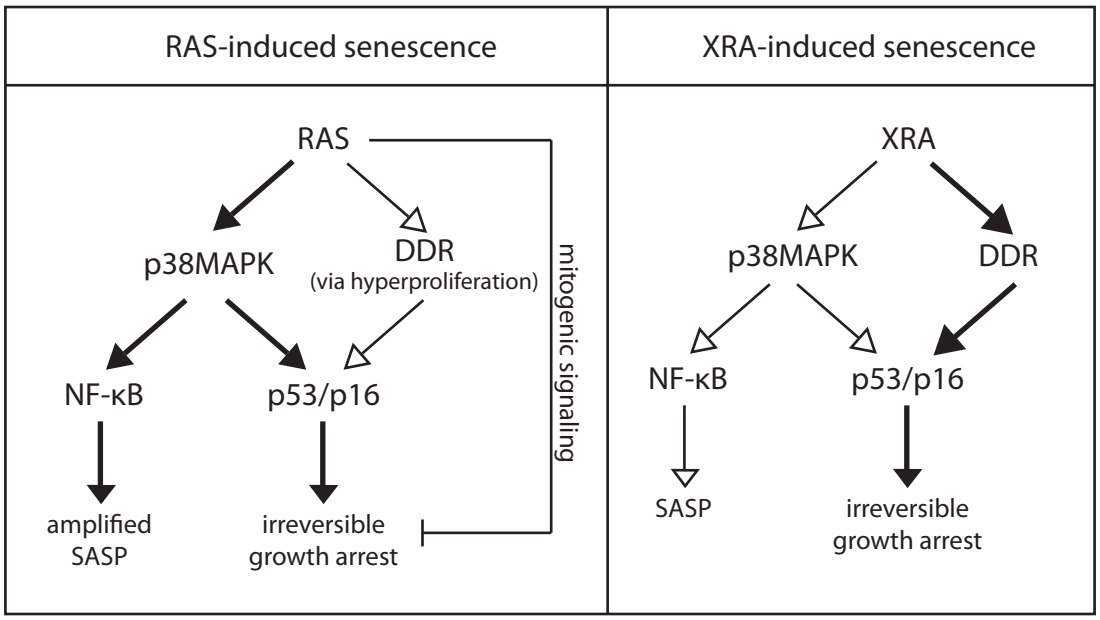

CHAPTER 3

Lamin B_1 loss is a biomarker of cellular senescence

“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

-J.R.R. Tolkien

CHAPTER 3 ABSTRACT

Cellular senescence is an important tumor suppressive mechanism, but studies of senescence have been impeded by the lack of specific, exclusive biomarkers of the senescent state. Senescence is associated with widespread morphological changes, including an enlarged and irregular nucleus and chromatin reorganization. Alterations to the nuclear lamina are implicated in regulation of both nuclear morphology and gene expression. Consequently, we asked whether changes to the nuclear lamina might be associated with senescence. We show here than lamin B_1 is lost from multiple cell strains during senescence caused by DNA damage, replicative exhaustion, or oncogene expression. Interestingly, this loss was not dependent on growth arrest, morphological changes, or other senescence-regulatory factors such as p38MAPK, NF-κB, ATM, or ROS signaling. Lamin B_1 was decreased at the mRNA level rather than degraded by caspase cleavage as it is during apoptosis, suggesting transcriptional regulation. The functional role of lamin B_1 loss at senescence remains unclear, but preliminary evidence suggests that it may reinforce DNA damage signaling and growth arrest. As lamin B_1 loss has not previously been associated with any viable cellular state, we suggest that lamin B_1 loss may serve as a specific and highly applicable biomarker of senescence and may have a functional role in regulating senescence phenotypes.

CHAPTER 3 INTRODUCTION

Senescent cells undergo widespread morphological changes. Among these is an increase in average nuclear size, an irregular nuclear envelope, and changes in chromosome distribution, with larger chromosomes migrating toward the nuclear periphery (Mehta et al, 2007; Narita et al, 2003). This nuclear rearrangement is correlated with global gene expression changes, and recent work has highlighted the important role of the nuclear envelope in the regulation of gene expression, particularly in gene repression (Reddy et al, 2008).

The inner surface of the nuclear envelope is lined by the nuclear lamina, which contributes to the size, shape, and stability of the nucleus (Lammerding et al, 2006). The major structural proteins of the lamina are the nuclear lamins, which are Type V intermediate filaments with a nuclear localization sequence, ranging from 60 to 80 kDa (Krohne & Benavente, 1986; Mehta et al, 2007). The lamins are categorized as either A-type (lamin A, C) or B-type (lamin B_1, B_2) based on their isoelectric points (Gerace & Blobel, 1980; Krohne & Benavente, 1986). Despite forming a relatively impenetrable nuclear boundary, lamin assembly is a dynamic process – each time a cell enters mitosis, phosphorylation events leads to disassembly and reassembly of the nuclear lamina (Gerace & Blobel, 1980; Goldman et al, 2002).

The lamins are all members of the same class of structural protein; however, there are important differences between the A-type and B-type lamins. Lamin A and C, which are alternatively spliced isoforms of the same gene, LMNA (Lin & Worman, 1993; Wydner et al, 1996), are only expressed when cells have committed to a particular differentiation pathway. Depletion of lamin A in HeLa cells does not perturb cell growth (Harborth et al, 2001), and mice that express lamin C but not lamin A are entirely healthy (Fong et al, 2006). However, lamin A mutations that lead to an accumulation of incorrectly processed or misfolded lamin A are associated with a diverse spectrum of diseases, appropriately termed “laminopathies”. These include muscular dystrophy, cardiomyopathy, lipodystrophy, and Hutchinson-Gilford progeria (Worman et al, 2010). This last laminopathy is a caused by an overproduction of progerin, a mutant form of lamin A, which causes premature aging (Broers et al, 2006; De Sandre-Giovannoli et al, 2003; Eriksson et al, 2003), and fibroblasts taken from Hutchinson-Gilford patients undergo more rapid senescence in culture than normal fibroblasts (Bridger & Kill, 2004). Interestingly, the sporadic expression of progerin in wild type cells has been linked to normal aging (Scaffidi & Misteli, 2006).

The B-type lamins, on the other hand, arise from two different genes – LMNB1 and LMNB2. Studies have suggested that both B-type lamins must be expressed for cell survival (Harborth et al, 2001); however, further analysis has revealed that only one or the other may be required for individual cell survival (Broers et al, 1997; Goldman et al, 2002; Lammerding et al, 2006). However, lamin B_1 seems to be required for organism survival: mice mutant for LMNB1 die minutes after birth, and fibroblasts extracted from these mice have misshapen nuclei and enter crisis several passages early (Mehta et al, 2007; Vergnes et al, 2004). Probably due to this lethal phenotype, lamin B null mutations have never been implicated in any human disease, though lamin B overexpression causes leukodystrophy (Padiath et al, 2006; Vergnes et al, 2004).

As mentioned above, lamin A mutations are associated with premature senescence. Lamin A dysfunction results in DNA damage, chromosomal abnormalities, increased sensitivity to DNA damaging agents, and p53-dependent senescence (Liu et al, 2005; Varela et al, 2005). The relationship between lamin B and senescence is less studied; however, recent reports demonstrate that depletion of lamin B_1 causes mitotic defects that correlated with a general

deterioration in nuclear compartmentalization and chromatin structure (Martin et al, 2010). Further, evidence suggests that changes in lamina organization occur during the onset of cell senescence, and mutant forms of lamin B promote telomeric aggregates (Mehta et al, 2007; Raz et al, 2008). These changes may lead to an increase in the association of heterochromatin at the nuclear periphery, as is characteristic of cell senescence (Mehta et al, 2007). Lastly, lamin B_1 deficient cells were more sensitive to oxidative stress and harbored higher levels of reactive oxygen species (ROS), which play an important role in some types of senescence (Jun & Lau, 2010; Lu & Finkel, 2008; Malhas et al, 2009; Passos et al, 2010).

Here we demonstrate that lamin B_1 loss is a biomarker of senescence. Lamin B_1 decreased in all types of senescence examined (DNA damage-induced, replicative, RAS-induced, and MKK6-induced), with the exception of p16 overexpression, and in all cell strains examined (HCA2, WI-38, BJ), but not in proliferating or quiescent cells. This decrease was independent of other pathways that have been implicated in senescence-regulation, including the DNA damage response (DDR), p38MAPK and NF-κB activation, and ROS signaling. Additionally, though lamins are often regulated by caspase cleavage during apoptosis, we found that lamin B_1 cleavage products were not evident in senescent cells, nor did caspase inhibition have any effect on lamin B_1 loss at senescence. Instead, lamin B_1 seems to be regulated via a transcriptional program, as LMNB1 mRNA levels were decreased at senescence. Interestingly, preliminary results suggest that lamin B_1 loss at senescence may somehow reinforce DDR signaling and growth arrest: lamin B_1 overexpression, but not lamin C overexpression decreased DNA damage foci in senescent cells and increased the percent of cells in S-phase after DNA damage-induced senescence. To the best of our knowledge, lamin B_1 loss has not previously been associated with any cellular state other than apoptosis, which is transient (Kivinen et al, 2005; Neamati et al, 1995; Rao et al, 1996; Vergnes et al, 2004); consequently, we suggest that lamin B_1 loss may serve as a specific and highly applicable biomarker of senescence in viable cell populations, though further studies are needed to determine whether its loss has a functional role in regulating senescence phenotypes.

CHAPTER 3 RESULTS

Unless indicated otherwise, error bars represent standard deviation.

Lamin B_1 loss is associated with multiple types of cellular senescence

To determine whether lamin B_1 expression was altered in senescent cells, we examined whole cell lysate from both presenescent (PRE), proliferating normal human fibroblasts (strain HCA2) and fibroblasts made senescent (SEN) by treatment with 10 Gy X-radiation (XRA), which induces DNA double strand breaks, leading to senescence (Chapters 1 and 2). Using two independent lamin B_1 antibodies, we found that the protein level of lamin B_1, but not lamin A or C was markedly decreased in SEN(XRA) cells as compared to PRE controls (Figure 3-1A). Our initial lamin antibodies mapped to the C-terminus of lamin B_1; to control for any cleavage artifacts, we verified our findings using a third independent antibody that maps to an internal region of lamin B_1 (Figure 3-1B). We tested this third antibody in a different normal human fibroblast strain (BJ) (Figure 3-1B). All three antibodies showed a similar reduction in lamin B_1, demonstrating that this loss is not cell strain-specific or an antibody artifact. We chose to use the first antibody (lamin B_1 #1) for subsequent experiments, as it gave the strongest signal.

10 Gy of X-radiation causes massive DNA damage that could give rise to gene expression changes that are not part of the general senescence program, *per se*. To determine

whether a more canonical type of senescence is also associated with lamin B_1 loss, we examined cells that had been cultured to replicative senescence and found that lamin B_1 but not lamin A or C was decreased in these cells as well (Figure 3-1C, SEN(REP)). Senescence can also be driven by the activation of certain oncogenes, including the oncogenic form of H-RAS (RAS^{V12}) (Serrano et al, 1997). We stably expressed RAS^{V12} in cells via lentiviral infection and allowed them to senescence (SEN(RAS)). We found that lamin B_1 was lost from these cells also, though in this case lamin A was lost as well (Figure 3-1D). We also found that lamin B_1 and lamin A were lost in cells expressing MKK6EE (Figure 3-1E), a constitutively active form of MAP kinase kinase 6 (MKK6), the upstream kinase of p38MAPK (Ishikawa, 2003; Raingeaud et al, 1996). These cells become senescent from constitutive p38MAPK activity (Chapter 2). We also found that lamin B_1 was decreased after XRA in a third cell strain, WI-38 (Figure 3-1F). Interestingly, the one inducer of irreversible growth arrest that did not cause lamin B_1 loss was overexpression of $p16^{INK4A}$. Ectopic expression of $p16^{INK4A}$ via lentiviral delivery causes cells to undergo rapid senescence (Beausejour et al, 2003; Coppe et al, 2006; Serrano et al, 1997). However, despite strong overexpression, $p16^{INK4A}$-induced senescence (SEN(p16)) did not cause lamin B_1 loss (Figure 3-1F).

We reasoned that lamin B_1 loss may have been a result of mitotic arrest, rather than senescence *per se*; however, cells made quiescent by 48 hours of serum starvation (QUI), which were not dividing and had negligible BrdU incorporation (data not shown), had lamin B_1 levels equal to proliferating, presenescent cells (PRE) (Figure 3-1G).

Many senescence markers, such as SASP activation and senescence-associated βgal staining, take 7-10 days to become fully expressed after direct DNA damage (Chapter 1 and 2) (Campisi & d'Adda di Fagagna, 2007). To determine whether lamin B_1 loss followed similar kinetics, we performed a timecourse on nuclear extract from X-irradiated cells (Figure 3-1H). Whereas lamin B_1 loss after XRA was slower than DDR activation, which occurs with in the first hour (Chapter 1), the lamin B_1 loss was essentially complete two days after XRA, earlier than many senescence markers.

Taken together, these data suggest that lamin B_1 loss is part of the general senescence program, irrespective of the upstream effector of senescence. Interestingly, this loss occurs within two days of the senescence-inducing insult, earlier than activation of the senescence-associated secretory phenotype (SASP), senescence-associated βgal staining, or the majority of morphological changes (Chapter 1 and 2). However, neither quiescence nor direct activation of the p16INK4A growth arrest pathway drove lamin B_1 loss, demonstrating that growth arrest alone is insufficient.

Lamin B_1 loss at senescence is independent of other senescence-regulatory pathways such as p38MAPK, NF-κB, ATM, and ROS signaling

We were interested in whether pathways that play a causative role in aspects of the senescence phenotype mediated lamin B_1 loss. The p38MAPK pathway has been implicated in senescence growth arrest (Chapter 2) as well as SASP regulation (Chapter 1). To determine whether p38MAPK was involved in lamin B_1 loss, we inhibited p38MAPK signaling with the well-characterized small molecular SB203580 (SB) (Cuenda et al, 1995; Wilson et al, 1997; Young et al, 1997), which we have previously shown to be effective at a working concentration of 10 uM (Chapters 1 and 2). When added to already-SEN(XRA) cells, SB was unable to reverse lamin B_1 loss (Figure 3-2A). Additionally, continuous treatment with SB, starting before XRA, was unable to prevent or delay lamin B_1 loss (Figure 3-2B).

We found that this was true in SEN(RAS) cells as well. We treated RAS cells with either of two modes of p38MAPK inhibition by SB. In one case, we added SB for 48 h before sample collection, starting 8 d post-RAS infection (RAS+SB post). In the other case, we treated cells with SB for a total of 12 d, starting 2 days pre-RAS infection and continuing until sample collection (RAS+SB pre). In both cases, SB was replaced daily. Neither treatment regimen had any effect on lamin B_1 loss (Figure 3-2C): p38MAPK inhibition could not reverse the RAS-induced lamin B_1 loss (+SB post), and even when p38MAPK was inhibited continuously starting before RAS infection (+SB pre), lamin B_1 was lost to the same extent as in untreated SEN(RAS) cells. In addition to suggesting that lamin B_1 loss is independent of p38MAPK, these data demonstrate that lamin B_1 loss does not require growth arrest or senescence-associated morphological changes, as both of these phenotypes are prevented in RAS-expressing cells by continuous p38MAPK inhibition (Chapter 2).

We applied the same SB treatment regimen to MKK6EE-expressing cells, which mediates senescence via constitutive p38MAPK activation (Figure 3-2D). In this case, p38MAPK inhibition could not reverse the lamin B_1 loss (+SB post), but continuous p38MAPK inhibition starting before MKK6EE infection (+SB pre) prevented lamin B_1 loss, simply verifying that lamin B_1 loss can be mitigated by complete blockage of senescent signals. We further demonstrated that the p38MAPK pathway was not involved in lamin B_1 loss by examining the role of NF-κB, which acts downstream of p38MAPK to regulate the SASP (Chapter 1). Stable NF-κB depletion by either of two unrelated shRNAs against RelA, an NF-κB subunit required for NF-κB activity (Chapter 1), did not prevent XRA-induced lamin B_1 loss (Figure 3-2E). Taken together, these data demonstrate that lamin B_1 loss at senescence is independent of the SASP and the p38MAPK/NF-κB pathway.

The classic DNA damage response (DDR) pathway, particularly activation of ATM, has been implicated in various aspects of the senescence phenotype (Kuilman et al, 2010; Mallette et al, 2007; Rodier et al, 2009; Zglinicki, 2005). Additionally, we have shown that this pathway is regulated independently of p38MAPK (Chapter 1). To determine whether the classic DDR was required for lamin B_1 loss, we stably depleted ATM via RNAi and then irradiated cells. We found that, despite efficient knockdown of ATM, the ATM shRNAs had no effect on lamin B_1 loss. We next asked whether ROS signaling, which has been implicated in various aspects of senescence (Jun & Lau, 2010; Lu & Finkel, 2008; Passos et al, 2010; Rai et al, 2010), was required for lamin B_1 loss. We inhibited ROS signaling by continuously treating cells with 10 mM N-acetyl cysteine (NAC), starting before senescence induction. However, NAC treatment had no effect on lamin B_1 loss induced by either XRA (Figure 3-2G) or MKK6EE expression (Figure 3-2H). We conclude that lamin B_1 loss at senescence is independent of the classic DDR or ROS signaling.

Senescence-associated lamin B_1 loss is regulated at the mRNA level, rather than post-translationally by caspase-mediated degradation

Senescence and apoptosis are often mentioned in the same sentence, as both are tumor suppressor mechanisms, and the decision whether a cell undergoes one or the other may depend on the levels of only a few factors, such as p53 and PTEN (Bargonetti & Manfredi, 2002; Campisi & d'Adda di Fagagna, 2007; Lane et al, 2010; Lee et al, 2010). During apoptosis, breakdown of the cellular structure is mediated largely by caspases, and the nuclear lamins are primary caspase targets; their degradation promotes the nuclear disruption seen during apoptosis (Kivinen et al, 2005; Neamati et al, 1995; Rao et al, 1996). Senescent cells also often have disrupted nuclei (Mehta et al, 2007), leading us to hypothesize that the lamin B_1

loss seen at senescence is a post-translational process resulting from caspase-mediated degradation.

Lamin cleavage can be visualized by the presence of smaller cleavage products on a western blot (Gajdusek et al, 2001; Kivinen et al, 2005; Rao et al, 1996). To determine whether caspase-mediate degradation of lamin B_1 played a role in senescence, we examined whether we could detect lamin B_1 cleavage products at senescence. As a positive control, we induced apoptosis with staurosporine (500 nM) (Kivinen et al, 2005). Staurosporine treatment effectively induced apoptosis, lamin degradation, and characteristic lamin B_1 and lamin A/C cleavage products; however, despite a lamin B_1 decrease equivalent to staurosporine-treated cells, no cleavage products were detectable in SEN(XRA) cells (Figure 3-3A).

However, it was possible that XRA treatment induced lamin B_1 cleavage products that were only visible during certain timepoints – others have shown that lamin cleavage products disappear 24 hours after apoptosis induction (Gajdusek et al, 2001). Therefore, to functionally determine whether caspase degradation mediates lamin B_1 loss at senescence, we treated cells with z-VAD-fmk, an irreversible pan-caspase inhibitor that blocks caspase degradation of lamins during apoptosis (Kivinen et al, 2005). Though treatment of cells with 100 uM z-VAD-fmk prevented staurosporine-induced lamin B_1 degradation, treatment with z-VAD-fmk starting before XRA did not prevent XRA-induced lamin B_1 loss (Figure 3-3B). We conclude that lamin B_1 is not degraded by caspase cleavage during senescence.

Given that the primary post-translational mechanism of lamin regulation (caspase cleavage) was not responsible for lamin B_1 loss at senescence, we next asked whether lamin B_1 was decreased at the mRNA level. Quantitative RT-PCR showed that lamin B_1 mRNA was significantly decreased within 2 days after XRA and remained decreased for the length of the experiment, whereas Lamin A mRNA level was not significantly affected (Figure 3-3C). Combined with the inability of caspase-inhibition to prevent lamin B_1 loss, we conclude that lamin B_1 is regulated at the mRNA level during senescence, rather than post-translationally by caspase cleavage, as it is during apoptosis.

Lamin B_1 loss at senescence may be important for DNA damage sensing and/or efficient growth arrest

Because lamin B_1 has been implicated in proper mitosis (Martin et al, 2010), we hypothesized that lamin B_1 loss at senescence might be important for growth arrest signaling. To counteract lamin B_1 loss, we infected cells with a lentiviral vector carrying a copy of the wildtype LMNB1 open reading frame (ORF). We also infected cells with a lentiviral vector carrying a copy of the wildtype LMNA ORF as a control for general increase of protein at the nuclear envelope. LMNB1 infection led to strong overexpression of lamin B_1 protein, though there was an unexplained size shift in the final protein product as compared to endogenous lamin B_1 (Figure 3-4A). LMNA infection led to an increase in lamin C protein but not lamin A, likely due to alternative splicing regulation (Figure 3-4A).

We first examined the effect of lamin B_1 overexpression on DNA damage foci after XRA. X-radiation induces widespread DNA double strand breaks; cells then attempt to repair these breaks by localization of DNA damage sensing and repair proteins to the site of damage, such as p53 binding protein (53BP1), which facilitates cell cycle checkpoint activation and repair. 53BP1 localizes into discrete foci that can be easily visualized by immunofluorescence, and serve as a measure of DNA damage sensing (Beausejour et al, 2003; Rodier et al, 2009; Rodier et al, 2010). We treated lamin-overexpressing cells with XRA. Five days later, lamin C

overexpressing cells had a 53BP1 foci profile that was not significantly different from normal XRA cells (Figure 3-4B, top, $p>0.05$). However, lamin B_1 overexpressing cells had, on average, significantly fewer 53BP1 foci than normal XRA cells (Figure 3-4B, bottom, $p<0.0001$). Interestingly, this decrease in 53BP1 foci was accompanied by a small, but significant increase in the percent of cells in S-phase (Figure 3-4C, $p<0.01$), as measured by bromodeoxyuridine (BrdU) incorporation. In contrast, lamin C overexpression did not cause a significant increase in the number of cells in S-phase, as compared to control XRA cells (Figure 3-4C, $p>0.05$). These data are preliminary, but they suggest that the loss of lamin B_1 at senescence may be important for continued DDR signaling and subsequent growth arrest, at least in a subset of cells.

CHAPTER 3 DISCUSSION

Research into senescence has been constrained by the lack of senescence-specific biomarkers (Campisi & d'Adda di Fagagna, 2007; Kuilman et al, 2010). For example, growth arrest, while necessary for a cell to be labeled senescent, is not sufficient to define the state – many cells *in vivo* are post-mitotic (terminally differentiated) or quiescent, which also prevent proliferation (Kuilman et al, 2010). Conversely, $p16^{INK4A}$ is thought to be an almost-exclusive marker of senescence, though reports have demonstrated a general increase in *in vivo* $p16^{INK4A}$ expression with age, which may not be confined to senescent cells (Krishnamurthy et al, 2004; Ohtani et al, 2010; Zindy et al, 1997). However, $p16^{INK4A}$ expression is not necessary for senescence, as some cell types senesce after DNA damage via activation of the p53 pathway alone (Beausejour et al, 2003). Additionally, the most widely used senescence marker, senescence-associated βgal staining, while a useful and general tool, is neither necessary nor sufficient for senescence (Dimri et al, 1995; Lee et al, 2006). This lack of biomarkers has led some to propose a "senescence score" – a rating of senescence based on a combination of senescence-associated gene expression changes and phenotypes (Lafferty-Whyte et al, 2010). This may turn out to be the most effective strategy, though the search from biomarkers is far from over – any additional senescence biomarkers improve the scoring algorithm; additionally, if specific senescence biomarkers are eventually found, it will remove the need to analyze multiple markers in the same sample, a process that can be technically difficult, if not impossible.

We have shown here that lamin B_1 is lost in multiple types of senescence, in multiple human fibroblast cell strains. Lamin A, though lost in RAS and MKK6-induced senescence, was not lost in DNA damage or replicative senescence, and lamin C was not lost in any type of senescence examined. Consequently, we conclude that lamin B_1 loss, but not lamin A or lamin C loss, is a general biomarker of senescence, though these findings should be verified in other cell types and in tissue. Interestingly, lamin B_1 was not lost in cells induced to senesce by direct $p16^{INK4A}$ overexpression, demonstrating that the mechanisms regulating lamin B_1 loss are upstream or independent of the $p16^{IN4A}$/Rb growth arrest pathway. Further, lamin B_1 loss was not prevented by continuous p38MAPK inhibition in RAS expressing cells, which prevents both RAS-induced growth arrest and the morphological changes associated with senescence; these data suggest that lamin B_1 loss is not simply a byproduct of cell cycle arrest or changes to nuclear morphology.

Similarly, lamin B_1 loss was independent of senescence and SASP regulatory pathways such as the p38MAPK/NF-κB pathway, the classic DDR (as measured by ATM), and ROS signaling. Surprisingly, lamin B_1 regulation at senescence seems to be distinct from the caspase-mediated lamin degradation seen at apoptosis, as no lamin cleavage products were evident in senescent cells, and caspase inhibition did not prevent senescence-associated lamin B_1 loss. Rather, lamin B_1 seems to be regulated at the mRNA level – lamin B_1 mRNA was

decreased within 2 days after XRA, a timeframe similar to lamin B_1 protein loss. We hypothesize that lamin B_1 is regulated transcriptionally, though further mechanistic studies are required.

We found that lamin B_1 loss at senescence may play a role in regulating DNA damage signaling and growth arrest. Prevention of lamin B_1 loss by overexpression significantly reduced the number of DNA damage foci in cells after XRA, and this was accompanied by a small but significant increase in the percent of cells in S-phase, suggesting that growth arrest was impaired. This effect may be amplified in cells with a weaker form of senescence growth arrest, such as RAS-expressing cells. These results are preliminary and thus far confined to DNA damage-induced senescence, but they are supported by an increasing body of work demonstrating that the nuclear lamina plays a role in gene regulation and chromatin organization (Reddy et al, 2008), and that lamin B_1 depletion sensitizes cells to oxidative stress and increases ROS signaling (Malhas et al, 2009). Cellular senescence is associated with an increase in average nuclear size, an irregular nuclear envelope, and changes in chromosome distribution (Mehta et al, 2007; Narita et al, 2003). The role that these changes play at senescence is not completely clear, though chromatin reorganization and the formation of senescence-associated heterochromatic foci is important for establishing efficient growth arrest (Narita et al, 2003). It is possible that lamin B_1 loss imbues the nuclear envelope with a degree of plasticity that allows many of these senescence-associated structural changes to occur.

CHAPTER 3 ACKNOWLEDGMENTS

We thank Drs. Eisuke Nishida (Tokyo University) for the pSRalpha-myc-MKK6-EE vector. This work was supported by grants from the National Institutes of Health (research grants AG09909 & AG017242 to JC; center grant AG025708 to the Buck Institute) and a National Science Foundation Graduate Research Fellowship (AF).

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CHAPTER 3 FIGURE LEGENDS

Figure 3-1: Lamin B_1 loss is associated with multiple types of cellular senescence

(A) Lamin B_1 is decreased in DNA damage-induced senesence. HCA2 cells were mock irradiated (PRE) or treated with 10 Gy X-radiation (XRA) and allowed to senesce (SEN(XRA)). Whole cell lysates were then collected and analyzed by western blotting with either of two unrelated Lamin B_1 antibodies.

(B) Lamin B_1 is decreased in SEN(XRA) cells, verification. BJ cells were mock irradiated (PRE) or treated with XRA and allowed to senesce (SEN(XRA)). Whole cell lysates were then collected an analyzed by western blotting with a third Lamin B_1 antibody raised against an internal part of the protein.

(C) Lamin B_1 is decreased in replicative senescence. HCA2 cells were cultured until replicative senescence (SEN(REP)) (~70 population doublings). Whole cell lysates were then collected an analyzed by western blotting.

(D) Lamin B_1 is decreased in RAS-induced senescence. HCA2 cells were infected with a lentivirus lacking an insert (PRE) or expressing oncogenic RAS^{V12} and allowed to senesce (SEN(RAS)). Whole cell lysates were then collected and analyzed by western blotting.

(E) Lamin B_1 is decreased in MKK6-induced senescence. HCA2 cells were infected with a lentivirus lacking an insert (PRE) or expressing a constitutively active MAP kinase kinase 6 mutant (MKK6EE) and allowed to senesce (SEN(MKK6). Whole cell lysates were then collected and analyzed by western blotting.

(F) Lamin B_1 is decreased in WI-38 cells after XRA, but not after $p16^{INK4A}$ overexpression. WI-38 cells were irradiated (SEN(XRA)) or infected with a lentivirus expressing $p16^{INK4A}$ (SEN(p16)) and allowed to senesce. PRE cells were mock irradiated and infected with a lentivirus lacking an insert. Whole cell lysates were collected and analyzed by western blotting.

(G) Lamin B_1 is not decreased in quiescent cells. HCA2 cells were treated with normal media (PRE), serum free media for 48 hours to induce quiescence (QUI), or irradiated and allowed to senesce (SEN(XRA)). Whole cell lysates were collected and analyzed by western blotting.

(H) Lamin B_1 is fully decreased within 48 hours after DNA damage. HCA2 cells were mock irradiated (PRE) or treated with XRA. Nuclear (N) and cytoplasmic (C) extract were collected at the indicated timepoints thereafter and analyzed by western blotting. RPA serves as a loading control for the nuclear fraction; tubulin serves as a loading control for the cytoplasmic fraction.

Figure 3-2: Lamin B_1 loss at senescence is independent of other senescence-regulatory pathways such as p38MAPK, NF-κB, ATM, and ROS signaling

(A) p38MAPK inhibition cannot reverse lamin B_1 loss in DNA damage-induced senescence. The p38MAPK inhibitor SB203580 (SB) (10 uM) was added to SEN(XRA) HCA2 cells for 48 hours. Whole cell lysates were then collected and analyzed by western blotting. PRE cells were mock irradiated.

(B) p38MAPK inhibition cannot prevent lamin B_1 loss in DNA damage-induced senescence. SB was added to HCA2 cells before XRA. Cells were then mock irradiated (PRE) or treated with XRA. Whole cell lysates were collected at the indicated timepoints thereafter and analyzed by western blotting. SB was replaced daily.

(C) p38MAPK inhibition cannot reverse or prevent RAS-induced lamin B_1 loss. HCA2 cells were infected with a lentivirus expressing oncogenic RAS^{V12} and allowed to senesce

(SEN(RAS)). SB was either not added to cultures (-), added to cultures for 48 h before collection, after RAS was expressed for 8 days (+SB post), or added to cultures pre-RAS infection and continued until sample collection (+SB pre). In all cases, presenescent controls (PRE) were infected with an insertless vector. Whole cell lysates were collected and analyzed by western blotting.

(D) p38MAPK inhibition can prevent but not reverse MKK6-induced lamin B_1 loss. HCA2 cells were infected with a lentivirus expressing MKK6EE and allowed to senesce (SEN(MKK6)). SB was either not added to cultures (-), added to cultures for 48 h before collection, after MKK6EE was expressed for 8 days (+SB post), or added to cultures pre-MKK6EE infection and continued until sample collection (+SB pre). In all cases, presenescent controls (PRE) were infected with an insertless vector. Whole cell lysates were collected and analyzed by western blotting.

(E) RelA depletion does not prevent DNA damage-induced lamin B_1 loss. HCA2 cells were infected with a lentivirus expressing either of two shRNAs against RelA (shRelA) or GFP (shGFP; control) and selected. Cells were then irradiated and allowed to senesce (SEN(XRA)). Presenescent controls (PRE) were mock irradiated. Whole cell lysates were collected and analyzed by western blotting.

(F) ATM depletion does not prevent DNA damage-induced lamin B_1 loss. HCA2 cells were infected with a lentivirus expressing an shRNA against ATM (shATM) or GFP (shGFP; control) and selected. Cells were then irradiated and allowed to senesce (SEN(XRA)). Presenescent controls (PRE) were mock irradiated. Whole cell lysates were collected and analyzed by western blotting.

(G) ROS inhibition by NAC does not prevent DNA damage-induced lamin B_1 loss. N-acetyl cysteine (NAC) (10 mM) was added to HCA2 cells before irradiation and continued until sample collection. Whole cell lysates were collected 10 d after XRA (SEN(XRA)). Presenescent controls (PRE) were mock irradiated. NAC was replaced daily. Whole cell lysates were collected and analyzed by western blotting.

(H) ROS inhibition by NAC does not prevent MKK6-induced lamin B_1 loss. NAC (10 mM) was added to HCA2 cells before infection and continued until sample collection. Whole cell lysates were collected 10 d after infection with a lentivirus expressing MKK6EE (SEN(MKK6)). Presenescent controls (PRE) were infected with a lentivirus lacking an insert. NAC was replaced daily. Whole cell lysates were collected and analyzed by western blotting.

Figure 3-3: Senescence-associated lamin B_1 loss is regulated at the mRNA level, rather than post-translationally by caspase-mediated degradation

(A) Lamin cleavage products are present in apoptotic, but not senescent cells. HCA2 cells were treated with 500 nM staurosporine for 24 hours to induce apoptosis (Stauro) or irradiated and collected 4 days later (SEN(XRA)). Presenescent controls (PRE) were mock irradiated. Whole cell lysates were collected and analyzed by western blotting.

(B) Caspase inhibition prevents staurosporine-induced lamin B_1 degradation, but not senescence-associated lamin B_1 loss. HCA2 cells were treated with 500 nM staurosporine for 24 hours to induce apoptosis (Stauro) or irradiated and collected 4 days later (SEN(XRA)). Presenescent controls (PRE) were mock irradiated. Where indicated, the pan-caspase inhibitor z-VAD-fmk (Z-VAD) (100 uM) was added starting before staurosporine/XRA and continuing until whole cell lysates were collected. Z-VAD-fmk was replaced daily.

(C) Lamin B_1 mRNA is decreased in senescent cells. HCA2 cells were mock irradiated (PRE) or treated with XRA. Total RNA was isolated at the indicated timepoints thereafter and analyzed by quantitative RT-PCR. Signal was normalized to Tubulin.

Figure 3-4: Lamin B_1 loss at senescence may be important for DNA damage sensing and/or efficient growth arrest

(A) Efficacy of lamin overexpression. HCA2 cells were infected with a lentivirus lacking an insert (Vector), containing the LMNA ORF (Lamin C OE), or containing the LMNB1 ORF (Lamin B1 OE) and selected. Whole cell lysates were collected and analyzed by western blotting.

(B) Lamin B_1, but not lamin C, overexpression decreases DNA damage foci after XRA, as measured by 53BP1. HCA2 cells were infected with a lentivirus lacking an insert (Vector), containing the LMNA ORF (Lamin C OE, top), or containing the LMNB1 ORF (Lamin B1 OE, bottom) and selected. Cells were then irradiated (SEN(XRA)), fixed 5 d later, and immunostained for 53BP1. Foci were quantitated using CellProfiler. Top and bottom SEN(XRA) Vector histograms are identical.

(C) Lamin B_1, but not lamin C, overexpression increases the percentage of BrdU positive cells. HCA2 cells were infected with a lentivirus lacking an insert (Vector), containing the LMNA ORF (Lamin C OE, top), or containing the LMNB1 ORF (Lamin B1 OE, bottom) and selected. Cells were then mock irradiated (PRE) or irradiated (SEN(XRA)). 4 days after XRA, BrdU was added to the media. Cells were fixed 24 hours later and immunostained for nuclear BrdU.

Figure 3-1
(1 of 1)

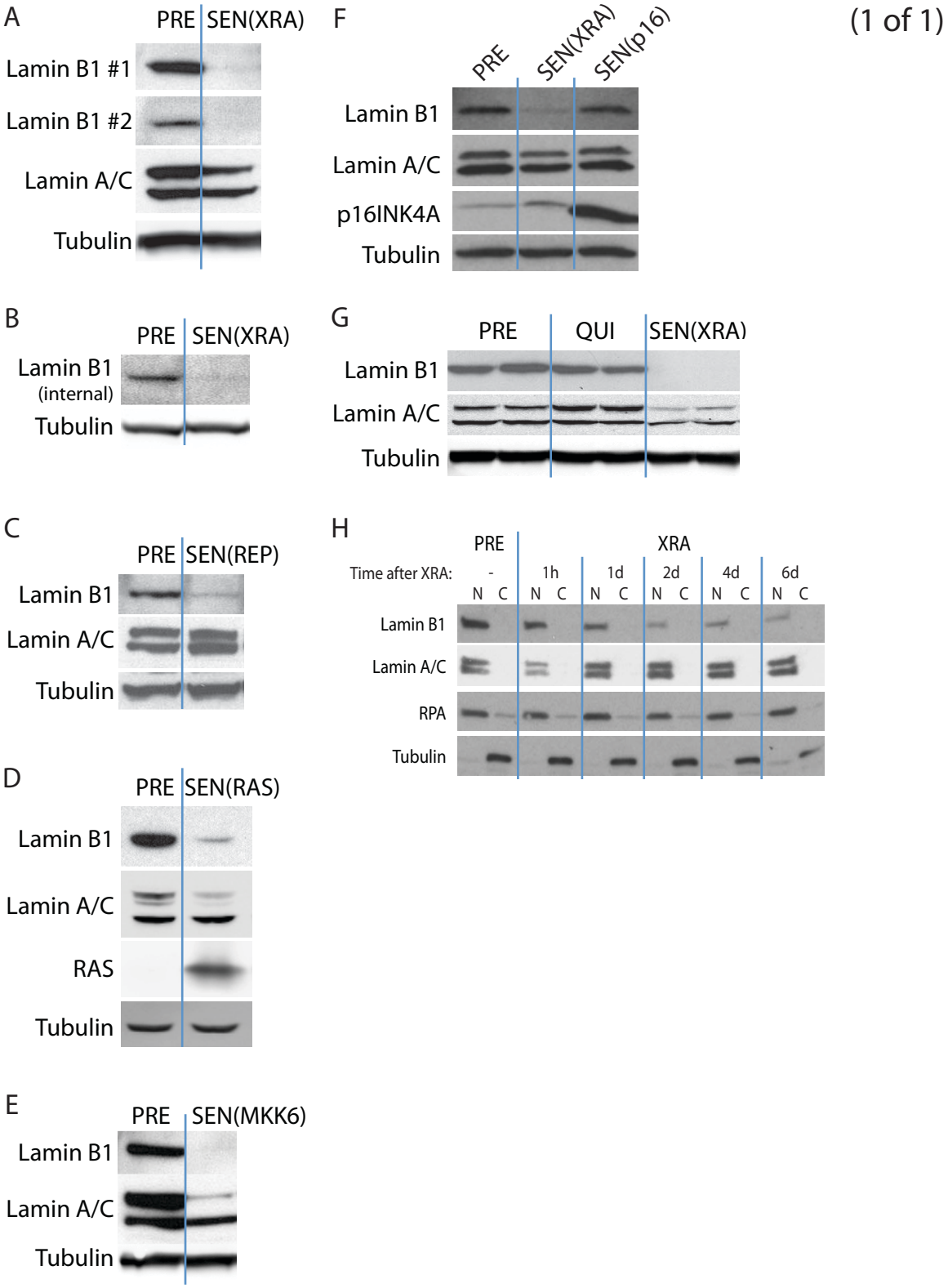


Figure 3-2
(1 of 1)

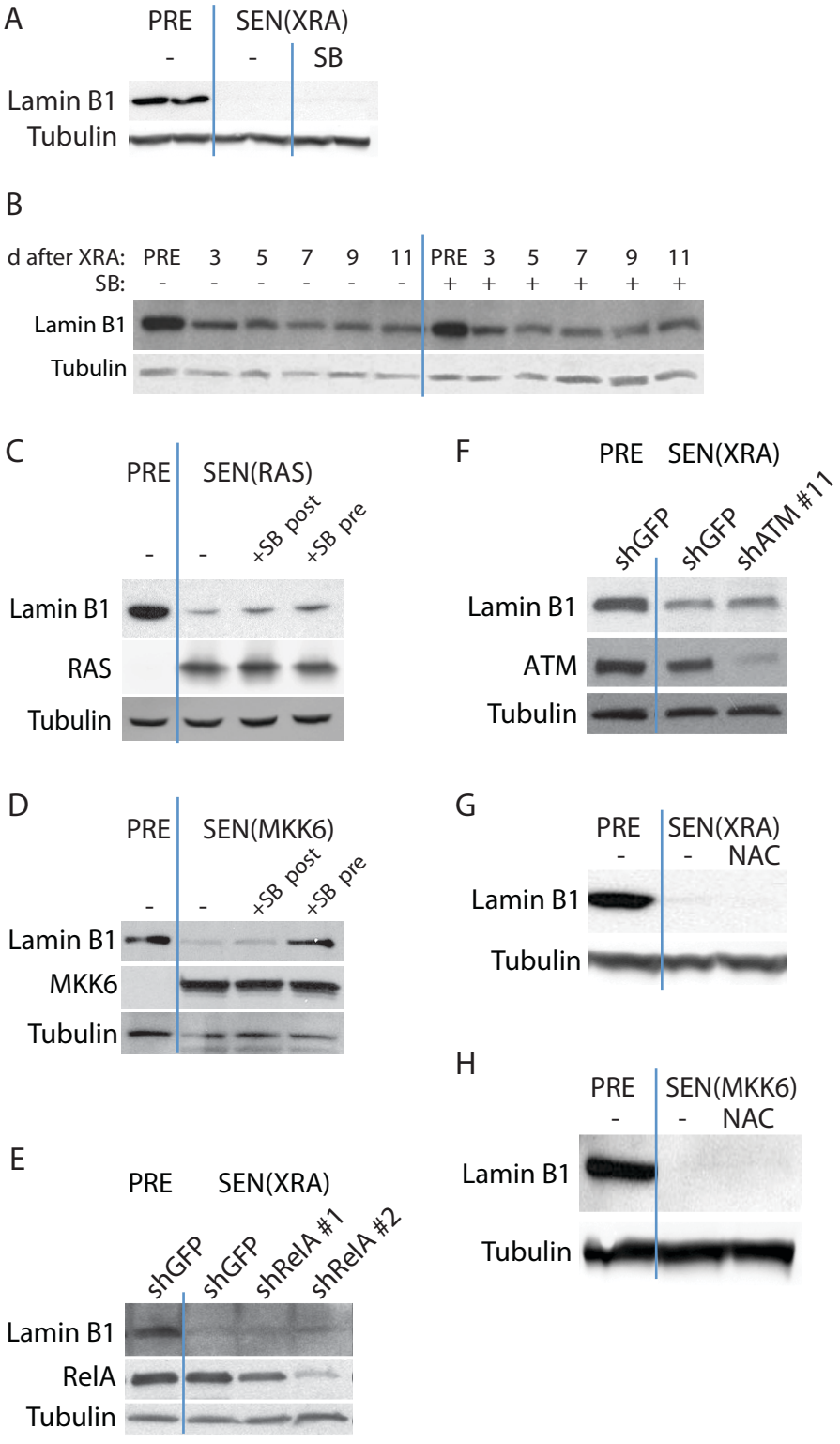


Figure 3-3
(1 of 1)

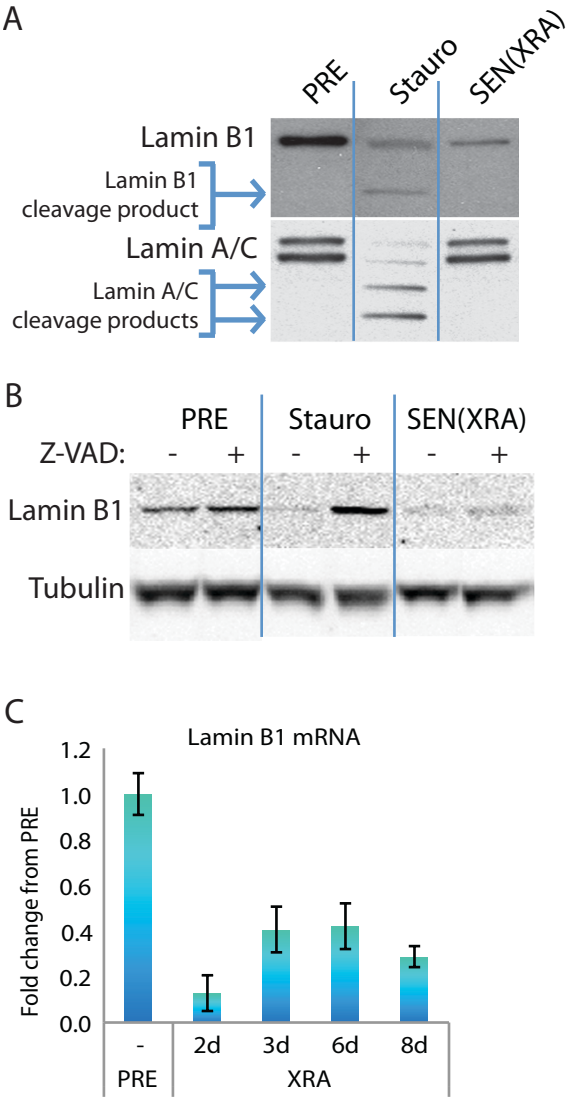
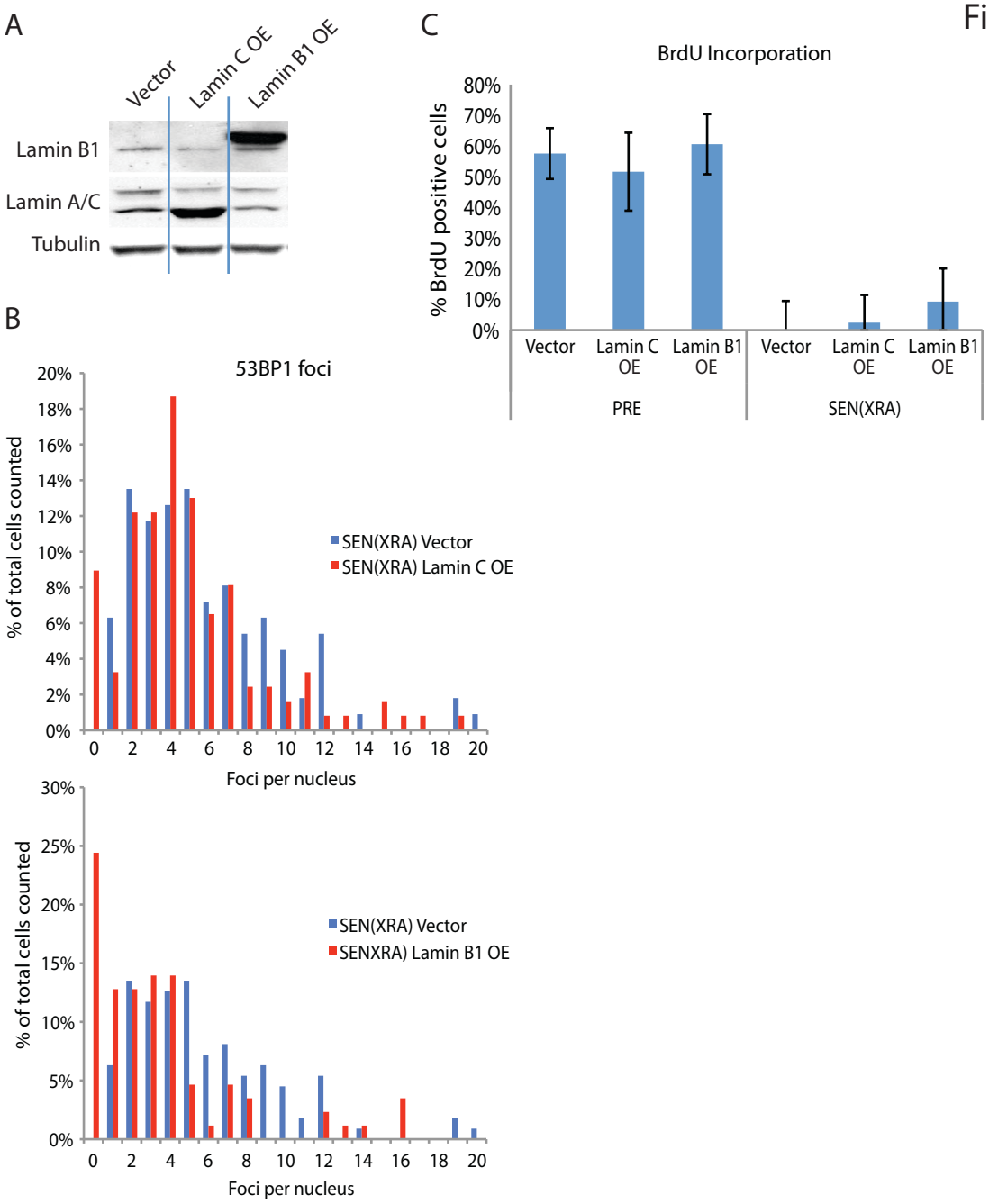


Figure 3-4
(1 of 1)



OVERALL DISCUSSION

“I don’t want to achieve immortality through my work. I want to achieve immortality through not dying.”

-Woody Allen

Chronological versus biological aging

"Aging" has a confusingly dual usage: chronological versus biological. Chronological aging is the realm of physics – the definition of the passage of time. This is immutable, barring relativistic speed, but in no way compels biological aging. Biological aging refers to the physiological changes in an organism with time, and for all relevant purposes, implies a probabilistic increase in mortality with time. Chronological aging may have turned you fifty-five this year, but the way your body feels at fifty-five, and the fact that you are more likely to develop cancer at fifty-five than you were at twenty-five, are results of biological aging. Notably, extrinsic mortality, that is, the probability that you have been killed by an extrinsic factor (i.e. lion) before reaching fifty-five is a consequence of chronological aging (assuming a constant probability of lion-related death), not biological aging.

Chronological aging results in unavoidable effects such as the entropy of the entire universe being higher when you die than it was when you were born. Biological aging, on the other hand, is not a necessarily unavoidable consequence of biological processes. This is a common misconception, often attributed to the 2nd law of thermodynamics – that aging is an unavoidable increase in entropy with time, a signal to noise problem with a relentlessly rising baseline. However, unlike the universe, organisms are not closed thermodynamic systems, and thus the 2nd law does not place restrictions on lifespan. Whether biological aging is caused by aberrant gene expression, chronic inflammation, DNA damage, the accumulation of cellular debris, or a combination of all of these factors, they are processes that occur differently in different organisms, leading to hyper-aging in some, and what appears to be negligible senescence in others (Finch, 2009). Consequently, they are tunable, flexible, and possibly preventable. The search for anti-aging therapies is a fight against biology, not physics.

The evolution of senescence

Throughout this dissertation, I have argued that cellular senescence may have deleterious effects on organismal fitness over time. At first glance it seems paradoxical that senescence, which was presumably evolutionary selected for its tumor-suppressing role, could also promote cancer and other age-related diseases. Evolution, it is argued, selects against processes that reduce organismal fitness; consequently, any negative aspects of senescence should have been eliminated, leaving only the positive effects. However, it is an underappreciated subtlety of evolution that the force of natural selection declines with chronological age. This has nothing to do with biological aging; rather, it is a consequence of extrinsic mortality. Due to predation, disease, and scarcity of resources, the average lifespan of an organism in the wild is often much shorter than its maximal lifespan. Consequently, even without invoking biological aging, old individuals of any given species are more rare than young individuals, and thus are unable to produce as many offspring as young individuals. This means that the heritable traits promoting longevity are not as positively selected as, for example, heritable traits promoting early-life fecundity. Thus, while species, particularly those with high extrinsic mortality, evolve a predisposition for shuttling resources towards early development and reproduction, they do not generally develop long lifespans. An important prediction of this theory is that species with low extrinsic mortality should have more old individuals, and thus would evolve to have longer lifespans. As it turns out, this prediction matches observed trends – animals that live in protected environments such as caves or below ground (bats, naked mole rats) (Austad, 2005; Buffenstein, 2008), that spend significant time above the earth's surface, away from ground predators (birds) (Holmes et al, 2001), that have developed protective carapaces (lobsters, tortoises) (Carnes, 2007), or that are intelligent enough to evade predation (humans) live significantly longer than animals of the same size and complexity (de Magalhaes

& Toussaint, 2002; Finch, 2009; Partridge, 2001). From this understanding of evolution, it follows that, though early-life selective pressures are not *necessarily* anti-longevity, a given trait may be selected for its early-life benefits (e.g. rapid development, increased fecundity, cancer prevention), but late in life may have no benefit, or even deleterious effects, and this arrangement would not be strongly selected against. This theory is termed "antagonistic pleiotropy", and it reconciles the apparent paradox of senescence (Campisi, 2010; Caruso et al, 2004; Franceschi et al, 2000; Goto, 2008; Sedivy, 2006). I hypothesize that senescence, both the growth arrest and the associated secretory phenotype, plays an important role in early life fitness by suppressing cancer and promoting tissue repair. However, over time, this process may trigger or increase chronic inflammation, drive aberrant tissue remodeling, limit stem cell regenerative capacity, and in part, lead to biological aging.

The physiological roles of cellular senescence

Cellular senescence is a tumor suppressing mechanism, and one that is gaining recognition. Animal models in which senescence has been impaired are invariably more cancer prone than their wild type counterparts (Braig et al, 2005; Chen et al, 2005; Donehower et al, 1992; Ohtani et al, 2004; Takeuchi et al, 2010), and senescent cells are found in pre-cancerous lesions (Castro et al, 2003; Chen et al, 2005; Collado et al, 2005; Michaloglou et al, 2005). Even replicative senescence (driven by telomere shortening) seems to play a role in limiting tumorigenesis, providing long-awaited validation of Hayflick's original proposal (Cosme-Blanco et al, 2007; Feldser & Greider, 2007; Hayflick, 1965). In short, there are now numerous examples of senescence acting as a tumor suppressor *in vivo* (Collado & Serrano, 2010).

Senescent cells are also present around sites of fully malignant tumors (Charalambous et al, 2007; Studebaker et al, 2008). Arguably, this is not surprising; as malignant cells manage to escape senescence arrest via a strategic combination of intrinsic and extrinsic signaling – a process that, due to its random nature, involves many false starts – one would expect the failed attempts at malignancy (i.e. those cells that trigger senescence) to accumulate in the same spatial area as successfully malignant cells. Thus, the proximity of senescent cells to malignant tumors could be nothing more than correlation. However, evidence suggests that the relationship has a causal component. Cancer is well known to be exacerbated by inflammation (Grivennikov et al, 2010), and senescent cells are a particularly potent source of inflammatory factors (Coppe et al, 2008). In vitro and xenograft studies have demonstrated that senescent cells can induce aggressive, cancer-associated behavior in neighboring cells via the pro-inflammatory senescence-associated secretory phenotype (SASP) (Coppe et al, 2010a; Coppe et al, 2010b; Coppe et al, 2008; Krtolica et al, 2001; Liu & Hornsby, 2007).

Senescent cells and senescence markers accumulate *in vivo* with age, particularly in renewable tissues, as one would expect from a persistent cellular phenotype that is activated by oncogenic mutations and stress signaling (Dimri et al, 1995; Erusalimsky & Kurz, 2005; Jeyapalan et al, 2007; Wang et al, 2009). Interestingly, senescent cells have been preferentially found at sites of degenerative, age-related pathology (Erusalimsky & Kurz, 2005; Price et al, 2002; Roberts et al, 2006; Stanley & Osler, 2001; Vasile et al, 2001). As with tumorigenesis, this could be only correlative – both the senescent cells and the pathological condition could be triggered by the microenvironment; alternatively, the pathological condition could trigger senescence. As yet, the arrow of causality has not been clearly illuminated. However, there are two mechanisms by which senescence might drive age-related degeneration. First, the SASP may interfere with cell growth, tissue structure, differentiation, all of which are vital for proper tissue function (Campisi, 2010). Supporting this theory, chronic inflammation is correlated with aging and age-related disease (Bruunsgaard, 2006; Ferrucci et al, 2004; Vasto

et al, 2007), plays a causative role in at least some of these pathologies (Brennan et al, 1995; Brod, 2000; Caruso et al, 2004), and is widely hypothesized to be a driver of normal aging (Franceschi et al, 2000; Franceschi et al, 2007; Freund et al, 2010; Vasto et al, 2009). Second, senescence growth arrest itself may limit tissue regeneration – most adult stem cells are capable of undergoing senescence, and senescence may contribute to the decline in stem cell function and tissue regeneration seen in aging organisms (Carlson & Conboy, 2007; Drummond-Barbosa, 2008; Janzen, 2006; Krishnamurthy et al, 2006; Molofsky et al, 2006; Sharpless & DePinho, 2007; Zhou et al, 2008).

It would be a mistake, however, to assume that SASP signaling is necessarily deleterious, or deleterious in all contexts. The pro-inflammatory molecules produced by senescent cells can reinforce the tumor-suppressing growth arrest (Acosta et al, 2008; Kuilman et al, 2008; Wajapeyee et al, 2008; Yang et al, 2006), as well as promote clearance of senescent cells (Xue et al, 2007), and the matrix metalloproteases and matrix proteins secreted by senescent cells can limit tissue fibrosis and promote wound healing (Jun & Lau, 2010; Krizhanovsky et al, 2008).

The contribution of this dissertation

The true role(s) of senescence *in vivo* are still being determined, but clearly senescence has important physiological significance. Consequently, it is vital both to understand the pathways regulating senescence and to identify markers of senescence that can be utilized in multiple systems. In this dissertation, I have investigated three aspects of cellular senescence – the specificity and applicability of a novel biomarker of cellular senescence, the regulation of the senescence-associated secretory phenotype (SASP), the pathways and signals that establish irreversible growth arrest.

I identified lamin B_1 loss as a general marker of cellular senescence in multiple cell types and conditions. This loss was regulated at the mRNA level and, interestingly, did not require growth arrest, morphological change, or SASP activity. Consequently, lamin B_1 loss may serve as a particularly useful marker of upstream senescence signaling, even when downstream functional changes have been inhibited (e.g. by oncogenic transformation). While further research must be done to determine what, if any, functional significance this lamin B_1 loss has, it may play a role in regulating DNA damage signaling and growth arrest.

The majority of my thesis focused on the pathways regulating senescence growth arrest, the SASP, and the degree to which those pathways overlap. I found that the p38MAPK/NF-κB pathway is a key regulator of the SASP. We previously showed that the SASP requires classic DDR signaling such as ATM and CHK2, but that DDR signaling is insufficient to induce the SASP (Rodier et al, 2009). Therefore, other, DDR-independent molecular events must be involved in SASP regulation. I found that p38MAPK phosphorylation was the rate-limiting step in SASP induction, and the level of p38MAPK phosphorylation was directly responsible for the degree of SASP activity. Both p38MAPK signaling and the classic DDR impinged on NF-κB activity, but did so via independent mechanisms. NF-κB, in turn, was required for the expression of virtually all SASP factors.

High levels of p38MAPK phosphorylation were sufficient to drive SASP activity in the absence of DDR signaling, but lower levels of p38MAPK, such as those found in DNA damage-induced senescence, were not sufficient, as the DNA damage-induced SASP also requires DDR signaling (Rodier et al, 2009). While the molecular mechanism behind this p38MAPK regulatory threshold remains unknown, I hypothesize that p38MAPK and/or downstream targets

phosphorylate/acetylate several sites of the NF-κB transcriptional complex with varying degrees of affinity, as has been demonstrated in other contexts (Saha et al, 2007). Multiple post-translational modifications are necessary for NF-κB to have full transcriptional activity (Karin, 2006; Perkins, 2007). High p38MAPK activity may lead to phosphorylation/acetylation of all the necessary sites on NF-κB, even those for which the p38MAPK pathway has low affinity, leading to full SASP activation. Lower levels of p38MAPK may not be able to modify those low-affinity sites; consequently, DDR signaling, which presumably has higher affinity for those sites, is required.

Both p38MAPK and the DDR are required for the SASP and NF-κB activity under normal conditions; combined with the identification of other SASP regulators such as C/EBPβ (Acosta et al, 2008), IL-1α (Orjalo et al, 2009), mTOR (Laberge, unpublished data), etc, these results suggest that combined endogenous induction of many semi-independent pathways is necessary for SASP activation. At the risk of over simplifying a complex network, the data summarized here are consistent with an "AND" logic gate to model SASP regulation. That is, there is a set of pathways that regulate most SASP factors, and it seems that all of these pathways must be active at senescence in order for the factors to be expressed. Molecularly, this may take the form of a large transcriptional complex involving NF-κB, C/EBPβ, and a host of cofactors that are dependent on the individual pathways; without all the components, the complex cannot be fully active. Unlike a simple "AND" logic gate, disruption of the final complex can feed back onto the input signals, causing the collapse of the entire network. In this respect, the SASP is regulated in much the same way as the inflammatory response to other stresses (such as viral infection), and further clues about SASP regulation will almost certainly be found in analyses of the inflammatory response in other contexts.

I also determined that p38MAPK is required for oncogene-induced growth arrest, but not for DNA damage-induced growth arrest, and that DNA damage activates $p16^{INK4A}$ via a p38MAPK-independent mechanism, whereas oncogene-induced $p16^{INK4A}$ requires p38MAPK (Kwong et al, 2009). This demonstrates that oncogene-induced senescence and DNA damage-induced senescence have distinct regulatory mechanisms, rather than simply being different flavors of the same phenomenon. Additionally, oncogene-induced mitogenic signaling counteracted DNA damage-induced p53 growth arrest signals; as a result, oncogene-induced growth arrest, but not DNA damage-induced growth arrest, required activation of the $p16^{INK4A}$ pathway. This has implications for our general understanding of senescence growth arrest: I suggest that growth arrest is not simply a guaranteed consequence of p53 or $p16^{INK4A}$ signaling, but rather is modulated by the net effect of growth arrest signals versus mitogenic signals. In the context of strong mitogenic signaling, both growth arrest pathways must be active to drive senescence. This may explain why the path to tumorigenesis often involves suppression of only one growth arrest pathway.

Despite being vital for the SASP and downstream of p38MAPK, NF-κB was not required for oncogene- or DNA damage-induced growth arrest. While it is unclear why the SASP feedback loop was not required for efficient growth arrest in our system, as others have reported (Acosta et al, 2008; Kuilman et al, 2008), it may be a consequence of the degree of p53/$p16^{INK4A}$ expression, or the balance between mitogenic and growth arrest signaling. Regardless, p38MAPK and NF-κB clearly have different roles in growth arrest regulation, which suggests that p38MAPK bifurcates the senescence pathways, shuttling growth arrest signals through p53/$p16^{INK4A}$ while sending SASP regulatory signals through NF-κB.

It remains to be proven that senescent cells and the SASP are drivers of normal aging and age-related diseases. However, as researchers come closer to understanding the roles of

senescence *in vivo*, it is possible to envision therapies aimed at mitigating the deleterious effects of the SASP without affecting beneficial aspects of senescence. The identification of p38MAPK as the final common node between SASP regulation and growth arrest pathways provides a means to modulate one aspect of senescence without directly affecting the other. Both NF-κB and p38MAPK have been suggested as potential targets for therapeutic suppression of inflammation (Coulthard et al, 2009; Pasparakis, 2009). However, if cells have acquired oncogenic mutations, p38MAPK inhibition might compromise their ability to senesce; therefore, targeting NF-κB may be a safer strategy. Of course, precise treatment regimens may be required - disruption of the cytokine network during the early stages of senescence can prevent efficient growth arrest in some contexts (Acosta et al, 2008; Kuilman et al, 2008). Additionally, it may be necessary to temporally separate the induction of localized senescence (e.g. by chemotherapy or radiation) from therapies designed to mitigate the SASP so that the clearance of senescent cells occurs unimpeded (Xue et al, 2007) and only residual senescent cells are targeted by anti-SASP therapies. Additionally, NF-κB is an important regulator of the immune system and cannot be globally inhibited without dangerous repercussions (Pasparakis, 2009). However, targeted NF-κB inhibition -- in specific tissues or for short durations -- may be efficacious in ameliorating age-related pathologies to which senescent cells contribute, without interfering with tumor suppression.

A comparison of cellular senescence to other potential drivers of aging

With rare exceptions such as the programmed senescence of salmon after reproduction (Finch, 1998), aging is a consequence of evolutionary neglect, not a selected process. This almost guarantees that there is no single cause of aging; rather, the processes that drive aging are predictably numerous and complex – a diverse network of stochastic, deregulated mechanisms, each the byproduct of some selected, early-life resource allocation toward survival or reproduction. This does not, however, mean that anti-aging therapy is doomed to failure unless a hundred causes of aging can be mitigated independently and simultaneously. It has already been demonstrated that single gene mutations in model organisms can cause dramatic lifespan extension by modulating the expression of hundreds of genes and affecting the entire metabolic process (Murphy et al, 2003). Additionally, there is reason to suspect that one of the primary suspects of aging – DNA damage-induced dysfunction on a cell autonomous level – may not play as important a role as commonly thought. First, if aging were primarily caused by dysfunction on a cell autonomous level, that dysfunction should occur at a relatively equal rate throughout the body, as tissues accumulate aging phenotypes in a relatively coordinated fashion (Zahn et al, 2007). However, DNA mutations accumulate at rates that are highly organ-specific (Vijg & Dolle, 2002). Consequently, true DNA mutation-driven conditions (i.e. cancer) also have quite varied organ-specific incidence rates (Jemal et al, 2009). Age-related changes, on the other hand, are as dramatic in tissues with low DNA damage accumulation (e.g. brain) as in tissues with high DNA damage accumulation (e.g. small intestine) (Vijg & Campisi, 2008; Zahn et al, 2007). Second, in order to disrupt tissue function on a cell-autonomous level, DNA damage would have to occur in a large proportion of cells, particularly progenitor cells, and would be effectively irreversible. However, while the function of progenitor cells such as hematopoietic stem cells and muscle satellite cells is perturbed in aged animals, those cells are no different than young cells in terms of their ability to form colonies and proliferate *in vitro* (Morrison et al, 1996). Further, exposing aged progenitor cells to systemic factors from young animals (both *in vitro* and *in vivo*) reverses many, if not all, of the aged phenotypes of those cells (and vice versa) (Carlson & Faulkner, 1989; Conboy, 2005; Harrison et al, 1977; Hotta et al, 1980; Mayack et al, 2010), arguing that some type of systemic extracellular signaling, rather than cell-intrinsic DNA damage, drives cellular dysfunction in aged animals. Extracellular signaling, e.g. from cytokines, chemokines, and growth factors, has to potential to regulate

almost all cellular processes, including division and metabolism, as well as the clearance of cellular "junk" such as misfolded, crosslinked, or aggregated proteins. While these processes may function normally in young animals, accumulating extracellular signaling molecules could, over time, affect the efficiency of these cellular processes and cause cellular dysfunction. Indeed, extracellular signaling molecules and the pathways that regulate them seem to play an important role in longevity: taking NF-κB as an example, age-associated gene expression in the skin can be reversed by NF-κB inhibition (Adler et al, 2007), and longevity-promoting genes such as SIRT1 suppress NF-κB signaling (Salminen & Kaarniranta, 2009a). NF-κB signaling, in turn, affects inflammation, autophagy, cell division, and metabolism, which also play roles in longevity (Franceschi et al, 2007; Madeo et al, 2010; Salminen & Kaarniranta, 2009b; Takacs-Vellai et al, 2006).

An increase in extracellular signaling molecules throughout life would arise from any inequality between the rate of production and the rate of active clearance. This could be a constant inequality beginning at birth, or could arise later in life from an increase in the rate of production or a decrease in the rate of clearance. The first explanation is simple and requires no additional regress; however, as the half-life of most extracellular signaling molecules is short (and concentration-independent), it seems unlikely that a constant inequality would be sufficient to cause a substantial accumulation of signaling molecules. The latter two explanations, however, require a cause for the shifting homeostasis. The immune system is a potential candidate – immune cells generate large quantities of signaling molecules in response to antigenic stimuli, and others have argued that the continuous antigenic challenge throughout life progressively raises the level of immune activation (Franceschi et al, 2007). However, while the immune system certainly plays a vital role in propagating extracellular signaling such as inflammation, it is unclear whether the instigator of that response is continuous antigenic stress: the theory predicts that decreased exposure to antigens would reduce the rate of aging, and there is currently no evidence for this (delayed aging being different from extended lifespan, which would be a natural consequence of fewer fatal infections). Alternatively, cellular senescence is a potential instigator of chronic extracellular signaling, both because senescent cells persist and because senescence induction does not rely on foreign factors. As stated previously, there is currently no direct evidence that the SASP drives aging, and the SASP likely has a beneficial purpose at some stage of senescence; however, interventions that induce cellular senescence *in vivo*, such as forced telomere dysfunction, induce pro-inflammatory signals that inhibit stem cell function (Ju et al, 2007), and interventions that likely reduce cellular senescence can reverse aging phenotypes in certain mouse models (Jaskelioff et al, 2010). It is particularly optimistic to imagine that senescent cells are a primary driver of chronic extracellular signaling, as they are likely only a small subset of cells and thus may be more easily eliminated than, say, the immune system.

Aberrant extracellular signaling such as chronic inflammation, once established, often acquires momentum because of positive feedback loops in the immune system: cytokines activate leukocytes, which produce more cytokines, etc. Therefore, even a small pro-inflammatory stimulus, such a population of senescent cells scattered throughout organs and tissues, could seed a more systemic chronic inflammatory response. As we learn more about the role of senescent cells *in vivo*, we might find that the tradeoff between tumor suppression and longevity can be manipulated.

Final thoughts on aging

Healthy aging is an oxymoron. Distinctions are often drawn between "normal" aging and age-related disease; these are semantic categories. A disease is merely a pathophysiological

process that has been named. The concept that aging is a natural part of life leads to the insistence that age-related “diseases” such as Parkinson’s, Alzheimer’s, diabetes, atherosclerosis, osteoarthritis, etc are mechanistically distinct from the relentless increase in mortality with time. This narrative generates a fallacious social conscience in which we saddle ourselves with concepts like “healthspan”, arguing that we want to prevent diseases of aging without extending lifespan, and rendering it is acceptable to provide therapies for age-related diseases, but not “normal aging”. While certain pathological characteristics of aging are undeniably exacerbated in segments of the population, leading to visible symptoms that are sources of much suffering, their genesis is rooted in the same processes that create phenotypes as benign as wrinkles, whether those processes are inflammation, cellular senescence, or another other of the panoply of theories of aging. In researching aging, we should attempt to understand and mitigate *all* the suffering associated with biological aging, irrespective of its medical name. In order for the field to reach its potential, this false dichotomy between disease and healthy aging must be eliminated, both in the minds of researchers and the public. Aging is a disease – it deserves a cure.

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MATERIALS AND METHODS

"That which I cannot create, I do not understand"
-Richard Feynman

Cell culture

Primary human fibroblasts (HCA2, WI-38, BJ strains) and MDA-MB-231 cells were obtained and cultured as previously described (Coppe et al, 2008). Unless noted otherwise, "fibroblast" or "cells" in the text and legends refer to HCA2 fibroblasts. Briefly, cells were cultured in a 10% CO_2, 3% O_2 atmosphere in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serums (FBS). When cells reached confluence, they were passaged at 1:3-1:4. Presenescent (PRE) HCA2 cells completed <35 population doublings (PDs) and had a 24 h BrdU labeling index of >60%. Cells were made replicatively senescent (SEN(REP)) by repeated subculture, as described (Dimri et al, 1995; Krtolica et al, 2001). For DNA damage-induced senescence (SEN(XRA)), cells were grown to confluence, exposed to 10 Gy X-ray and, unless noted otherwise, analyzed 8-10 d later; PRE cells were mock-irradiated. For oncogene-induced senescence (SEN(RAS)), cells were infected with a lentivirus expressing RAS^{V12} and analyzed 8-10 d after infection.

Chemical inhibitors and inducers

Where indicated, cells were given 10 μM SB203580 (SB) (Calbiochem, 559395) dissolved in water for the specified intervals with daily media changes. Where indicated, cells were given 10 mM N-acetyl cysteine (NAC) (SigmaAldrich, A7250) dissolved in water for the specified intervals with daily media changes. Where indicated, cells were treated with 100 uM z-VAD-fmk (Z-VAD) (R&D Systems, FMK001) dissolved in DMSO for the specified intervals with daily media changes. Staurosporine (500 nM) was from SigmaAldrich (S4400). Actinomycin D (10 ug/mL) was from SigmaAldrich (A1410).

Vectors, viruses, and infections

MKK6EE (provided by Dr. Eisuke Nishida of Kyoto University), Genetic suppressor element 22 (GSE) and RAS^{V12} were subcloned into Gateway destination vector 670-1, as previously described (Beausejour et al, 2003; Campeau et al, 2009). p38MAPK cDNA was from Open Biosystems, and a drug resistant mutant of p38MAPK (p38MAPK DR) was created by three amino acid substitutions using Stratagene's QuikChange kit and protocol (#200519): Thr 106 to Met, His 107 to Pro, Leu 108 to Phe (Eyers et al, 1998; Gum et al, 1998). p38MAPK constructs were then subcloned into Gateway destination vector 670-1. The PPM1D (Wip1) full-length open reading frame (ORF) was from Open Biosystems (#OHS4559-99856749) and was subcloned into Gateway destination vector 670-1. LMNB1 and LMNA full-length open reading frames (ORF) in the pENTR211 vector were from Open Biosystems and were also subcloned into Gateway destination vector 670-1. In all cases of infection with a 670-1 vector, infection with an insertless vector was used as a control for PRE cells.

Lentiviral vectors encoding shRNAs against GFP (RHS4459), p38α (TRCN0000000509, TRCN0000010051), and RelA (TRCN0000014686, TRCN0000014687) were from Open Biosystems. RNAi against PPM1D (Wip1) was accomplished by creating a pool of three separate shRNAs that target unrelated segments of PPM1D, as described (Chew et al, 2009). shRNA sequences with appropriate loop sequences and overhangs (caccGTGCATCTGGGAAATGAGGTgtgtgctgtccACCTCATTTCCCAGATGCA, caccGTCGAAGTAGTGGTGCTCAgtgtgctgtccTGAGCACCACTACTTCGAC, caccGATGTCAACTCCTGGCCAAgtgtgctgtccTTGGCCAGGAGTTGACATC, as well as their reverse complements (with appropriate overhangs), were ordered as single strand

oligonucleotides, annealed, and subcloned into pENTR/H1/TO via Invitrogen's BLOCK-iT Inducible H1 RNAi Entry Vector kit (#K4920-00). shRNAs were then subcloned into the pLenti X2 vector w16-1 (Campeau et al, 2009). As a control, a scrambled version of shRNA #1 was created (caccAGTGTGTATAAGCGTGCGAgtgtgctgtccTCGCACGCTTATACACAC) and viral stocks were adjusted to so that both the 3 shRNA pool and the scrambled control were at equivalent titer.

Lentiviral vectors encoding ATM shRNAs, CHK2 shRNAs, p53 shRNA (Rodier et al, 2009) and virus production were described (Beausejour et al, 2003; Naldini et al, 1996). Briefly, lentivirus was produced by transiently transfecting 293FT packaging cells (Invitrogen, R700-07) in Opti-Mem media with Lipofectamine 2000 (Invitrogen, 11668-019) at 1:370 dilution, with 4 µg each of 3 packaging vectors and 8 µg of the plasmid carrying the gene of interest. Viral supernatant was collected 48 hours later, filtered at 0.45 µm, and concentrated by ultracentrifugation. Viral titers were adjusted to infect ~90% of cells. Cells were infected overnight with Polybrene, allowed to recover for 48 h, selected for 48 h, and allowed to recover for at least another 48 h before use.

Immunofluorescence

Cells were cultured on glass chamber slides, fixed with Formalin for 10 min, and permeabilized with 0.1% Triton in PBS. Slides were blocked for 30 min at room temperature with 1% BSA, 4% normal donkey serum in PBS, washed, and incubated with primary antibody overnight at 4˚ C in 1% BSA in PBS. Slides were washed and incubated with Alexa Fluor (Molecular Probes, Alexa 350, 488, 594) secondary antibodies in 1% BSA in PBS at 1:750 for 45 min. DAPI was used to label DNA. Slides were washed and mounted with Vectashield (Vector Labs, H1000). Primary antibodies were from R&D Systems (IL6, 1:60, AF206NA), Novus Biologicals (53BP1, 1:2000, NB 100-305), and BD Biosciences (BrdU, 1:100, 347580). Where indicated in the figure legends, images were quantitated using CellProfiler, an open-access image analysis program (www.cellprofiler.org).

Senescence-associated β-galactosidase assay

Cells were fixed and stained for SA-βgal using BioVision's Senescence Detection Kit (#K320-250) for 24 h. Staining was visualized by light microscopy and the positive cells were counted manually.

Bromodeoxyuridine (BrdU) incorporation

Subconfluent cells were incubated for 24 h with 10 µM BrdU (Roche, 10280879001) in growth medium, fixed, permeabilized and washed with ExoIII reaction buffer (66 mM Tris-HCl, pH 8.0, 0.66 mM $MgCl_2$). DNA was partially digested with 200 U/mL Exonuclease III (Promega, M1815, 1:1000) and 10 U/mL DNAse I (Roche, 10104159001, 1:1000) in 0.75x ExoIII reaction buffer for 30 min at 37˚ C. Cells were washed with PBS and visualized by fluorescence microscopy. Mean BrdU fluorescence in each nucleus was quantitated using CellProfiler, and a single "BrdU positive" cutoff value was established for each independent experiment and applied to all images in that experiment.

Measurement of reactive oxygen species

The level of reactive oxygen species (specifically, H_2O_2) was measured by CM-H2DCFDA fluorescence and quantitated via flow cytometry. CM-H2DCFDA (Invitrogen, #C6827) was added to cells at a final concentration of 10 uM for 30 min. Cells were then washed with PBS, trypsinized, and strained to remove cell clumps. CM-H2DCFDA fluorescence was measured with a guava EasyCyte Mini flow cytometer.

Western blot analysis

Cells were washed with warm PBS and quickly lysed and scraped with either denaturing (5% SDS, 10 mM Tris) or non-denaturing (Cell Lysis Buffer, Cell Signaling 9803) buffer containing protease (Sigma, P8340, 1:200) and phosphatase inhibitors (200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate, 400 mM sodium tartrate, 1:100). Lysates were needle-sheared, clarified by centrifugation, subjected to SDS-PAGE using 4-12% Bis-Tris gels and transferred onto PVDF membranes. Membranes were blocked and incubated overnight at 4˚ C with primary antibodies: Cell Signaling (p38, 9212, 1:1000; p38α, 9228, 1:2000; p38β, 2339, 1:1000; Hsp27, 2402, 1:1000; Hsp27-P, 2401, 1:1000; p53-P-Ser15, 9284, 1:1000; Chk2, 2661, 1:1000; Chk2-P, 2662, 1:1000), Santa Cruz Biotechnology (NF-κB p65, SC-8008, 1:1000; RelB, SC-226, 1:500; C-Rel, SC-70, 1:500; MKK6, SC-1992, 1:1000; p53, SC-126, 1:1000; ATM-P, SC-47739, 1:1000; lamin B_1 (C-20), SC-6216, 1:1000; lamin B_1 (S-20), SC-30264, 1:1000), Abcam (p38-P, AB4822, 1:1000; ATM, AB32420, 1:2000; lamin B_1, AB16048, 1:000), SigmaAldrich (Tubulin, T-5168, 1:4000), BD Transduction Labs (RAS, R02120-050, 1:1000), BD Biosciences (p21, 556430, 1:1000; $p16^{INK4A}$, 554070, 1:1000; lamin A/C, 612162, 1:4000), Novus Biologicals (Wip1, NB100-2110, 1:5000), Phospho Solutions (p38-P, p190-1802, Lot #CYP309Y, 1:1000). Membranes were washed and incubated with HRP- (Cell Signaling, 1:5000) or IR-dye- (LI-COR, 1:20000)-conjugated secondary antibodies for 45 min at room temperature, washed, and signals detected by enhanced chemiluminescence or LI-COR Odyssey, respectively. Signals were quantified with LI-COR Odyssey software.

ELISAs and conditioned media

ELISA kits to detect IL-6 (D6050), IL-8 (D8000C) and GM-CSF (DGM00) were from R&D Systems. MMP1 and MMP3 were detected with AlphaLISA kits from Perkin Elmer (AL242C, AL284C). Conditioned media (CM) were prepared by washing with serum-free DMEM and incubating in serum-free DMEM for 24 h. CM were collected, clarified by centrifugation and stored at -80° C. Cells were trypsinized and counted, and CM were normalized for cell number.

Antibody arrays

CM samples were diluted to equivalent cell numbers in serum-free DMEM. Antibody arrays from Raybiotech (AAH-CYT-G1000-8) were used according to the manufacturer's instructions. Arrays were scanned using a GenePix 4200A Professional microarray scanner at 10 μm resolution. Signal intensities were quantitated using LI-COR Odyssey software, and normalized to positive controls for each sample, which were then normalized across all samples.

Invasion assay

CM were diluted to equivalent cell numbers in serum-free DMEM. Invasion assays were performed as described (Coppe et al, 2006) using Matrigel Invasion Chambers (8 mm pore, BD Biosciences 354480). Briefly, 50,000 MDA-MB-231 cells were plated in the upper chamber and CM was added to the lower chamber. After 16 h, cells were fixed (2.5% glutaraldehyde/PBS) and stained (0.5% Toluidin Blue/2% Na_2CO_3). Cells that remained atop the Matrigel were removed. Cells that invaded the Matrigel were counted by light microscopy.

Quantitative RT-PCR

RNA was isolated using the RNeasy Mini kit (Qiagen, 74104) and cDNA generated with iScript cDNA synthesis kit (Bio-Rad, 170-8891). Taqman analyses were performed by the UCSF Genome Core. Samples were normalized to β-glucuronidase (GUS). Primers were from Applied Biosystems: IL-1α (Hs00174092 m1), IL-1β (Hs01555410 m1), IL-6 (Hs 00174131 m1), IL-8 (Hs 00174103 m1), GROα (Hs 00236937 m1), GM-CSF (Hs 00929873 m1), MCP-2 (Hs 99999026 m1). LMNB1 and LMNA mRNA levels were determined via the UPL system from Roche (probes #31 and #17, respectively) using the following primers: LMNB1, left: aagcagctggagtggttgtt; LMNB1, right: ttggatgctcttggggttc; LMNA, left: agcaaagtgcgtgaggagtt; LMNA, right: tcaggtcaccctccttcttg. PPM1D (Wip1) mRNA levels were determined via the UPL system from Roche (probe #123, left: tcctataatagtcaagaaacctgtgtg, right: ccatggatcctcctccagt). Samples analyzed via the UPL system were normalized to Tubulin (probe #58, left: cttcgtctccgccatcag, right: ttgccaatctggacacca).

NF-κB assays

NF-κB DNA binding activity was assayed using the TransAM™ NF-κB p65 Transcription Factor Assay Kit from Active Motif (40096). Whole cell lysates were collected under non-denaturing conditions. Values were normalized to cell number. To assay transcriptional activity, cells were infected with Cignal NF-κB Lentiviral Reporter (SABiosciences, CLS-013L) and selected with puromycin. After senescence induction, cells were lysed and assayed for luciferase using the Luciferase Assay System (Promega, E1500). Values were normalized to cell number.

Analysis of transcription factor binding sites

TFM-Explorer (http://bioinfo.lifl.fr/TFME/form) (Defrance & Touzet, 2006) was used to identify the top ten statistically overrepresented partial weight matrices (PWMs) in the 200 bp upstream of the transcription start sites of genes encoding proteins significantly induced by MKK6EE expression. We searched all vertebrate PWMs available in the TRANSFAC database using a ratio (density of clusters) of 2.5. P-values were calculated by TFM-Explorer, compared to a background model incorporating 10kb upstream and 5kb downstream of the transcriptional start sites of all genes with RefSeq identifiers (24,328), as described (Defrance & Touzet, 2006).

Statistical analyses

Except where indicated, statistical significance was evaluated using a two-tailed Student *t*-test and assumption of equal variance. Statistical significance between binary assays (i.e., positive and negative scores) was evaluated using a Chi-squared test. Except where indicated, error bars represent standard deviation. Error bars for single cell

analyses (e.g. DNA damage foci analysis) represent the standard error of the mean (standard deviation/√(# nuclei). Error bars for binary assays represent the margin of error at 95% confidence (approximated to 1/√(# nuclei)).

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