

Aging and Apoptosis



SUMMARY

Many anti-aging creams and lotions are available to consumers these days. Some are quite expensive. The goal is to help prevent or mask the effects of aging. The biotechnology industry is also interested in aging for similar reasons. Aging can be split into two branches: genomic instabilities and cellular dysfunction. Genomic instabilities include genetic changes, telomere shortening, and epigenetic changes. Cellular dysfunction includes intercellular communication, nutrient sensing, protein function, and mitochondrial function.

DNA can be damaged from environmental factors such as chemicals, UV radiation, and biological agents. Additionally, DNA damage and mutation can come from within the cell either through errors introduced during DNA replication or chemical reactions attacking DNA. Despite the actions of various repair systems within the cell, some changes persist. Somatic mosaicism occurs when somatic cells have different sequences than the rest of the organism, such as in the case of blood cells between two different age groups. Adults over the age of 70 had 1% to 2% of blood cells with different genomic sequences. By comparison, adults younger than age 50 had only less than 1% of blood cells with differences. Loss of chromosomes, gene copy number variations, base changes and mutations, and chromosomal translocations all contribute to aging at the genetic level.

Additionally, oxidative stress produces mutations in both nuclear and mitochondrial DNA that accumulate with age. The mitochondria functions in aerobic metabolism, which produces many reactive oxygen species. Reactive oxygen species (ROS) include peroxides, superoxide anions, and hydroxyl radicals, which can cause damage to many cellular structures but particularly cause damage to mtDNA. The reason is that mtDNA is not protected by histones, and the mtDNA is also in close proximity to the metabolic reactions. Eventually, the accumulated damage reaches a threshold that sends the cells into senescence or apoptosis.

Telomeres are repetitive sequences at the ends of linear eukaryotic chromosomes. They are shortened after every round of cell division because DNA polymerase has difficulty replicating linear ends. The lengths of the telomeres are directly related to the fate of the cell. Longer telomeres mean more cell divisions. Shorter telomeres mean senescence. Senescence is a state in which the cells remain metabolically active but are no longer dividing. Telomerase is an enzyme that adds back nucleotides at the telomeres in some cells. Loss of function of telomerase results in many symptoms of aging, suggesting a role for telomere length and stability in the aging process.

Epigenetic changes are modifications to DNA and the associated proteins. These modifications include DNA methylation, histone modification, and chromatin remodeling. DNA methylation results in the silencing of genes in that region. DNA methylation decreases with age, which in turn causes increased gene expression. Some genes are even hypermethylated during aging.

In eukaryotes, DNA wraps around histone proteins. Several modifications of histones occur, including methylation, phosphorylation, ubiquitylation, and acetylation. These modifications control expression of the genes in those areas. Sirtuins are histone deacetylases that function to remove acetyl groups from histone proteins. Defects in the genes for sirtuins have shown to increase the life span of not only yeast, but also mammalian cell lines and mice. Sirtuins might one day become a target for anti-aging drugs.

Chromatin structural changes also contribute to aging. Heterochromatin is highly condensed and primarily associated with repetitive sequences near telomeres and centromeres. The quantity of these highly condensed regions declines with age, which results in expression of nonfunctional mRNA from those regions and destabilizes the genome.



Metabolism and nutrient sensing also play roles in aging. Aging studies in the nematode, *Caenorhabditis elegans*, have revealed that mutations in the *daf-2* and *age-1* genes can cause worms to enter a state of hibernation called dauer and increase their life spans. The protein products of these two genes are involved in metabolic rate regulation and encode homologs of mammalian insulin signaling pathways. Restrictions in dietary and caloric intake also extend the life span of many organisms so long as the nutrition is complete. This is likely due to a decrease in the activity of the IIS pathway, which is involved in insulin signaling although the molecular details are not yet elucidated.

Degradation of proteins occurs when proteins lose their shapes, become modified by chemical reactions, or are targeted by the cell for destruction. Chaperones correct the folding of proteins stressed during high heat conditions. Furthermore, they are impaired in aged tissues, which causes an increase in the quantity of incorrectly folded proteins. The misfolded proteins accumulate in the membranous endoplasmic reticulum and form aggregates.

Contrary to fixing misfolded proteins, defective proteins are also subjected to degradation by three systems: the ubiquitin-proteasome system, microautophagy-lysosome system, and chaperone-mediated autophagy. The ubiquitin-proteasome system adds a ubiquitin tag to the defective protein, which signals the proteasome to degrade the ubiquitinated protein. The microautophagy-lysosome system and chaperone-mediated autophagy identify defective proteins and move them to lysosomes. Lysosomes contain hydrolytic enzymes that degrade the defective protein. The difference between the two systems is in the delivery of the defective protein. The lysosome in the microautophagy system gulps regions of cytosol. In the more specific chaperone-mediated autophagy, the defective protein is identified by a chaperone, which then shuttles it to the lysosome. Both of these systems are defunct in aging cells.

Mitochondria, the powerhouse of the cell, also accumulate damage that contributes to aging, particularly in cells that are highly metabolically active like neurons. Reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive lipid species (RLS) accumulate in the brains of individuals with Parkinson's disease and Alzheimer's. Superoxide dismutase and catalase are antioxidant enzymes that help combat oxidative stressors. In terms of aging, the accumulation of oxidative stressors and the decrease in antioxidants add to the aging process.

Defective and damaged organelles and cell components are recycled by a process called autophagy, or mitophagy when the mitochondria is specifically targeted. Autophagy includes macroautophagy, microautophagy, and chaperone-mediated autophagy. Microautophagy and chaperone-mediated autophagy are involved in protein degradation during aging. Macroautophagy targets whole organelles.

Adult cells grown in culture undergo only a set number of cellular divisions, even in the presence of exogenous growth factors. The cells do not immediately die, though. They remain metabolically active as long as they can acquire nutrients. This is called cellular senescence. Senescence ensures that the damaged cells do not divide and produce damaged descendants. The number of cell divisions that occurs before senescence depends on the cell type and species, with some cells never entering senescence, such as intestinal, immune, and skin cells. The key characteristics associated with cellular senescence are (1) cells are arrested in the cell cycle; (2) cells have active tumor suppressor molecules; (3) senescent cells produce molecules only associated with an arrested growth state; (4) cells have an altered chromatin structure that can be visualized with dyes; and (5) senescent cells secrete more factors, such as cytokines and extracellular matrix factors.

Programmed cell death is a controlled process to eliminate cells that have accumulated damage beyond repair. Apoptosis or type I programmed cell death involves the disruption



of the cell membrane, nuclear shrinkage and division, and fragmentation of the cellular material into apoptotic bodies that are engulfed by either nearby cells or macrophages through phagocytosis. The bits and pieces of each apoptotic cell are recycled. The actual mechanism of apoptosis was first identified in *C. elegans*, but genetic analysis of mammalian genomes has revealed many homologs to the same proteins in the nematodes. In mammals, there are two cascading pathways that initiate apoptosis. The death receptor pathway is initiated by an external signal that binds to a death receptor on the cell surface, which in turn begins the signal transduction pathway, recruiting of various proteins, and finally activation of a specific protease called caspase. The mitochondria death pathway is initiated by an internal signal, probably extensive DNA damage. Proteins in the mitochondria activate caspase. In both pathways, the caspase digests the cellular components in order to form apoptotic bodies. The apoptotic bodies are removed by phagocytosis from either nearby cells or macrophages (in mammals). Macrophages are part of the immune system and usually recruit other immune system cells. Interestingly, macrophages do not activate other immune system cells during the phagocytosis of apoptotic bodies. Therefore, macrophages differentiate between apoptotic bodies and foreign material.

There are two possible outcomes for defective apoptosis. If too much tissue dies, the organism may die or have abnormalities. If too little tissue undergoes apoptosis, then there could be an excess that interferes with normal functions. Surplus immune system cells are eliminated in mammals through the death receptor pathway following an immune response. Elimination of too many white blood cells renders the organism immunocompromised. Additionally, uncontrolled apoptosis may be involved in the onset of Alzheimer's, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS).

Necroptosis, another type of programmed cell death, involves signals that overlap with the death receptor pathway of apoptosis. In the case of necroptosis, caspase is not activated and cell destruction occurs when membranes rupture. The exact mechanism of action is not well known.

Progression through the cell cycle is mediated by cyclins and cyclin-dependent kinases. An enzyme that functions in the glycolytic pathway regulates the expression of some cyclins. The redox state of cytochromes (electron carriers in the electron transport chain of respiration in the mitochondria) also dictates whether the cell will undergo apoptosis. Finally, some caspases are inhibited by intermediates and reduced electron carriers from the pentose phosphate pathway. Therefore, apoptosis is under metabolic control.

Cancer cells have accumulations of mutations, yet the senescence and apoptotic pathways are often suppressed. This leads to progeny cells that also have damage. Oncogenic mutations are a major factor promoting cellular senescence. Premature senescence occurs without shortening of the telomeres due to a mutation in Ras. Other defective proteins, including p53 and pRb, cause the cell to replicate more cycles and lead to greater accumulations of damage. Mutations in Ras, p53, pRb, and others prevent senescence and often cause uncontrolled cell growth, causing cancer.

Programmed cell death also occurs in single-celled organisms, although it is not by the same mechanism, nor is it called apoptosis. Under stressful conditions, the MazEF system of *Escherichia coli* functions by using a toxin/antitoxin approach. The toxin is stable, but the antitoxin is not. If the antitoxin is not made, the toxin kills the cell. Programmed cell death in *E. coli* may be advantageous to the other cells if the cellular components of the dead cell can be recycled during a shortage of nutrients. Also, the death of the cell may limit the ability of some viruses to replicate and kill off the population of cells.



Case Study Senescence is a Developmental Mechanism that Contributes to Embryonic Growth and Patterning

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Senescence is cell cycle arrest that functions in tumor suppression and aging. The presence of oncogenes, DNA damage, oxidative stress, and chemotherapeutic drugs can contribute to a cell's entry into a senescent state. Senescent cells have some key characteristics, including an enlarged and flat morphology, production of senescence-associated β -galactosidase, and production of tumor-suppressor proteins p53/p21 and p16/RB. In addition to these characteristics, the numbers of senescent cells increase with age, suggesting a role for senescence in the aging process.

Senescent cells are able to communicate with the external environment through the production of growth factors, cytokines, and extracellular matrix remodeling proteins, all contributing to the senescence-associated secretory phenotype (SASP). Contrary to the tumor-suppressor role of senescent cells, these cells are resistant to apoptotic stimuli. Apoptosis eliminates damaged and defective cells in an attempt to suppress tumor formation.

The authors examined the role of senescence in embryo development and determined that senescent cells were detected in many tissues within the embryo.

How did the authors determine that senescent cells were present in many different tissues and locations within the embryo?

Since senescent cells produce a senescence-associated β -galactosidase, the authors stained various mouse and chick embryonic tissues for the presence of this enzyme. Many different regions were stained, including the developing limbs, tip of the tail, otic vesicle, brain vesicles, closing neural tube, fusing sternum midline, and gut endoderm. Furthermore, when anatomical regions were sectioned to identify the specific tissues and cells that were senescent as observed from the staining procedure, the authors found that cells and tissues were stained within only a specific time window.

What are the AER and RP?

The AER and RP are two major signaling centers in the embryo. The authors determined that both centers contained senescent cells. The AER, or the apical ectodermal ridge, is a specialized ectoderm zone that marks the dorsoventral boundary of a limb bud. The AER is involved in regulation of limb outgrowth and patterning. With the help of growth factors, the AER is a signaling center that directs the growth of mesenchymal (embryonic tissues) cells at the tip of the limb. The RP, or roof plate, of the hindbrain is involved in directing central nervous system (CNS) cell fate, inducing neural crest activity, and contributing to blood vessel patterning.

When testing for the presence of known senescence markers, the authors found that only high levels of p21, but not p53 or others, were expressed in the cells of the two signaling

centers, the AER and RP. What markers were not expressed, and what is a potential explanation for this?

Known markers for senescent cells include p21, p53, p16, p19, PML, γ H2AX, and H3K9me3. Out of these, in both the AER and RP, high levels of p21 were observed, but not of p53. Additionally, no significant levels (relative to adjacent mesenchymal cells) of PML and H3K9me3 were observed, and p16 and p19 expression were not detected. Since these markers are known adult senescent cell markers, this suggests that senescence might have originally been intended as a developmental mechanism, a more primitive role for senescence, and further evolved into an adult role, such as tumor suppression.

What is oncogene-induced senescence (OIS)? Were there any similarities in markers between the AER or RP and OIS?

OIS occurs in the presence of oncogenes within cells. This is an attempt by cells to decrease the propensity of replicating damaged or defective cells, potentially leading to cancer. The authors used senescent human fibroblasts induced into OIS to compare with developmental senescence in the targeted tissues. In addition, the authors used gene expression profiling of AER and senescent human fibroblasts and determined that multiple genes were upregulated in both. These genes were the known senescence inducers p21, p15, as well as many others. Within the RP, p15 was also found to be upregulated. This suggests that p15 is involved specifically in embryonic developmental senescence.

From the gene expression profiles of the OIS versus developmental senescence targets, were there any developmental genes expressed in the senescent human fibroblasts induced into OIS? What is the significance of this, if any?

Yes. Genes involved in developmental pathways were upregulated within OIS. This further suggests that senescence might have evolved primarily in a developmental capacity and is a more primitive form of senescence.

The p21 senescence marker is a common marker for both developmental senescence and OIS and is involved in preventing apoptosis. The authors used a p21-deficient mouse model to examine the effects of p21 impairment on senescence. What were the results of this experiment?

In the p21-deficient mouse model, the authors found a significant increase in cell death within the AER, suggesting that apoptosis prevails when senescence is impaired. Furthermore, although the rate of proliferation of AER cells was not affected, the rate of proliferation for the underlying mesenchymal cells was significantly decreased. Further analysis revealed that p21 is required for proper patterning of the limb and that AER signaling to the mesenchymal cells is impaired in the p21-deficient mouse model.

(Continued)

Case Study Senescence is a Developmental Mechanism that Contributes to Embryonic Growth and Patterning—cont'd

Developmental senescence occurs within a specific time frame. Senescence within the AER could be observed by staining procedures from the developmental time frame E9.5–E14.5. By E15.5, no staining was observed. How were the senescent cells cleared?

The AER is removed by apoptosis and phagocytosis. During the time frame, the senescent cells are redistributed. Between E11.5 and 12.5, the senescent cells were present in the ectoderm of the AER. At E13.5, fewer senescent cells were detected in the ectoderm and more were detected in the mesenchyme. At E14.5, there were no senescent cells detected in the ectoderm. Any remaining senescent cells were found surrounded by immune-system cells within the posterior mesenchyme. These immune system cells were found to be macrophages. Additionally, apoptotic cells were found within the tissues in a similar pattern over the course of the same time frame. All of these results suggest that during the time frame, senescent cells are redistributed and then cleared by a combination of apoptosis and macrophage-mediated phagocytosis.

The AER and RP are both signaling centers within the embryo that direct the growth and proliferation of underlying mesenchymal cells. The authors determined that developmental senescence occurs within some regions of both mouse and chick models, follows a specific course over a specific timeline, and represents a conserved developmental pathway. Impairment of the system and/or factors influencing the signals between the AER and underlying mesenchyme leads to developmental abnormalities. In this study, p21 was identified as a mediator of developmental senescence, yet the exact mechanism is still unknown. It was also found that clearance of senescent cells depends on both apoptosis and macrophage-mediated phagocytosis. In terms of development, these areas are often disrupted, leading to developmental abnormalities. Further understanding of developmental senescence within these regions might give insight into the role of senescence and signaling in developmental abnormalities.



Senescence Is a Developmental Mechanism that Contributes to Embryonic Growth and Patterning

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SUMMARY

Senescence is a form of cell-cycle arrest linked to tumor suppression and aging. However, it remains controversial and has not been documented in nonpathologic states. Here we describe senescence as a normal developmental mechanism found throughout the embryo, including the apical ectodermal ridge (AER) and the neural roof plate, two signaling centers in embryonic patterning. Embryonic senescent cells are nonproliferative and share features with oncogene-induced senescence (OIS), including expression of p21, p15, and mediators of the senescence-associated secretory phenotype (SASP). Interestingly, mice deficient in p21 have defects in embryonic senescence, AER maintenance, and patterning. Surprisingly, the underlying mesenchyme was identified as a source for senescence instruction in the AER, whereas the ultimate fate of these senescent cells is apoptosis and macrophage-mediated clearance. We propose that senescence is a normal programmed mechanism that plays instructive roles in development, and that OIS is an evolutionarily adapted reactivation of a developmental process.

INTRODUCTION

Senescence is a form of cell-cycle arrest first identified in cells at the end of their replicative lifespan (Hayflick, 1965). Subsequently, it was demonstrated that senescence could be induced prematurely by oncogenes in primary cells, inducing oncogene-induced senescence (OIS) (Serrano et al., 1997). This was followed by the discovery in adult cells of other senescence-inducing stimuli, including DNA damage, oxidative stress, and chemotherapeutic drugs (Kuilman et al., 2010; Narita et al., 2003; Schmitt et al., 2002).

Senescent cells usually share a combination of signature features, including a characteristic, enlarged, and flat morphology.

Primarily, they are nonproliferating, exhibit activation of senescence-associated beta-galactosidase (SA β -gal) activity, and have enhanced expression of hallmark regulatory proteins (Dimri et al., 1995; Kuilman et al., 2010). Among the key proteins that are induced are members of the p53/p21 and p16/RB tumor-suppressor networks that function to arrest proliferation and contribute to the irreversibility of the senescent state (Narita et al., 2003; Serrano et al., 1997). This involvement of tumor-suppressor pathways led to the identification of senescence as a potent tumor-suppressor mechanism in vivo (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). More recently, however, broader roles for senescence in nontumorigenic pathologies are emerging. Senescent cells are suggested to increase in number with age and to contribute significantly to age-related decline (Baker et al., 2011; Dimri et al., 1995). Senescent cells also contribute to the process of liver fibrosis and wound repair (Jun and Lau, 2010; Krizhanovsky et al., 2008). However, to date, senescence has not been described in nonpathologic states.

An emerging hallmark feature of senescent cells is the senescence-associated secretory phenotype (SASP) that allows extensive signaling from the nonproliferating cells to the external environment (Acosta et al., 2008; Coppé et al., 2010; Kuilman et al., 2008, 2010). This secretome is largely composed of growth factors, cytokines/chemokines, and extracellular-matrix-remodeling proteins secreted by the arrested cells (Freund et al., 2010). Some of these secreted factors have functional roles compatible with senescence, such as the reinforcement of the arrested state (Acosta et al., 2008; Kuilman et al., 2008) or the recruitment of immune cells to remove senescent cells (Xue et al., 2007). However, there are paradoxical aspects of the SASP and senescence that argue against a sole tumor-suppressive role. In some instances, the SASP can induce proliferation or epithelial-to-mesenchymal transformation (EMT) and invasion in neighboring cells, exerting protumorigenic influence (Krtolica et al., 2001; Parrinello et al., 2005). In others, senescent cells instruct angiogenesis and vessel patterning (Coppé et al., 2006) or alter the local environment with the production of matrix-remodeling factors (Coppé et al., 2008; Parrinello et al., 2005). In addition, senescent cells are resistant to apoptotic stimuli, a finding at odds with their tumor-suppressive role (Mooi and Peeper, 2006; Wang, 1995).

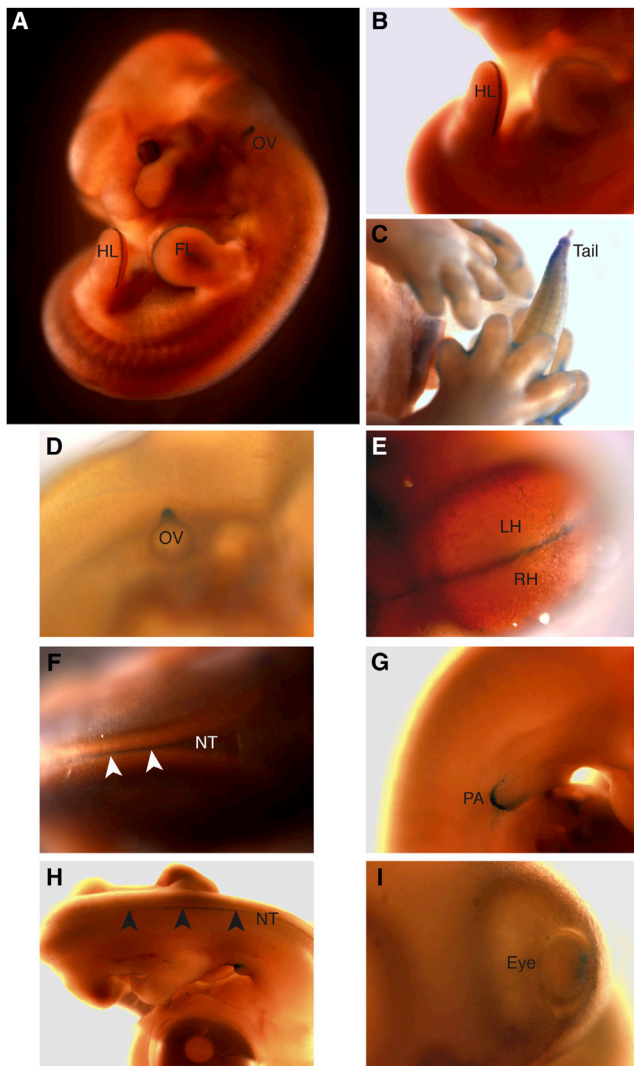


Figure 1. Senescence-Associated β -Galactosidase Is Expressed in Many Tissues during Embryonic Development

Mouse and chick embryos were stained at the whole-embryo level for SA β -gal. Representative images of regions that stained positive are shown.

(A) Mouse embryo, E11.5.

(B) mouse hindlimb, E11.5.

(C) The distal tip of the mouse tail, E14.5.

(D) Mouse otic vesicle, E10.5.

(E) Fusion zone of the vesicles of the mouse hindbrain, E11.5.

(F) Fusing neural tube of the mouse, E11.5.

(G) Chick pharyngeal arches, Hamburger and Hamilton (HH) stage 28.

(H) Chick neural tube, HH28. Arrows denote the line of positive staining along the length of the neural tube.

(I) Chick eye, HH28.

Total numbers examined at each stage listed in Table S1. OV: otic vesicle, HL: hindlimb, FL: forelimb, LH: left hemisphere, RH: right hemisphere, NT: neural tube, PA: pharyngeal arch. See also Figure S1 and Table S1.

In many ways, as a distinct cellular process, senescence can be equated to apoptosis, a tumor-suppressive mechanism that acts to functionally remove cells at risk of aberrant growth. In addition, both processes have demonstrated roles in tissue

pathology and repair. Given that apoptosis also has critical functions during normal embryonic development, we asked whether senescence might also play a role in the embryo. Surprisingly, we found that senescent cells are detectable in many different tissues and locations throughout the embryo, including known signaling centers. We describe that senescence is a normal programmed component of embryonic development and uncover a role in tissue development and patterning.

RESULTS

Senescent Cells Are Widespread throughout the Developing Embryo

To investigate whether senescence is expressed in the developing embryo, we used whole-mount staining for SA β -gal activity. We had previously demonstrated that this approach identifies senescent cells in a model where senescence was activated prematurely (Keyes et al., 2005). Embryos at mid to late stages of development (embryonic days [E] 9.5–17.5) were stained with SA β -gal and were examined for the appearance of positively staining cells. Surprisingly, distinct regions were immediately visible (Figure 1), and it was clear these were specific anatomical structures and locations throughout the embryo. Examples of positive areas included the developing limbs (Figures 1A and 1B), the tip of the tail (Figure 1C), the otic vesicle (Figure 1D), the brain vesicles (Figure 1E), the closing neural tube (Figure 1F), the fusing sternum midline, and the gut endoderm (Table S1 and Figure S1A available online). Sectioning of these embryos that had been stained with SA β -gal further identified specific stained populations of cells and tissues (Figure S1A). Interestingly, the staining in each location was detectable in 100% of animals but was only detectable during a specific time window, with all senescence detectable in this manner disappearing by E17.5 (sites and timeline are detailed in Table S1). Staining with SA β -gal identifies senescent cells at acidic pH 5.5. To support that the staining in the embryo indicated senescence, we stained embryos at pH 6.5 and 7.0. In each case, no staining was detectable, validating the senescent phenotype of the positive cells (Figure S1B). To further investigate these findings, we tested to see whether SA β -gal was detectable in chick embryos. Interestingly, similar distinct patterns of staining were visible throughout the embryo, including at the limbs, in the pharyngeal arches (Figure 1G), along the closed neural tube/spinal column (Figure 1H), and in the developing eye (Figure 1I). Together, this identifies that SA β -gal staining is found in distinct structures and tissues during particular time windows in the normal developing embryo.

Embryonic Structures Express Molecular Mediators of Senescence

To verify that SA β -gal indicated senescence, we examined for expression of known markers and mediators of senescence in two separate tissues that presented with pronounced SA β -gal at E11.5. Senescence in the limb appeared as a distinct layer around the edge of the developing limb bud in a pattern reminiscent of the apical ectodermal ridge (AER) (Figure 2A). The AER is a zone of specialized ectoderm marking the dorsoventral boundary of the limb bud and is a major signaling center regulating limb

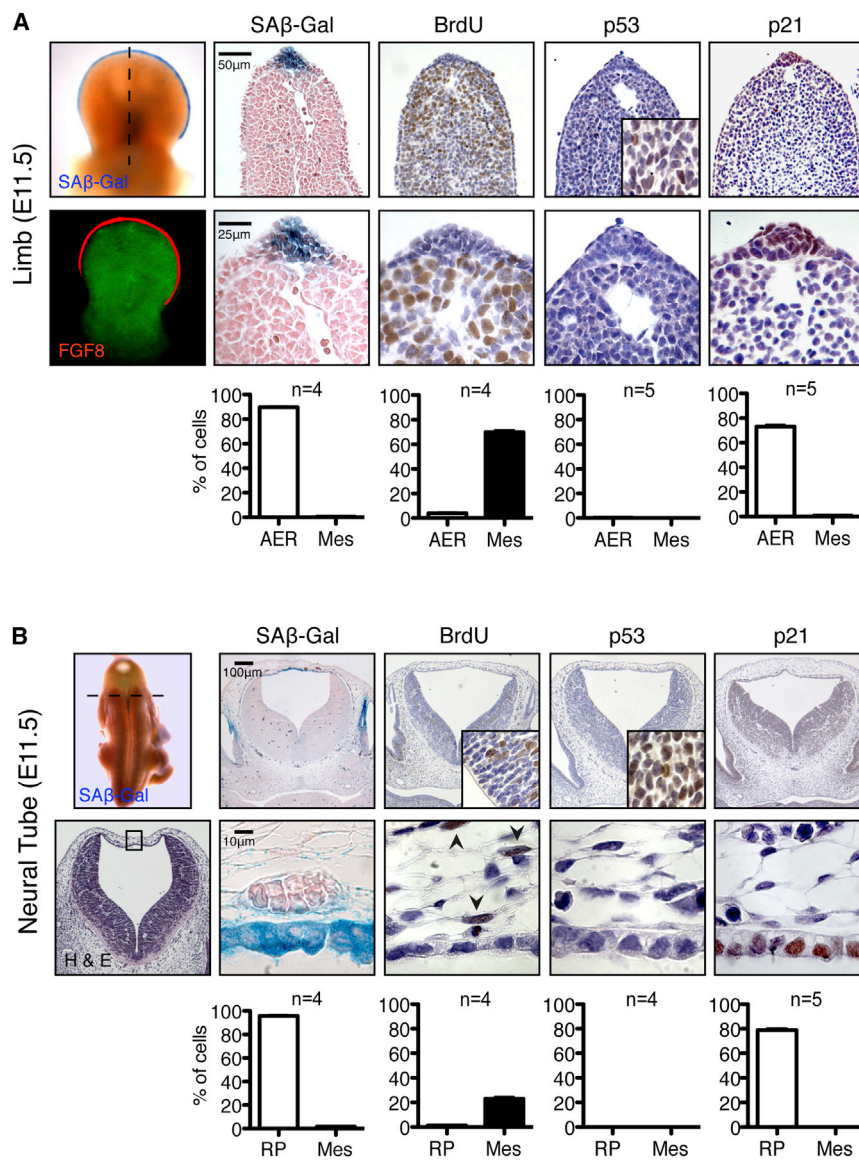


Figure 2. Senescence Markers Are Expressed in the Developing Forelimb and Neural Tube

(A) Forelimb stained for SA β -gal, BrdU incorporation, p53, and p21 by immunohistochemistry. The insert panel of positive p53 staining corresponds to limb mesenchymal tissue of irradiated wild-type (WT) embryos. Top left image shows SA β -gal whole-mount staining with a line showing the plane of section for the staining. Bottom left image was processed for in situ hybridization with FGF8 and scanned by OPT to allow visualization of the AER. The graphs at the bottom show the percentage of positively stained cells in the AER and underlying mesenchyme (mes).

(B) Left panels show whole-mount embryo highlighting plane of section, and below, box indicates area shown in high-magnification images. Neural tube stained for SA β -gal, BrdU incorporation, p53, and p21 by immunohistochemistry. The BrdU insert corresponds to an enlarged area in the neural tube. The graphs at the bottom show the percentage of positively stained cells in the neural RP and adjacent mesenchyme (mes).

All samples stained were WT embryos at E11.5. Values are expressed as mean \pm SEM; n = total number of embryos analyzed. See also Figure S2.

markers. In addition, the AER expressed PML and H3K9me3 (Figure S2A) but at levels similar to the those of proliferating mesenchymal cells, whereas p16 (not shown) and p19 expression were not detectable in the limb.

To identify whether similar results were seen in other regions of the embryo, we looked at the closing neural tube, where a line of senescent cells was visible (Figures 1F and 2B). Transverse sections of SA β -gal-stained E11.5 embryos identified a narrow band of epithelial cells, the roof plate (RP) of the hindbrain, that were stained with SA β -gal (Figure 2B).

The RP is another signaling center that instructs central nervous system (CNS) cell fate, induces neural crest activity, and contributes to blood vessel patterning along the embryonic axis (Chizhikov and Millen, 2004; Gammill and Bronner-Fraser, 2003; Lee et al., 2000). Analysis of senescence markers confirmed that these cells are also nonproliferative and express high levels of p21 but do not have detectable levels of p53, p16 (not shown), or p19 (Figures 2B and S2B). Interestingly, in the chick, this structure expresses the transcript for p15, another key regulator of senescence (Kim et al., 2006). Together, this identifies that two major signaling centers in the embryo contain senescent cells.

outgrowth and patterning (Cohn et al., 1995; Niswander et al., 1993). It lies directly above and, via secreted growth factors including fibroblast growth factors (FGFs), instructs the progress zone (PZ), an area of mesenchymal proliferation at the tip of the limb. In turn, the PZ is reciprocally required to maintain AER function (Kanegae et al., 1998). The AER can be identified with in situ hybridization for FGF8, which develops in a pattern of expression that exactly matches that of SA β -gal (Figure 2A).

Sectioning of the whole-mount SA β -gal-stained embryos confirmed that the AER stains positive for senescence. Next, we tested for the expression of known senescence markers, including p53, p21, p16, p19, PML, γ H2AX, and H3K9me3, as well as the ability to incorporate BrdU. Interestingly the cells of the AER were nonproliferative and expressed high levels of p21, but not p53 (Figure 2A), whereas the adjacent zone of proliferative mesenchyme was negative for these senescence

markers. In addition, the AER expressed PML and H3K9me3 (Figure S2A) but at levels similar to the those of proliferating mesenchymal cells, whereas p16 (not shown) and p19 expression were not detectable in the limb.

The AER Shares an Expression Signature with OIS

We wanted to assess whether these structures expressed any other markers of senescence or shared similarity with OIS. As a first step, we performed gene-expression profiling on the

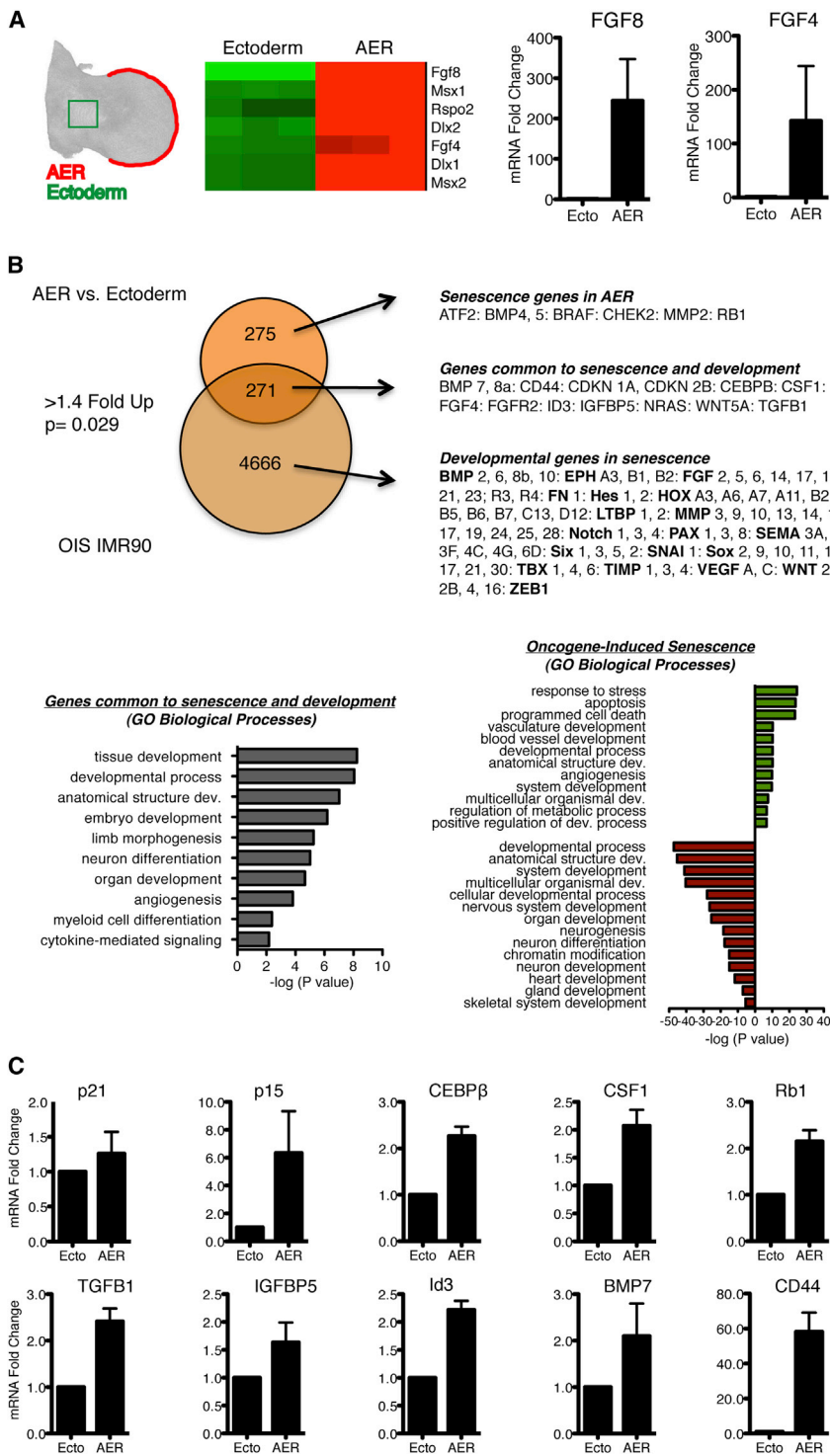


Figure 3. Developmental Senescence Shares a Molecular Signature with OIS

(A) Schematic demonstrating regions in which tissue samples were collected for microarray (red = AER, and green = ectoderm). Representative heatmap of the microarray profile on AER compared to nonsenescent adjacent ectoderm is shown. Example genes are shown to demonstrate that known markers of the AER were expressed at higher levels (red) in the AER compared to the ectoderm (green) (left). qPCR expression on separate biological replicates for FGF8 and FGF4 is shown. Values are expressed as mean ± SEM. (B) Representational overlap of the microarray profile of gene probes that were upregulated >1.4-fold in the AER versus ectoderm compared to those that were upregulated >1.4-fold in two pooled arrays of IMR90 human fibroblasts undergoing OIS. Statistical analysis denotes Fisher's test of enrichment, $p = 0.029$. OIS data are from Collado et al., 2005. Representative examples of genes from each section are denoted with arrows. The full list of genes and their overlap is shown in Table S2, along with the AER expression values for the genes common to OIS and AER. Underlying graphs represent GO analysis of upregulated genes common to senescence and development (left), downregulated genes (Figure S3), and genes in OIS (right). (C) qPCR validation on separate biological replicates for selected genes identified in the microarray. Values are expressed as mean ± SEM. Ecto, Ectoderm; AER, apical ectodermal ridge. See also Figure S3 and Table S2.

(DLX) 1 and 2, and R-Spondin 2 (RSPO2), were among the most highly detected (Figure 3A), validating that this is an AER-enriched gene-expression profile. We used real-time quantitative polymerase chain reaction (qPCR) for FGF8 and FGF4 genes to validate these results on separate biological samples (Figure 3A).

Next, we used published data sets to retrieve gene-expression signatures of senescent cells. Collado et al. (2005; GEO accession number GSE2487) profiled senescent IMR90 human fibroblasts induced into OIS (Collado et al., 2005). This study provides two separate profiles of senescent cells, which we merged to extract a representative gene-expression signature of senescence induced by oncogenic stimulation. This was then overlapped with the signature of the

AER. The AER was microdissected from E11.5 mouse forelimbs, and pooled replicates were compared to samples of nonsenescent surface ectoderm from the dorsal-proximal limb bud (Figure 3A). Importantly, many known AER genes, including FGF8 and 4, Msh homeobox (MSX) 1 and 2, distal-less homeobox

AER-enriched genes to identify those unique to each state and those genes common to both the AER and OIS (Figure 3B and Table S2). Surprisingly, from 546 AER-enriched genes that were common to both studies, 50% (271 genes; $p = 0.029$; Fishers enrichment test) exhibited increased expression in

senescent human fibroblasts (Figure 3B and Table S2), whereas 60% (273 genes; $p = 0.0004$; Fishers enrichment test) of the downregulated genes also overlapped (Figure S3A).

Interestingly, among the upregulated genes common to both senescent human fibroblasts and the AER were the senescence inducers p21 (*CDKN1A*) and p15 (*CDKN2B*) (Figure 3B). However, many other genes that were previously implicated in the regulation of senescence and the SASP, such as CCAAT/enhancer-binding protein beta (*CEBPB*), insulin-like growth factor-binding protein 5 (*IGFBP5*), wingless-type MMTV integration site family, member 5A (*WNT5A*), and the macrophage-recruiting factors colony-stimulating factor 1 (*CSF1*) and *CD44*, were also increased in the AER. Gene Ontology (GO) analysis of the signature common to the AER and OIS highlights the developmental profile of the genes that are up- and downregulated (Figures 3B and S3). The increased expression in the AER for a number of these common genes was confirmed by qPCR on separate biological samples (Figure 3C). In addition, we used the embryonic midline, another site of *in vivo* senescence, to validate expression of some senescence markers. Interestingly, in this tissue, p15 was also prominently expressed (Figure S3B), suggesting that it might be a common regulator of developmental senescence.

Furthermore, among the genes that were expressed in the AER but did not overlap with this profile of OIS were other mediators of senescence, including Retinoblastoma (*RB1*), B-Raf protooncogene serine/threonine-protein kinase (*BRAF*), checkpoint kinase 2 (*CHEK2*), and activating transcription factor 2 (*ATF2*) (Figure 3B). Together, this identifies that the AER shares a significant gene-expression signature with OIS, in particular expressing critical mediators of the senescence program and the SASP.

However, it was striking to also note that in OIS, there was a strong expression of genes that comprise many well-known developmental pathways (Figure 3B). Indeed, we used GO analysis on the OIS signature to identify biological processes that are altered in senescent cells. Surprisingly, this identified that many developmental processes are among the main signatures and pathways of the genes that change expression (Figure 3B). This identifies that the induction of OIS involves changes in expression of many different developmental pathways.

p21-Deficient Mice Exhibit Alterations in Senescence and Limb Patterning

As a first step toward identifying a possible role for senescence in the embryo, we examined mouse models that are genetically deficient in known senescence mediators. We identified p21 as a common marker of senescent cells in the developing embryo and OIS. Although mice deficient in p21 are described as being developmentally normal (Brugarolas et al., 1995; Deng et al., 1995), we sought to examine this more closely and assess whether p21 deficiency might cause defects in embryonic senescence. Initially, we subjected p21-deficient mice at E11.5 to whole-embryo SA β -gal staining. Interestingly, we noticed that these mice had a significant reduction in the total number of SA β -gal-positive cells in the AER (Figure 4A). In addition to inhibiting the cell cycle, a major function of p21 is to prevent apoptosis. To identify the impact of p21 deficiency and impaired senescence, we looked for changes in proliferation or cell death.

Surprisingly, we found a significant increase in cell death in the SA β -gal-positive cells of the AER as measured by TUNEL staining (Figure 4B), suggesting that in the absence of p21, these cells adopted a fate of cell death. Interestingly, there was no difference in the rate of proliferation in the AER in the absence of p21 (Figure 4C). However, there was a significant decrease in proliferation in the mesenchyme directly below the AER, suggesting that the function of the AER in promoting proliferation in the underlying tissue was impaired as a result of impaired senescence (Figure 4C, right graph).

To extend this analysis and to determine whether loss of senescence in p21-deficient mice might affect the integrity and function of the AER, we assayed for FGF expression. Interestingly, in the AER, these mice exhibited decreased expression of FGF8 and FGF4, two key proliferation-inducing signals from the AER to the mesenchyme and limb (Figure 4D) (Capdevila and Izpisua Belmonte, 2001; Sun et al., 2002). This suggests that the function of the AER in instructing pattern formation and proliferation to the mesenchyme might be compromised in p21-deficient animals. To evaluate this, we dissected the PZ from limbs at E11.5 and performed qPCR for known patterning genes (Figure 4E). Interestingly, p21-deficient mice had a significant disruption of the normal pattern for many of these genes, including *GLI3*, *MSX2*, *MEIS1*, and *SHH*, and a decrease in the proliferation marker *Klf6* (Figure 4E; unmerged graphs of biological replicates in Figure S4). Together, these data demonstrate that p21 is required for normal senescence and patterning in the limb.

Interestingly, p21 directly regulates the expression of some SASP components (Chang et al., 2000; Devgan et al., 2006). To ask whether p21 might also regulate developmental patterning genes in OIS, we used the tissue culture model of OIS induced in IMR90 human fibroblasts. In these cells, we used small interfering RNA (siRNA) to knock down p21 levels in senescent cells and, as proof-of-principle, assayed for the expression of some of the developmental patterning genes that were identified in OIS (Figures 4F and S4C). Interestingly, we found that a deficiency of p21 directly in senescent cells significantly disrupted the pattern of expression of select developmental mediators. Together, this suggests that p21 is a critical mediator of developmental senescence, and that these functions might be partially conserved in OIS.

Senescence Overlaps with the Pattern of the AER

In the embryo, we identified that developmental senescence appears during particular time windows but disappears before birth. To identify the ultimate fate of embryonic senescent cells, we performed a time-course staining on the limb. During development, the limb bud develops from reciprocal signaling between the ectoderm and underlying mesenchyme to initiate outgrowth at E9.5 (Capdevila and Izpisua Belmonte, 2001). Limb development proceeds through well-defined stages to reach the state of a mature appendage by E17.5. We examined the developing limb for SA β -gal staining at each stage. Staining was visible in the distal tip of the initiating limb bud at E9.5 (Figure 5A). By E10.5–11.5, the pattern of senescence became restricted to the tip of the limb in the developing AER (Figures 5B and 5C). The AER is a transient structure that persists until later stages of development, before ingressing into the

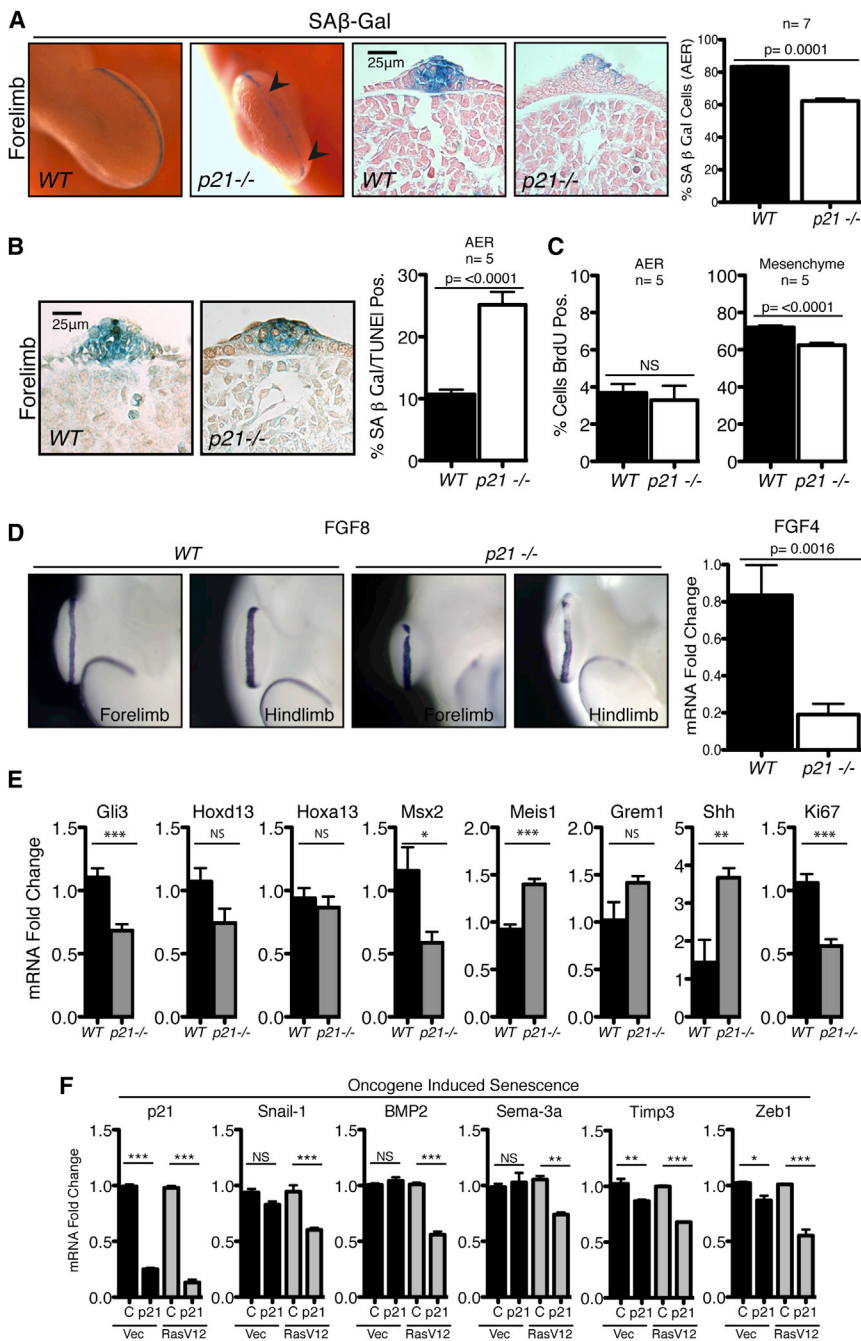


Figure 4. p21 Deficiency Impairs Developmental Senescence and Induces Patterning Defects in Limbs and OIS Cells

(A) Representative images of limbs from WT and p21-deficient embryos that had been stained at the whole-embryo level for SAβ-gal at E11.5 and corresponding sections. Graph shows percentage of SAβ-gal-positive cells in the AER in WT and p21-deficient embryos (n = 7 embryos; values are ± SEM, t test).

(B) Sections of whole-mount stained SAβ-gal embryos, costained with TUNEL. Graph shows percentage of the SAβ-gal population that are TUNEL positive in the AER in WT and p21-deficient embryos (n = 5 embryos; values are ± SEM, t test). (C) Graphs shows the percentage of BrdU incorporation in the AER and mesenchyme in WT and p21-deficient embryos (n = 5 embryos; values are ± SEM, t test).

(D) In situ hybridization for FGF8 on forelimbs and hindlimbs of WT and p21-deficient mice at E11.5. qPCR for FGF4 expression in the AER from WT and p21-deficient embryos is shown (n = 5 embryos; values are ± SEM, t test).

(E) The anterior limbs of WT and p21-deficient embryos were removed at E11.5, and qPCR was performed for representative genes involved in limb patterning (n = 4 embryos; values are ± SEM, t test).

(F) Growing IMR90 cells (black columns; Vec: vector) or Ras-infected senescent IMR90 cells (gray columns; RasV12) were infected with control siRNA (c) or a siRNA targeting p21 (p21) and were assayed by qPCR for the indicated genes (values are ± SEM, Anova and t tests). See also Figure S4.

5H and 5I). In addition, a similar pattern of expression and timing was seen in the AER of developing chick wing bud (Figure S5). Altogether, this demonstrates that senescent cells and the AER follow the same pattern and distribution during a precise developmental window.

Senescent Cells in the Limb Are Removed by Apoptosis and Immune-Mediated Clearance

In stress-induced senescence, senescent cells are removed by immune-mediated clearance (Kang et al., 2011; Xue et al.,

2007). In the embryo, the AER is removed by apoptosis and macrophage-mediated phagocytosis (Guo et al., 2003; Wood et al., 2000). To uncover the cause of senescence disappearance in the limb, we sectioned limbs from embryos at E11.5–14.5 that had been whole-mount stained for SAβ-gal. At the histological level, the redistribution of the pattern of senescent cells was clearly visible. At E11.5–12.5, senescent cells were largely detectable in the ectoderm of the AER (Figures 6A, 6B, and 6E). However, by E13.5, there were fewer positive cells in the surface ectoderm/AER, and the remaining senescent cells

interdigital spaces (Guo et al., 2003). Interestingly, the pattern of staining with SAβ-gal seemed to very closely follow that of the AER, with staining persisting through E12.5 and E13.5 (Figures 5D and 5E). By E13.5, senescent cells were apparent in the developing interdigital spaces, and by E14.5, following disappearance of most of the interdigital tissue, senescence staining was prominent on the posterior flanks of the digits (Figure 5F). By E15.5, staining had virtually disappeared, with only small focal regions remaining on the tips of the developing nails (Figure 5G), and was no longer detectable by E16.5–17.5 (Figures

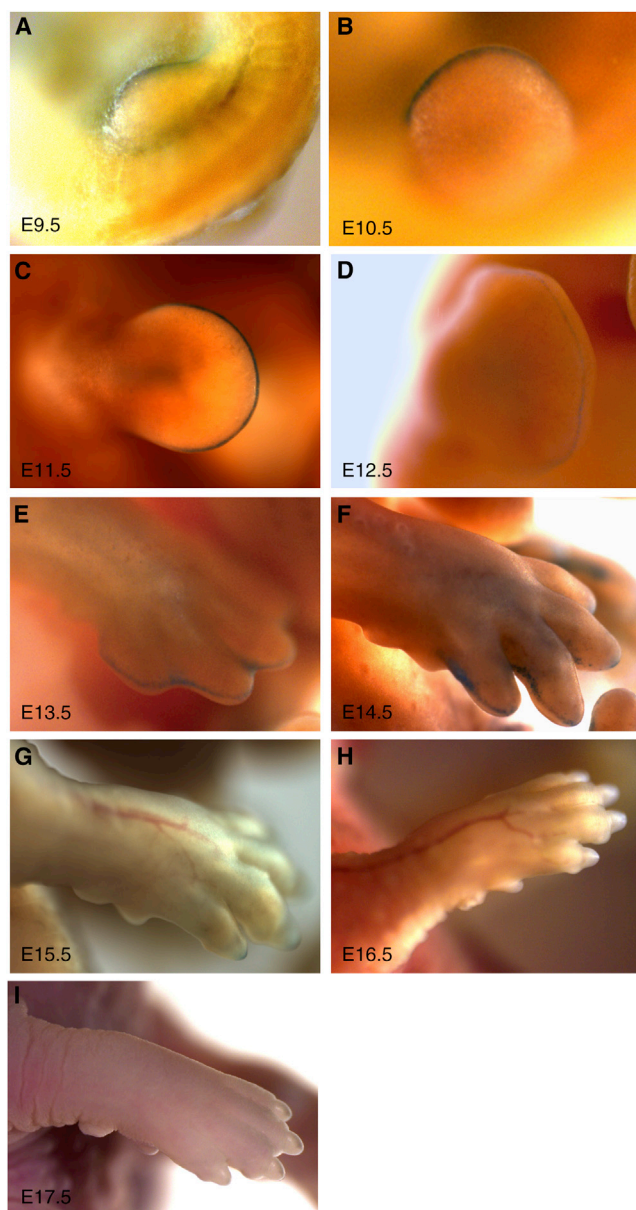


Figure 5. Timeline of Senescence during Mouse Forelimb Development

SA β -gal staining showing the localization of senescent cells during mouse forelimb development. (A) E9.5; (B) E10.5; (C) E11.5, showing prominent staining in the AER; (D) E12.5; (E) E13.5; (F) E14.5; (G) E15.5; (H) E16.5; (I) E17.5. See also [Figure S5](#).

were predominantly detected in the adjacent mesenchyme ([Figures 6C and 6E](#)). By E14.5, senescent cells had practically disappeared from the digit ectoderm ([Figures 6D and 6E](#)), and the remaining cells were predominantly in the posterior mesenchyme. Interestingly, once senescent cells appeared in the mesenchyme, they were frequently surrounded by clusters of immune cells ([Figures 6C and 6D, II'–III'](#)). Immunostaining for the macrophage marker F4/80 revealed that, consistent with

previous reports ([Wood et al., 2000](#)), there is an infiltration of macrophages in the developing limb, and that by E13.5–14.5, these are predominantly located in the mesenchyme surrounding the senescent cells, suggesting that developmental senescent cells might also be removed by macrophage-mediated clearance ([Figure S6](#)).

Next, we costained SA β -gal sections with TUNEL to identify whether these cells might be undergoing apoptosis. At E11.5–12.5, only a small percentage of the senescent cells in the AER were apoptotic ([Figures 6A, 6B, and 6E](#)). By E13.5 however, this percentage increased in both the ectoderm and the mesenchyme ([Figures 6C and 6E](#)), and by E14.5, almost all of the remaining senescent cells, now located in the interdigital mesenchyme, were apoptotic ([Figures 6D and 6E](#)). Interestingly, the clusters of immune cells surrounding the senescent cells also stained positive for TUNEL, presumably identifying macrophages containing apoptotic bodies ([Figures 6C and 6D](#)). At each stage, the number of apoptotic cells outnumbered the quantity of dying senescent cells, suggesting that there are separate populations of cells undergoing apoptosis at this stage. Altogether, this identifies that, at least in the AER, senescent cells undergo a coordinated process of redistribution to the interdigital mesenchyme, apoptosis, and macrophage-mediated clearance.

Senescence in the AER Is Instructed by Paracrine Signaling from the Underlying Stroma

The AER initiates through instructive signaling from the underlying mesenchyme. In turn, the established AER instructs proliferation through growth factor signaling, which activates phospho-ERK (pERK) pathways in the mesenchyme ([Corson et al., 2003](#)). Reciprocally, the mesenchyme then maintains AER function, establishing interdependency of the two regions ([Capdevila and Izpisua Belmonte, 2001; Kanegae et al., 1998](#)). Surprisingly, adult stress-induced senescence is maintained in part by ERK signaling, with these growth-promoting signals paradoxically contributing to the arrested state. Given that senescence is synonymous with the AER, we asked whether it too might be mediated by signals arising from the mesenchyme. First, we determined pERK expression in the whole limb at E11.5, specifically detecting pERK only in the mesenchyme underlying the AER ([Figure 7A](#)) as previously demonstrated ([Corson et al., 2003](#)). Next, we wanted to determine whether transient *in vivo* inhibition of ERK signaling could affect senescence. To address this, we injected pregnant females at E11.5 with the MEK inhibitor U0126, which prevents ERK phosphorylation *in vivo* and blocks OIS in the adult ([Shukla et al., 2007](#)). Surprisingly, this led to a significant reduction of the number of senescent cells in the AER ([Figure 7B](#)), suggesting that pERK-mediated signaling in the mesenchyme establishes paracrine instruction of senescence in the AER. Next, to see whether prolonged inhibition of ERK signaling might have additional effects, we cultured E11.5 embryonic limbs for 15–24 hr in the presence of U0126 ([Figure 7C](#)). Remarkably, while control limbs developed normally and retained a normal pattern of senescence, inhibition of ERK signaling led to a significant loss of senescence in the AER concomitant with abnormal development of the limb. Together, this suggests that senescence is maintained in part

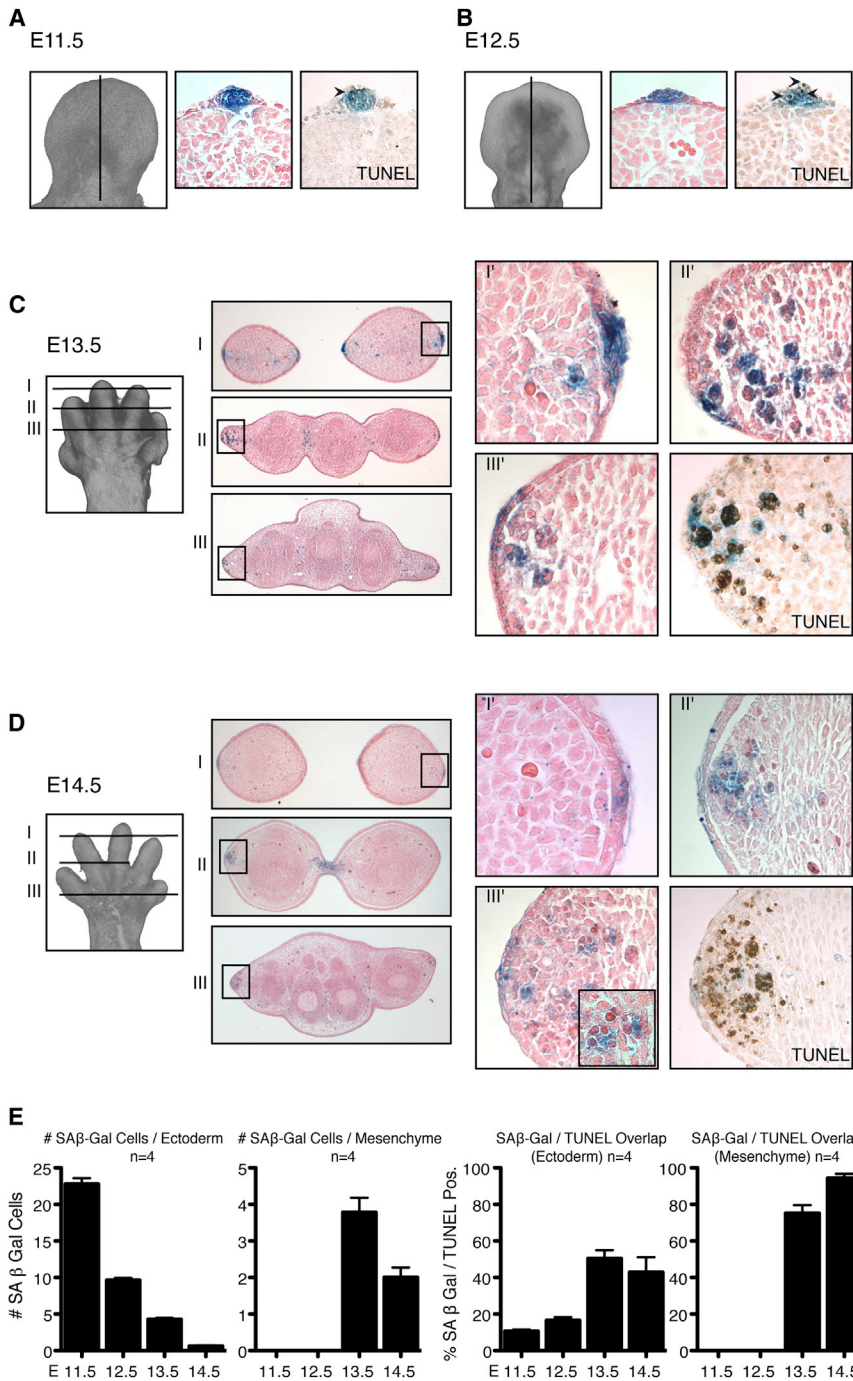


Figure 6. Senescent Cells in the AER Are Removed by Apoptosis and Immune-Mediated Clearance

Representative OPT scans of whole limbs at each stage are shown to illustrate plane of section.

(A) E11.5 SAβ-gal-stained forelimb and TUNEL overlap.

(B) E12.5.

(C) E13.5. Bars I, II, and III denote plane of section in images, and higher magnification of boxed areas is shown in I', II', and III'. Note that senescent cells are surrounded by clusters of immune cells in II' and III'.

(D) E14.5, similar notation of plane of section and high-magnification images as seen in (C). Inset in II' shows clusters of immune cells surrounding a senescent cell.

(E) Shown are the numbers of SAβ-gal-positive cells in the ectoderm and mesenchyme at each stage (left) and the percentages of the total SAβ-gal cells that are TUNEL positive at each stage in the ectoderm and in the mesenchyme (right) (values are ± SEM). See also Figure S6.

normal *in vivo* setting. We use chick and mouse models to show that senescence is a recurrent process in the embryo, is restricted to particular regions and structures, and follows a specific time course, identifying that developmental senescence is a conserved programmed mechanism.

We used SAβ-gal staining to identify senescent cells in the embryo because it is a proven and reliable marker of senescence *in vivo* in many settings, including premalignant lesions (Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005), chemotherapy and tumor regression (Schmitt et al., 2002; Xue et al., 2007), premature aging (Baker et al., 2011; Keyes et al., 2005), fibrotic tissue (Jun and Lau, 2010; Krizhanovsky et al., 2008), and aging human skin (Dimri et al., 1995). Furthermore, in addition to the many regions identified here with a whole-mount staining approach, we believe that many other developing structures will also use senescence to mediate correct patterning at distinct stages of

development, as exemplified in the accompanying manuscript (Muñoz-Espín et al., 2013).

DISCUSSION

Here we describe that senescence is a key feature of embryonic development and, importantly, identify senescent cells in a

via paracrine signals from the underlying mesenchyme, and that a deregulation of this process leads to developmental abnormalities.

Interestingly, we identified significant overlap between developmental and oncogene-induced senescence, including expression of p21, p15, and a number of SASP components (Kuilman et al., 2010). Subsequently, to characterize developmental senescence, we focused on the AER and the RP. Interestingly, these are both major signaling centers in the

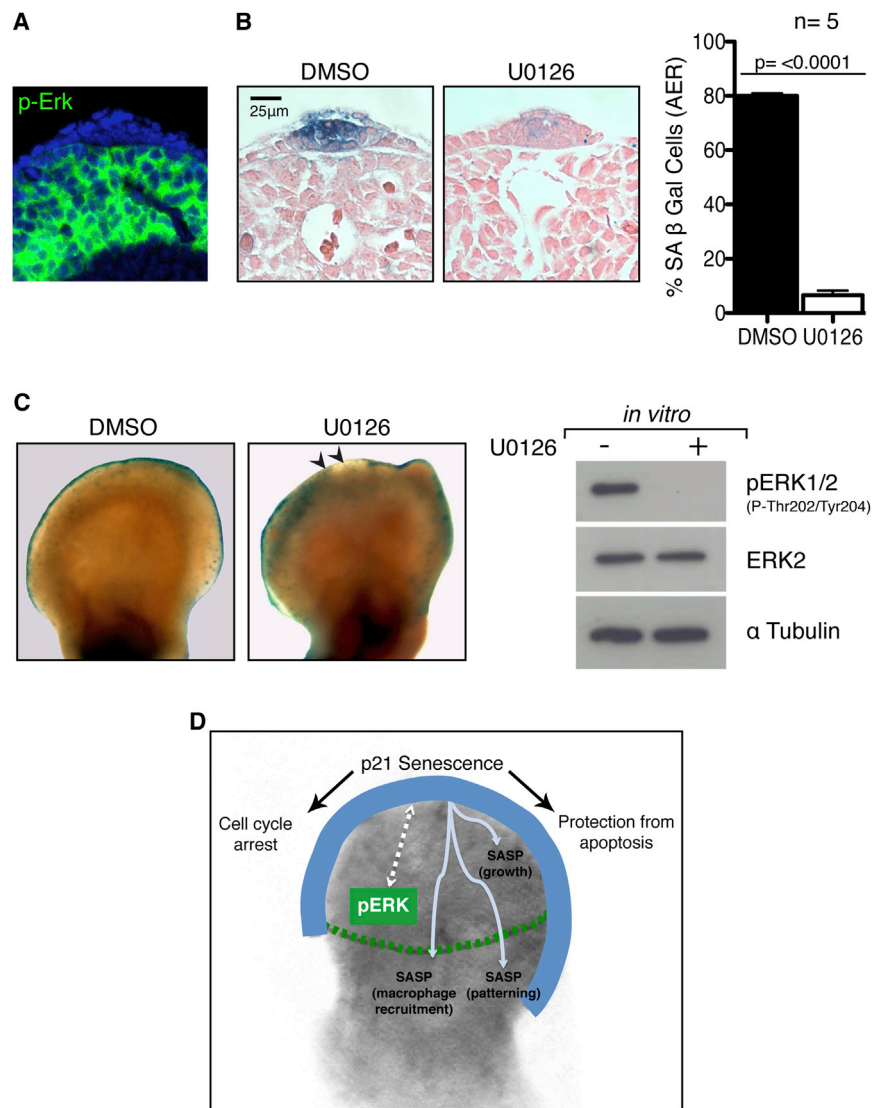


Figure 7. Instructive Signals from the Stroma Maintain Senescence in the AER

(A) Immunofluorescence of pErk showing localization in the mesenchyme in E11.5 forelimbs.

(B) Inhibition of pErk *in vivo* leads to a decrease in senescence in the AER. Quantification of percentage of SAβ-gal-positive cells in control (DMSO) and U0126-treated embryos ($n = 5$ embryos, values are \pm SEM, t test).

(C) Inhibition of pErk in cultured limbs shows a partial loss of senescence in the AER and abnormal patterning. Western blot shows a loss of pErk in cultured limbs.

(D) Model demonstrating proposed function of p21-mediated senescence in the AER: inducing senescence during development, protecting from apoptosis, and instructing local tissue patterning, which is in part maintained by pErk-mediated signals from the mesenchyme.

increased in our array (Acosta et al., 2008; Kuilman et al., 2008). One possible interpretation is that developmental senescence represents a more primitive form of senescence intended as a temporary signaling process during particular developmental stages, whereas the pathological aspects of OIS or aging necessitated the establishment of more complex regulation, requiring additional mediators of reinforcement (Kuilman et al., 2010). More detailed comparisons of senescence in each condition will be informative in classifying stimulus-specific mediators, as well as identifying new common markers of senescence.

While we, and the accompanying study (Muñoz-Espín et al., 2013), identify p21 as one mediator of developmental senescence, precisely how p21 contributes to

embryo that instruct growth and patterning through the release of secreted factors and whose disruption can significantly perturb normal development (Chizhikov and Millen, 2004; Lee et al., 2000; Mariani et al., 2008). Remarkably, in each case, the secreted proteins are common components of the SASP of adult stress-induced senescence, suggesting that some of the paradoxical functions of the SASP in OIS might involve a reactivation of developmental processes. Indeed, this common expression of key mediators and features suggests that senescence might primarily have arisen as a developmental mechanism that was later adapted during evolution to perform its adult roles.

However, there are also notable differences between developmental and adult senescence. Surprisingly, in senescent cells in the embryo, we failed to detect p16 or DNA damage, two central mediators of replicative and oncogene-induced senescence (Kuilman et al., 2010). Additionally, mediators of senescent reinforcement such as interleukin-6 (IL-6) and IL-8 were also not

the regulation of developmental senescence remains to be determined. A member of the CIP/KIP family of cyclin-dependent kinase inhibitors, p21 is a potent inhibitor of proliferation and an important mediator of senescence (Xiong et al., 1993). Despite primarily being regulated by p53 in stress-induced senescence, in the embryo, p21 function is p53 independent (Parker et al., 1995). However, an additional feature of p21 is to protect from apoptosis, both at the intracellular level and through paracrine effects mediated by the secretion of survival/SASP factors (Chang et al., 2000; Devgan et al., 2006; Roninson, 2002). Our data suggest that p21 contributes to many aspects of the senescent state, preventing proliferation and apoptosis, while regulating secretion of SASP factors. Interestingly, however, in OIS, p21 levels are not maintained and return to nearly normal levels as p16 increases to establish irreversibility (Alcorta et al., 1996). As loss of p21 is associated with apoptosis in the limb mesenchyme (Vasey et al., 2011 and data not shown), this suggests that the p21-p16 expression juncture might represent

a critical step in the senescence-apoptosis fate determination of cells.

Additionally, it might seem surprising that mice deficient in p21 do not have more pronounced developmental defects. However, embryos are particularly resilient and adaptive to alterations, often requiring compound deficiencies before a frank defect manifests, as in the case of the pRB-related p130 and p107 genes (Cobrinik et al., 1996) or FGF members in the AER (Mariani et al., 2008). Alternatively, adaptations can arise, as seen in mice deficient in the proapoptotic mediator Apaf1, which present with a delayed mid-stage limb development that is ultimately resolved by birth (Yoshida et al., 1998). Furthermore, whereas a deficiency of the hematopoietic lineage-specific transcription factor PU.1 prevents macrophage development, clearance of interdigital cell death is functionally replaced by mesenchymal cells adopting phagocytic properties (Wood et al., 2000). In the absence of p21, it is possible that other family members such as p57 or senescence mediators like p15 might play similar compensatory roles (Zhang et al., 1999).

Our study shows that, at least in the AER, senescent cells undergo a fate of cell death and immune-mediated clearance. The role of apoptosis and macrophage-mediated phagocytosis in interdigital cell death is well established, as is the immune-mediated clearance of adult senescence (Kang et al., 2011; Wood et al., 2000; Xue et al., 2007). However, whether senescent cells in the limb undergo apoptosis before clearance or macrophages directly induce apoptosis in the senescent cells remains to be shown (Lang and Bishop, 1993). Nonetheless, it is interesting to note that macrophages are recruited to lie adjacent to the senescent AER prior to the appearance of cell death (Rae et al., 2007), suggesting that a primary function of senescence is to contribute to the cellular balance of the tissue, recruiting macrophages to sites where they will ultimately be needed for phagocytosis. In addition, as embryonic macrophages can have trophic and patterning functions mediated by CSF1 instruction (Qian and Pollard, 2010; Rae et al., 2007), it is tempting to speculate that developmental senescence might also indirectly contribute to tissue patterning.

Notably, this study uncovers the senescence program as an integral and inseparable component of limb biology, with disruption of the normal homeostatic signals through genetic or chemical means, leading to a loss of the senescent state. This introduces the question of the identity of senescence-inducing signals. We identify that a disruption of pERK signaling in the underlying mesenchyme perturbs normal senescence and development. Interestingly, Rel/NF- κ B, a master regulator of OIS and the SASP, is expressed in the PZ and is required for proper AER maintenance, suggesting that this might be one such mediator of senescence instruction from the pERK-positive mesenchyme (Kanegae et al., 1998). However, our data also suggest that the AER may be subject to autocrine or local-paracrine influence on the senescent state within the AER, involving CEBP β , transforming growth factor β (TGF- β), and p15 pathways (Acosta et al., 2013; Gomis et al., 2006; Hannon and Beach, 1994).

In summary, this study and the accompanying manuscript from Serrano and colleagues (Muñoz-Espín et al., 2013) describe the identification of cellular senescence as a cellular process and an additional layer of regulation in the developing embryo. We

focused on two main areas where senescence is detected, the AER and the RP. Interestingly, these are among the most frequently disrupted areas affected by developmental birth defects, implying that an investigation of the regulatory mechanisms of developmental senescence might shed new light on the causes of developmental abnormalities. Additionally, this discovery helps to reconcile known and paradoxical functions of senescent cells in the adult with their biological role in the embryo, firmly establishing the importance of cellular senescence as a distinct regulatory and instructive cellular mechanism.

EXPERIMENTAL PROCEDURES

Animal Use and Genotyping

Mice were housed in accordance with the CEEA (Ethical Committee for Animal Experimentation) of the Government of Catalonia. The details and genetic models used are described in the [Extended Experimental Procedures](#). Fertilized hen's eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for specified times, and embryos were staged according to [Hamburger and Hamilton \(1992\)](#).

Histological Analysis, Immunohistochemistry, and Immunofluorescence

Embryos were fixed in 4% PFA for 3 hr at room temperature and washed in PBS, and tissues were processed for paraffin embedding and sectioning. Embryos for phospho-ERK (p44/42) immunostaining were fixed in EFA solution overnight at 4°C. Immunostaining was performed using standard procedures with a complete list of antibodies included in the [Extended Experimental Procedures](#). Whole-mount SA β -gal was detected as previously described (Keyes et al., 2005). Incubation with X-gal was restricted to 3 hr unless otherwise stated.

Culture and Infection of Human Lung IMR90 Fibroblasts

IMR90 fibroblast cultures were established and infected with oncogenic-Ras as per standard protocols. p21 deficiency was achieved through infection of pooled siRNA constructs against p21 (Thermo Scientific). Sequences are provided in [Table S3](#).

Microarray Analysis

Individual AER and limb ectoderm was microdissected from E11.5 forelimbs. Samples from 2–3 mice were pooled for each replicate, for 3–4 replicates. Total RNA was isolated using an RNA-easy plus micro RNA extraction kit (QIAGEN). RNA was subsequently labeled and hybridized to Agilent 8x60, 1-color gene expression arrays, with a detailed description of the analysis and statistical methods in the [Extended Experimental Procedures](#).

RT-qPCR and Real-Time qPCR Analysis

Total RNA from cells and embryonic tissues was purified using an RNA-easy micro RNA extraction kit (QIAGEN). Equal amounts of RNA were reverse-transcribed using random hexamer priming and SuperScript III (Invitrogen). Real time qPCR was performed using gene-specific primers (sequences in [Table S3](#)) and a LightCycler 480 (Roche).

ACCESSION NUMBERS

Data have been deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) with accession number GSE51877.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.10.041>.

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REFERENCES

- Acosta, J.C., O’Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., et al. (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133**, 1006–1018.
- Acosta, J.C., Banito, A., Wuestefeld, T., Georgilis, A., Janich, P., Morton, J.P., Athineos, D., Kang, T.W., Lasitschka, F., Andruelis, M., et al. (2013). A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat. Cell Biol.* **15**, 978–990.
- Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J.C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA* **93**, 13742–13747.
- Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232–236.
- Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dörken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436**, 660–665.
- Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**, 552–557.
- Capdevila, J., and Izpisua Belmonte, J.C. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Dev. Biol.* **17**, 87–132.
- Chang, B.D., Watanabe, K., Broude, E.V., Fang, J., Poole, J.C., Kalinichenko, T.V., and Roninson, I.B. (2000). Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc. Natl. Acad. Sci. USA* **97**, 4291–4296.
- Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725–730.
- Chizhikov, V.V., and Millen, K.J. (2004). Mechanisms of roof plate formation in the vertebrate CNS. *Nat. Rev. Neurosci.* **5**, 808–812.
- Cobrinik, D., Lee, M.H., Hannon, G., Mulligan, G., Bronson, R.T., Dyson, N., Harlow, E., Beach, D., Weinberg, R.A., and Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.* **10**, 1633–1644.
- Cohn, M.J., Izpisua-Belmonte, J.C., Abud, H., Heath, J.K., and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739–746.
- Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M., et al. (2005). Tumour biology: senescence in premalignant tumours. *Nature* **436**, 642.
- Coppé, J.P., Kauser, K., Campisi, J., and Beauséjour, C.M. (2006). Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J. Biol. Chem.* **281**, 29568–29574.
- Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* **6**, 2853–2868.
- Coppé, J.P., Desprez, P.Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu. Rev. Pathol.* **5**, 99–118.
- Corson, L.B., Yamanaka, Y., Lai, K.M., and Rossant, J. (2003). Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development* **130**, 4527–4537.
- Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**, 675–684.
- Devgan, V., Nguyen, B.C., Oh, H., and Dotto, G.P. (2006). p21WAF1/Cip1 suppresses keratinocyte differentiation independently of the cell cycle through transcriptional up-regulation of the IGF-I gene. *J. Biol. Chem.* **281**, 30463–30470.
- Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 9363–9367.
- Freund, A., Orjalo, A.V., Desprez, P.Y., and Campisi, J. (2010). Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol. Med.* **16**, 238–246.
- Gammill, L.S., and Bronner-Fraser, M. (2003). Neural crest specification: migrating into genomics. *Nat. Rev. Neurosci.* **4**, 795–805.
- Gomis, R.R., Alarcón, C., Nadal, C., Van Poznak, C., and Massagué, J. (2006). C/EBPbeta at the core of the TGFbeta cytosstatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* **10**, 203–214.
- Guo, Q., Loomis, C., and Joyner, A.L. (2003). Fate map of mouse ventral limb ectoderm and the apical ectodermal ridge. *Dev. Biol.* **264**, 166–178.
- Hamburger, V., and Hamilton, H.L. (1992). A series of normal stages in the development of the chick embryo. 1951. *Dev. Dyn.* **195**, 231–272.
- Hannon, G.J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**, 257–261.
- Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636.
- Jun, J.I., and Lau, L.F. (2010). The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat. Cell Biol.* **12**, 676–685.
- Kanegae, Y., Tavares, A.T., Izpisua Belmonte, J.C., and Verma, I.M. (1998). Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb. *Nature* **392**, 611–614.
- Kang, T.W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., et al. (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479**, 547–551.
- Keyes, W.M., Wu, Y., Vogel, H., Guo, X., Lowe, S.W., and Mills, A.A. (2005). p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev.* **19**, 1986–1999.
- Kim, S.H., Rowe, J., Fujii, H., Jones, R., Schmierer, B., Kong, B.W., Kuchler, K., Foster, D., Ish-Horowitz, D., and Peters, G. (2006). Upregulation of chicken p15INK4b at senescence and in the developing brain. *J. Cell Sci.* **119**, 2435–2443.
- Krizhanovsky, V., Yon, M., Dickens, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. *Cell* **134**, 657–667.

- Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc. Natl. Acad. Sci. USA* *98*, 12072–12077.
- Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* *133*, 1019–1031.
- Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. *Genes Dev.* *24*, 2463–2479.
- Lang, R.A., and Bishop, J.M. (1993). Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell* *74*, 453–462.
- Lee, K.J., Dietrich, P., and Jessell, T.M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* *403*, 734–740.
- Mariani, F.V., Ahn, C.P., and Martin, G.R. (2008). Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* *453*, 401–405.
- Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* *436*, 720–724.
- Mooi, W.J., and Peeper, D.S. (2006). Oncogene-induced cell senescence—halting on the road to cancer. *N. Engl. J. Med.* *355*, 1037–1046.
- Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S., Rodríguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M., et al. (2013). Programmed cell senescence during mammalian embryonic development. *Cell*. Published online November 14, 2013. <http://dx.doi.org/10.1016/j.cell.2013.10.019>.
- Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* *113*, 703–716.
- Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G.R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* *75*, 579–587.
- Parker, S.B., Eichele, G., Zhang, P., Rawls, A., Sands, A.T., Bradley, A., Olson, E.N., Harper, J.W., and Elledge, S.J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science* *267*, 1024–1027.
- Parrinello, S., Coppe, J.P., Krtolica, A., and Campisi, J. (2005). Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J. Cell Sci.* *118*, 485–496.
- Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* *141*, 39–51.
- Rae, F., Woods, K., Sasmono, T., Campanale, N., Taylor, D., Ovchinnikov, D.A., Grimmond, S.M., Hume, D.A., Ricardo, S.D., and Little, M.H. (2007). Characterisation and trophic functions of murine embryonic macrophages based upon the use of a Csf1r-EGFP transgene reporter. *Dev. Biol.* *308*, 232–246.
- Roninson, I.B. (2002). Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Lett.* *179*, 1–14.
- Schmitt, C.A., Fridman, J.S., Yang, M., Lee, S., Baranov, E., Hoffman, R.M., and Lowe, S.W. (2002). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* *109*, 335–346.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* *88*, 593–602.
- Shukla, V., Coumoul, X., Wang, R.H., Kim, H.S., and Deng, C.X. (2007). RNA interference and inhibition of MEK-ERK signaling prevent abnormal skeletal phenotypes in a mouse model of craniosynostosis. *Nat. Genet.* *39*, 1145–1150.
- Sun, X., Mariani, F.V., and Martin, G.R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* *418*, 501–508.
- Vasey, D.B., Wolf, C.R., Brown, K., and Whitelaw, C.B. (2011). Spatial p21 expression profile in the mid-term mouse embryo. *Transgenic Res.* *20*, 23–28.
- Wang, E. (1995). Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res.* *55*, 2284–2292.
- Wood, W., Turmaine, M., Weber, R., Camp, V., Maki, R.A., McKercher, S.R., and Martin, P. (2000). Mesenchymal cells engulf and clear apoptotic footplate cells in macrophageless PU.1 null mouse embryos. *Development* *127*, 5245–5252.
- Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* *366*, 701–704.
- Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovskiy, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* *445*, 656–660.
- Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M., and Mak, T.W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* *94*, 739–750.
- Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W., and Elledge, S.J. (1999). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev.* *13*, 213–224.