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Detection of Oncogene-Induced Senescence *In Vivo*

Kwan-Hyuck Baek¹ and Sandra Ryeom^{2,*}

¹Department of Molecular and Cellular Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi 440-746, Republic of Korea.

²Department of Cancer Biology, Abramson Family Cancer Research Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Summary

Oncogene-induced senescence or OIS is defined as a permanent state of proliferative arrest resulting from an activating oncogenic-lesion. OIS has been suggested to function as a cancer cell intrinsic mechanism to restrain tumor growth and has been implicated as a key mechanism preventing the progression of certain pre-malignant lesions in genetically engineered mouse models of cancer. The senescent phenotype can be defined by two criteria that include cell cycle arrest and resistance to mitogens and oncogenic transformation. While the phenotype and properties of senescent cells *in vitro* are well described, the morphological characteristics defining senescence *in vivo* have been controversial with no specific marker that definitively proves a senescent state. Indeed many of the published *in vivo* markers to identify and characterize senescence in an organism are unreliable and often times have been found to be non-specific. However, the use of multiple markers is accepted as confirmation of senescence *in vivo*. Here we describe protocols for some of the most commonly used indicators of senescence in oncogenic *Kras*-induced lung adenomas including the detection of senescence-associated beta-galactosidase, expression of the tumor suppressor p19^{ARF}, the presence of senescence-associated heterochromatic foci and *in vivo* BrdU uptake to confirm cell cycle arrest.

Keywords

Senescence; Oncogene-induced senescence; SA-beta galactosidase; Senescence-associated heterochromatin foci; p19^{ARF}; BrdU; Adenomas; Ras

1. Introduction

The term cellular senescence was first used by Hayflick to describe the limited replicative ability of primary cells in culture (1–2). The term now refers to the permanent growth arrest of viable and metabolically active cells and happens *in vivo* in response to various cellular stresses including telomere attrition, tumor suppressor activation, DNA damaging agents and oncogene activation among others (3–6). Telomere shortening triggers replicative senescence occurs when the Hayflick limit is reached in cultured cells. As cells continuously propagate, their telomeres are progressively shortened until they reach a critical minimal

*Corresponding Author, sryeom@upenn.edu.

length upon which they undergo stable proliferative arrest (7). Stable expression of the tumor suppressor p19^{ARF}-p53 or the p16^{INK4A}-retinoblastoma protein (RB) pathways has also been shown to induce senescence (8–9). Early studies found that expression of oncogenic HRAS (*Hras*^{v12}) alone into primary cells induced cell cycle arrest phenotypically very similar to replicative senescence (10). This concept has since become known as oncogene-induced senescence (OIS). However, unlike replicative senescence, OIS is independent of telomere shortening (11). Cells undergoing OIS require involvement of the p19^{ARF}-p53 and p16^{INK4A}-RB tumor suppressor pathways as functional inactivation of these tumor suppressor pathways permits cells to bypass oncogenic Ras-induced senescence (8–10). OIS has now been detected in pre-malignant lesions of a number of different genetically engineered mouse models of cancer driven by oncogenic RAS or its downstream effector RAF. These diverse tumors types include melanomas, papillomas, lung adenomas, mammary tumors and pancreatic tumors to cite a few examples (12–18).

While a number of studies support the notion that OIS is a tumor suppressive mechanism preventing tumor growth and progression to malignancy (18–19), a major obstacle in the senescence field is that the markers used to identify and characterize senescence *in vivo* are unreliable and non-specific. The challenges of detecting OIS *in vivo* are due in part to the heterogeneity of the senescence response in different tissues, the expression of senescence markers in non-senescent cells and reagents for senescence markers that have limited effectiveness in mouse tissues among other factors (3). Here we demonstrate detection of senescence in an oncogenic *Kras*-induced genetically engineered mouse model of lung cancer (14–15) through the detection of SA- β galactosidase (SA- β gal), p19^{ARF} expression, the presence of senescence-associated heterochromatin foci (SAHF) and the lack of BrdU uptake.

2. Materials

2.1. Induction of oncogenic *Kras*-induced senescence in a mouse model of lung cancer.

1. Animal: 6- to 8 weeks old *LSL-Kras*^{G12D} mice on a C57BL/6 background (15).
2. Recombinant adenovirus encoding Cre recombinase (Ad-Cre). (*Note:* The viral stock should be stored at –80°C until use for infection of mice.)
3. OPTI-MEM (Gibco BRL, catalog number: 31985).
4. 2M calcium chloride (CaCl₂).
5. Isoflurane and isoflurane vaporizer.
6. 2.5% avertin (2,2,2-tribromoethy alcohol). (*Note:* Prepare a stock of 100% avertin by dissolving 10g of 2,2,2-tribromoethy alcohol (Sigma, #T48402) with tert-amyl alcohol (Sigma, #152463) in a 50°C water bath and then, to make a working solution, dilute the stock solution to 2.5%, v/v, in phosphate buffered saline (PBS, pH 7.4) with vigorous stirring until it is completely dissolved followed by filtration through a 0.2 μ m filter. Both stock and working solutions should be stored in the dark at 4°C until use. The working solution is stable up to 6 months at 4°C.)

7. Protein gel loading tips.
8. Straight and curved operating scissors and forceps.
9. 1 and 3 ml syringes.
10. 26 gauge needles and 20 gauge blunt-ended needles.
11. 4-0 silk string suture.
12. OCT compounds and cryomolds (Tissue-Tek, #4583 and 4557).
13. 2-methylbutane ($\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)_2$) and liquid nitrogen (LN_2).
14. 10% neutral buffered formalin solution (NBF) and 70% ethanol.

2.2. Detection of senescence-associated beta-galactosidase (SA- β -Gal) activity in lung adenomas

1. Superfrost glass slides.
2. 37% formaldehyde and 50% glutaraldehyde solutions
3. Phosphate buffered saline (PBS, pH 7.4).
4. 0.2 μm filtered ultrapure water.
5. 400 mM Potassium Ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) solution dissolved in distilled water. (*Note:* Store the stock solution at -20°C until use.)
6. 400 mM Potassium Ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) solution dissolved in distilled water. (*Note:* Store the stock solution at -20°C until use. Potassium Ferricyanide and Potassium Ferrocyanide in the staining solution are irritants. Therefore wear nitrile or latex gloves, safety glasses and protective clothing when handling the solutions and discard in an appropriate container.)
7. 40 mg/ml 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) (Sigma, #B4252) solution dissolved in N,N-dimethylformamide (DMF) (Sigma, #33120). (*Note:* The stock solution should be stored in the dark at -20°C until use.)
8. 5M sodium chloride (NaCl) solution dissolved in distilled water.
9. 1M magnesium chloride (MgCl_2) solution dissolved in distilled water.
10. 100 mM citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) (pH 6.0) solution in distilled water. (*Note:* Citric acid may cause erosion of teeth and local irritation of mucosal membrane. Wear nitrile or latex gloves, safety glasses and protective clothing when handling the solutions and prepare the solutions in a fume hood.)
11. 200 mM sodium phosphate dibasic (Na_2HPO_4) solution dissolved in distilled water.
12. 200 mM citric acid/sodium phosphate buffer solution. (*Note:* Prepare the solution by mixing 36.85 ml of 100 mM citric acid solution with 200 mM sodium phosphate dibasic solution. The pH of the buffer solution should be at 6.0 as the enzymatic activity of SA- β -galactosidase is sensitive to pH.)

13. Coplin jars and a humidified 37°C incubator.
14. Hematoxylin, eosin, 95 and 100% ethanol, xylene.
15. Coverslips and mounting solution.

2.3. Detection of p19^{ARF} expression, a tumor suppressor upstream of p53, in lung adenomas.

1. Superfrost glass slides.
2. Ice-cold acetone.
3. PBS (pH7.4).
4. Triton X-100.
5. Normal goat serum.
6. Bovine serum albumin (BSA).
7. Thimerosal. (*Note:* Thimerosal is an anti-bacterial and antifungal compound used as preservative in research.)
8. Rat monoclonal anti-mouse p19^{ARF} antibody (Novus biologicals, #NB200–174).
9. Goat anti-rat IgG Alexa 594 secondary antibody (Invitrogen, #A11007).
10. 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) solution (ThermoFisher Scientific, #62248).
11. Coverslips and anti-fading mounting media.
12. PAP pen, Coplin jars and a humidified staining tray.

2.4. Detection of senescence-associated heterochromatin foci (SAHF) in lung adenomas

1. Superfrost glass slides.
2. Mouse on Mouse (M.O.MTM) fluorescein kit (Vector Labs, #FMK-2201).
3. PBS (pH 7.4).
4. Triton X-100.
5. Normal goat serum.
6. Mouse monoclonal anti-heterochromatin protein-1 gamma (HP-1 γ) antibody (Millipore, #MAB3450)
7. Goat anti-mouse IgG Alexa 594 secondary antibody (Invitrogen, #A11032).
8. 1 mg/ml DAPI solution.
9. Coverslips and anti-fading mounting media.
10. PAP pen, Coplin jars and a humidified staining tray.

3.5. Detection of bromodeoxyuridine (BrdU) uptake in lung adenomas

1. 1 ml syringes.
2. 26 gauge needles.
3. Superfrost glass slides.
4. 5-bromo-2'-deoxyuridine (BrdU) (Sigma, #B9285).
5. PBS (pH 7.4).
6. Acetone.
7. 30% hydrogen peroxide (H₂O₂) solution (Sigma, #H3410).
8. 10 mM sodium citrate buffer (pH 6.0).
9. BrdU *In-Situ* detection kit (BD Pharmingen, #550803).
10. Tween 20.
11. Distilled water, xylene and 100% ethanol.
12. PAP pen, Coplin jars, a humidified staining tray, a slide rack and a pressure cooker.
13. Coverslips and mounting solution.

3. Methods

3.1. Induction of oncogenic *Kras*-induced senescence in a mouse model of lung cancer

1. Thaw the Ad-Cre on ice and prepare calcium phosphate precipitates containing the viruses (Ad-Cre:CaPi) by mixing 2.5 μ l of 5×10^6 PFU Ad-Cre with 121.9 μ l of OPTI-MEM followed by addition of 0.6 μ l of 2M CaCl₂ to the mixture. Mix the solution well and allow the viral precipitates to be formed by incubating the mixture at room temperature for 20 minutes prior to use. (*Note:* High titer Ad-Cre (10^{10} PFU/ml) are commercially available from The University of Iowa Viral Vector Core Facility. Aliquot the Ad-Cre stock into appropriate amounts upon arrival and store the aliquots at -80°C . Avoid repeated freezing and thawing of Ad-Cre stock until use as the viral titer can drop as much as ten-fold with each freeze-thaw cycle.)
2. Anesthetize 6- to 8-weeks old *LSL-Kras^{G12D}* mice on a C57Bl/6 background (17) using isoflurane vaporizer. (*Note:* Alternatively the mice can be anesthetized by injecting 10 μ l of 2.5% avertin per gram mouse body weight intraperitoneally.)
3. Take 62.5 μ l of Ad-Cre:CaPi co-precipitates using a protein gel loading tip and administer the viral precipitates intranasally by slowly ejecting the mixture in a dropwise manner from the tip over the opening of one nostril. Ensure that the mouse inhales the viral drop completely and keep administering the viral precipitates until the entire volume of the mixture has been inhaled. (*Note:* Confirm that the mice are fully anesthetized by checking their response to pain

after pinching their toes with the forceps prior to administer the viral solution. If the mice are not fully anesthetized it will be difficult to instill the exact amount of the viral precipitates into the mice since they will spit out the viral solution.)

4. Allow the mice to fully recover for 10 minutes. They will cough for a while after inhaling the viral precipitates.
5. Once their breathing has returned to normal, repeat the intranasal administration of the remaining 62.5 μ l of viral mixture. (*Note:* Ad-Cre:CaPi co-precipitates should be used in 1 hour after preparation. If there are many mice to be infected, it is recommended to prepare the precipitates sequentially. The whole process of intranasal instillation of Ad-Cre:CaPi co-precipitates should be performed in the biosafety hood.)
6. *LSL-Kras^{G12D}* mice on a C56BL/6 background will develop lung adenomas within 20 weeks after induction of *Kras^{G12D}* expression in the lungs by intranasal delivery of Ad-Cre (Fig. 1).
7. At 20 weeks after intranasal instillation of Ad-Cre, anesthetize the mice with an intraperitoneal injection of 2.5% avertin.
8. Open the abdominal cavity by making a lateral incision just below the diaphragm and open the thoracic cavity by carefully cutting the rib cage to avoid cutting the lungs or any vessels.
9. Expose trachea by removing the rest of rib cage, heart, thymus and other tissues.
10. Separate the trachea from the esophagus using sharp-ended forceps, make a small hole on trachea, insert a blunt-ended 20 gauge needle into the trachea and secure the needle with a suture.
11. Inflate the lungs with a 1:1 mixture of PBS and OCT compound slowly through the needle. (*Note:* 2 to 3 ml of the mixture is enough to inflate one lung. For the preparation of paraffin embedded lung tissue sections, inflate the lungs with 10% neutral buffered formalin (NBF), tie off the trachea beyond the end of the needle with suture string to prevent the solution from escaping and fix the lungs in 10% NBF at 4°C for at least 24 hours followed by transfer to 70% ethanol.)
12. Remove the lungs and separate it into individual lobes. Lung tissues are ready to be processed for frozen sections.

3.2. Detection of senescence-associated beta-galactosidase (SA- β -Gal) activity in lung adenomas

1. Immediately after dissection, embed lung tissues in OCT compound in Tissue-Tek cryomolds and flash freeze the tissue blocks on 2-methylbutane in a metal tray immersed in LN₂. (*Note:* Placing the tissue blocks directly in LN₂ may cause cracks in the specimens. In addition, repeated freezing and thawing of frozen tissue blocks can destroy the enzyme activity of SA- β -Gal. When handling LN₂, wear cryo-gloves and goggles since skin or eye contact with liquid nitrogen results in severe cryogenic burns.)

2. Prepare 4- to 8- μ m-thick frozen lung tissue sections mounted onto superfrost glass slides. (*Note:* It is critical that the tissues are frozen and stained for SA- β -Gal activity immediately after isolation of tissues since the enzyme activity is not stable after freezing and thus can be diminished even after overnight storage of tissue samples at -80°C .)
3. Air dry the tissue sections at room temperature for 30 minutes to 1 hour.
4. While the sections are drying, prepare fixation solution containing 2% formaldehyde and 0.2% glutaraldehyde diluted in PBS (pH 7.4). (*Note:* It is recommended to use 0.2 μ m filtered ultrapure water to prepare the working solutions for staining. Formaldehyde and glutaraldehyde are toxic solutions and produce corrosive vapors. Therefore wear nitrile or latex gloves, safety glasses and protective clothing when handling the solutions and prepare the fixation solutions in a fume hood. Fixation solution can be stored at -20°C after preparation until use.)
5. Fix the tissue sections by immersing the slides in fixation solution in a Coplin staining jar for 6 to 7 minutes at room temperature.
6. Wash the slides by immersing in a large volume of PBS for 5 minutes at room temperature. Repeat two times for a total of three washes to completely remove any residual fixative.
7. During the wash step, prepare SA- β -Gal staining solution containing 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$, 150 mM NaCl, 2 mM MgCl_2 and 1 mg/ml X-gal in 40 mM citric acid/sodium phosphate buffer (pH 6.0). (*Note:* Before preparing the staining solution, incubate the X-gal stock solution at 37°C for 1 hour to avoid formation of aggregates which may interfere with the observation of SA- β -Gal-positive regions after staining. In addition, filter the staining solution using a 0.2 μ m filter to ensure that there are no aggregates in the solution.)
8. Immerse the slides in the staining solution in a Coplin staining jar with screw cap and wrap the jar with aluminum foil to protect from direct light. (*Note:* Close the jar tightly since the evaporation of staining solution may cause the formation of salt crystals that also interfere with the visualization of the stained regions. If salt crystals are formed, they can be removed with a brief incubation of stained tissue sections in dimethyl sulfoxide (DMSO).)
9. Incubate the slides at 37°C for 2 hours to overnight until senescent regions in tissues turn blue. (*Note:* Do not incubate the slide in CO_2 incubator since the staining of senescent cells is pH-sensitive and CO_2 can lower the pH of the staining solution under 6.0 through interchange between CO_2 and bicarbonate ion (HCO_3^-)).
10. Wash the tissue sections once with PBS and counterstain them with hematoxylin and eosin (H&E).

11. Mount the tissue sections and view the blue-stained senescent regions by bright-field microscopy. An example of SA- β -Gal staining in mouse lung adenomas is shown in Fig. 2A. (*Note:* Certain cell such as macrophages expresses β -galactosidase and can be also stained as positive.)

3.3. Detection of p19^{ARF} expression, a tumor suppressor upstream of p53, in lung adenomas

1. Prepare 8- μ m-thick frozen lung tissue sections as described above. (*Note:* It is recommended to store unstained frozen tissue sections at -80°C as thawing may affect the quality and staining of the sections.)
2. Air dry the tissue sections at room temperature for 30 minutes and fix the sections in ice-cold acetone in a Coplin jar at -20°C for 10 minutes. (*Note:* The frozen tissue sections can be also fixed with 4% formaldehyde in PBS at room temperature for 10 minutes. In this case, the tissue sections should not be dried out after fixation.)
3. Remove the slides from the jar and allow the acetone to evaporate completely from the sections at room temperature.
4. Encircle the sections with a PAP pen (a hydrophobic slide marker) and rehydrate the tissue sections twice with enough volume of PBS at room temperature for 5 minutes each. (*Note:* From this point on, be careful not to let the tissue sections dry out.)
5. Permeabilize the tissue sections with PBS containing 0.2% Triton X-100 at room temperature for 15 minutes. (*Note:* The necessity of the permeabilization step is dependent on the protein or cell type of interest.)
6. Briefly wash the tissue sections twice with PBS containing 0.5% normal goat serum at room temperature.
7. Block the tissue sections with 10% normal goat serum in PBS containing 0.2% Triton X-100, 0.01% bovine serum albumin (BSA) and 0.01% Thimerosal at room temperature for at least 1 hour.
8. Briefly wash the tissue sections once with PBS containing 0.5% normal goat serum at room temperature.
9. Incubate the tissue sections with an p19^{ARF} mAb diluted at 1:100 in blocking solution in a humidified staining tray at room temperature for 1 hour or at 4°C overnight. (*Note:* The dilution of anti-p19^{ARF} antibody differs depending on specificity, sensitivity, source and purification of the antibody. Therefore the dilution of anti-p19^{ARF} antibody should be optimized through control experiments. The dilution described in this method is using rat anti-mouse p19^{ARF} antibody obtained from Novus biological, clone 5-C3-1).
10. Wash the tissue sections with enough volume of PBS containing 0.5% normal goat serum and 0.2% Triton X-100 at room temperature for 5 minutes. Repeat

four times for a total of five washes to completely remove any unbound primary antibodies.

11. Incubate the tissue sections with anti-rat IgG Alexa 594 secondary antibody diluted at 1:1000 in blocking solution at room temperature for 1 hour.
12. Wash the tissue sections twice with enough volume of PBS containing 0.5% normal goat serum and 0.2% Triton X-100 at room temperature for 5 minutes each.
13. Counterstain the tissue sections with 1 µg/ml DAPI solution diluted in PBS at room temperature for 1 minute to visualize nucleus. (*Note:* As DAPI is a DNA binding compound, it is potentially mutagenic or carcinogenic. When handling DAPI, wear gloves and dispose of it properly.).
14. Briefly wash the tissue sections three times with PBS, mount the slides with anti-fading mounting media and examine under an immunofluorescence microscope. Example of immunofluorescence staining for p19^{ARF} expression in mouse lung adenomas is shown in Fig. 2B.

3.4. Detection of senescence-associated heterochromatin foci (SAHF) in mouse lung adenomas

1. Prepare, air dry and fix frozen lung tissue sections as described above. (*Note:* The whole staining procedure is performed using Mouse on Mouse (M.O.M™) fluorescein kit from Vector Labs as the primary antibody used in this staining is a mouse monoclonal IgG antibody against HP-1γ.)
2. Encircle the sections with PAP pen and rehydrate the tissue sections twice with enough volume of PBS at room temperature for 5 minutes each.
3. Permeabilize the tissue sections with PBS containing 0.2% Triton X-100 at room temperature for 15 minutes and briefly wash the tissue sections twice with PBS containing 0.5% normal goat serum.
4. Block the tissue sections with mouse IgG blocking reagent prepared by adding 90 µl of stock solution to 2.5 ml PBS at room temperature for 1 hour.
5. Wash the tissue sections twice with enough volume of PBS containing 0.5% normal goat serum at room temperature for 5 minutes each.
6. Incubate the tissue sections with M.O.M diluent buffer prepared by adding 600 µl of protein concentrate stock solution to 7.5 ml PBS at room temperature for 5 minutes and further incubate the sections with anti-HP-1γ antibodies diluted at 1:200 in diluent buffer in a humidified staining tray at room temperature for 30 minutes to 1 hour. (*Note:* The dilution described in this protocol is specifically for the mouse monoclonal anti-HP-1γ (clone 2MOD-1G6) antibody obtained from Millipore. Although anti-HP-1γ antibody is used for detecting SAHF in this protocol, the formation of SAHF can be assessed using specific antibodies against other components of SAHF such as histone H2A variant macroH2A,

HP-1 α , HP-1 β , lysine 9-dimethylated histone H3 (H3K9Me2) and lysine 9-trimethylated histone H3 (H3K9Me3).

7. Wash the tissue sections with enough volume of PBS containing 0.5% normal goat serum at room temperature for 5 minutes. Repeat two times for a total of three washes to completely remove any unbound primary antibodies.
8. Incubate the tissue sections with anti-mouse IgG Alexa 594 secondary antibody diluted at 1:1000 in diluent buffer at room temperature for 1 hour.
9. Wash the tissue sections three times with enough volume of PBS containing 0.5% normal goat serum at room temperature for 5 minutes each.
10. Counterstain the tissue sections with 1 μ g/ml DAPI diluted in PBS at room temperature for 1 minute to visualize nucleus, briefly wash the tissue sections three times with PBS, mount the slides with anti-fading mounting media and examine under an immunofluorescence microscope. An example of an immunofluorescence image of SAHF showing punctate DNA-stained dense foci co-localized with HP-1 γ in mouse lung adenomas is shown in Fig. 2C.

3.5. Detection of bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) uptake in lung adenomas

1. Prepare 5 mg/ml BrdU solution dissolved in PBS and inject 200 μ l of the solution intraperitoneally into mice bearing lung tumors. (*Note:* Alternatively, *in vivo* BrdU labeling can be achieved by supplying the mice with a drinking water containing 0.8 mg/ml BrdU at least for 9 days prior to sacrifice the mice. The drinking water should be prepared fresh, changed daily and protected from light.)
2. After 24 hours, harvest lung tissues from the mice and prepare 8- μ m-thick frozen lung tissue sections as described above. (*Note:* The BrdU-labeled tissues can be harvested between 2 to 24 hours post BrdU injection depending on the specific tumor type. Control experiments are necessary to optimize the time point for harvesting tissues or tumors after BrdU injection into mice.)
3. Air dry the tissue sections at room temperature for 30 minutes and fix the sections in ice-cold acetone in a Coplin jar at -20°C for 2 to 10 minutes. (*Note:* The frozen tissue sections can be also fixed with 4% formaldehyde in PBS at room temperature for 10 minutes. In this case, the tissue sections should not be dried out after fixation.)
4. Remove the slides from the jar and wash the sections twice with enough volume of PBS at room temperature for 5 minutes each. (*Note:* From this point on, be careful not to let the tissue sections dry out.)
5. Block endogenous peroxidase activity by incubating tissue sections in 3% H_2O_2 diluted in PBS at room temperature for 5 to 10 minutes.
6. Wash the sections three times with PBS at room temperature for 5 minutes each.
7. For heat-induced DNA denaturation, pre-heat the pressure cooker containing 10 mM sodium citrate buffer (pH 6.0), immerse the tissue sections placed in a slide

rack in the retrieval buffer when the temperature reaches 95 to 100°C, close the lid of the cooker tightly and incubate for 10 minutes (*Note*: Denaturation of DNA is critical for successful BrdU staining. This denaturation step can be also performed by incubating the tissue sections in 2M hydrochloric acid (HCl) at room temperature for 30 minutes followed by neutralization with 0.1M sodium borate buffer (pH 8.5) for 2 minutes.)

8. Turn off the cooker, release the pressure, open the cooker and cool down for 20 minutes.
9. Remove the tissue sections from the retrieval buffer and wash the sections three times with PBS at room temperature for 5 minutes each. (*Note*: It is crucial not to let the heated sections dry out.)
10. Encircle the tissue sections with PAP pen and incubate the sections with a biotinylated anti-BrdU antibody diluted at 1:10 in the dilution buffer in a humidified staining tray at room temperature for 1 hour or at 4°C overnight. (*Note*: The staining procedure is performed using BrdU *In-Situ* detection kit from BD Pharmingen.)
11. Wash the sections three times with PBS containing 0.05% Tween 20 at room temperature for 5 minutes each.
12. Incubate the tissue sections with three to four drops of the ready-to-use Streptavidin-HRP solution at room temperature for 30 minutes.
13. Wash the sections three times with PBS containing 0.05% Tween 20 at room temperature for 5 minutes each.
14. Incubate the sections with DAB solution at room temperature for 1 to 10 minutes until the desired color intensity is developed. (*Note*: The DAB solution is prepared by adding 1 drop of DAB chromogen to 1 ml of DAB buffer. Care should be taken in handling and disposal of DAB due its potential as a carcinogen.)
18. Immediately rinse the tissue sections three times in distilled water for 2 minutes each. (*Note*: The tissue sections can be counterstained with hematoxylin to visualize nucleus.)
19. Dehydrate the sections by immersing them sequentially in 100% ethanol three times for 30 seconds each and in xylene three times for 30 seconds each.
20. Mount the tissue sections and view by bright-field microscopy. Example of immunohistochemical staining of BrdU uptake in mouse lung adenomas is shown in Fig. 3 (15).

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Fig. 1. Time line of *Kras*^{G12D}-mediated lung tumor progression in *LSL-Kras*^{G12D} mice on a C57BL/6 background after intranasal instillation of Ad-Cre and representative lung tumor lesions in lungs isolated at the indicated time points followed by visualization with H&E staining. Scale Bar: 50 μm.

Fig. 2. Expression of senescence markers in lung adenomas undergoing oncogenic *Kras*-induced senescence.

Representative images showing lung tumor lesions positive for (A) SA-β-Gal activity, (B) p19^{ARF} and (C) SAHF showing co-localization of punctate DAPI-stained DNA and HP-1γ (arrowheads). Scale Bar: 50 μm.

Fig. 3. Absence of BrdU uptake in lung tumor lesions undergoing oncogenic *Kras*-induced senescence.

Representative images showing the absence of BrdU uptake in senescent lung adenomas as indicated by positivity for SA-β-Gal activity (*upper*) and extensive BrdU uptake in lung tumor lesions bypassing senescence (*lower*). Scale Bar: 50 μm (15).