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Detection of senescence-associated heterochromatin foci (SAHF)

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Summary

Senescence-associated heterochromatin foci (SAHF) are specialized domains of facultative heterochromatin that contribute to silencing of proliferation-promoting genes (such as E2F target genes) in senescent cells. Thus, methods for detection of SAHF are invaluable tools in the field of senescence to identify these distinct changes in heterochromatin that are associated with the senescence-associated cell cycle exit. Here, we describe fluorescent-based methods for the detection of SAHF in senescent cells, including examining reorganization of DNA structure by 4′, 6-diamidino-2-phenylindole (DAPI) staining and using specific antibodies to components of SAHF such as the histone H2A variant macroH2A, heterochromatin protein 1 (HP1) proteins and lysine 9 di-or tri-methylated histone H3 (H3K9Me2/3). Together, these markers identify SAHF, the distinct facultative heterochromatin structures that are associated with senescent cells.

Keywords

cellular senescence; senescence-associated heterochromatin foci; immunofluorescence staining; histone variant macroH2A; H3K9Me2/3; HP1 proteins; high-mobility group A proteins

1. Introduction

Cellular senescence is a state of irreversible cell growth arrest. Cellular senescence can be triggered by a variety of stimuli such as critically shortened telomeres, which occurs after extensive cell division, or activation of certain oncogenes (such as H-RAS^{G12V} or BRAF^{V600E}) (1–3). By definition, senescent cells are irreversibly growth arrested, and one of the necessary steps towards this irreversible cell cycle exit is the suppression of E2F target genes (4), which are mainly involved in promoting cell proliferation and S phase cell cycle progression (5). Promoters of E2F target genes typically acquire heterochromatic features during senescence (4). The heterochromatin associated with this process is specialized domains of facultative heterochromatin that often form in senescent human cells, senescence-associated heterochromatin foci (SAHF) (4, 6–12).

SAHF were first described in 2003 by Narita et al. who observed that the nuclei of senescent cells contain 30–50 bright, punctate DNA-stained dense foci that can be readily distinguished from chromatin in normal cells (4). Importantly, SAHF are not associated with cells undergoing quiescence, indicating that SAHF formation is not associated with reversible cell cycle exit (4). In addition, SAHF have also been shown to be distinct from constitutive heterochromatin because centromeres, telomeres, and other constitutive heterochromatin regions are not included in SAHF (4, 7, 13). Further, SAHF are also different from other facultative heterochromatin such as inactivated X chromosomes (Xi) in

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female human cells. For example, histone modifications such as lysine 27 trimethylated histone H3 (H3K27Me3) are associated with Xi but not SAHF (4).

SAHF play a role in sequestering proliferation-promoting genes (4), including E2F target genes such as cyclin A (7), which is required for the progression through S-phase of the cell cycle (14). Indeed, SAHF do not contain any active transcription sites (4), demonstrating their role in contributing to the senescence-associated cell cycle exit. Significantly, disruption of SAHF formation can cause cell transformation (15), which infers that SAHF contribute to the tumor suppressive function of senescence. Recently, there is evidence to suggest that SAHF may limit the extent of DNA damaging signaling which may prevent senescent cells from undergoing apoptosis induced by high DNA damage signaling, thereby maintaining the viability of senescent cells (12). Finally, there is emerging evidence to suggest that SAHF may play a role in the senescence phenotype *in vivo* (16–19).

A number of different inducers of senescence cause the formation of SAHF, including activated oncogenes such as H-RAS^{G12V} and BRAF^{V600E} (4, 20, 21), extensive passaging (4), chemotherapeutics such as etoposide (4) and hydroxyurea (10), and bacterial toxins (10). However, SAHF formation and senescence are not always coupled. Indeed, a number of studies have shown that senescence can occur in the absence of SAHF formation. For instance, activation of AKT and knockdown of PTEN do not cause SAHF formation (22, 23). It is also important to note that SAHF formation is cell-line dependent (10). For example, senescence induced by extensive passaging in the primary human embryonic fibroblasts cell lines IMR90 and WI38 cells is associated with SAHF, while senescence triggered by extensive passaging in BJ cells (primary human foreskin fibroblasts) is not associated with SAHF formation (4). The difference between these cell lines correlates with a variation in activation of the p16/pRb pathway after extensive passaging (10). Indeed, senescence induced by activated oncogenes (such as H-RAS^{G12V} and BRAF^{V600E}) in BJ cells triggers SAHF formation, which is associated with activation of the p16/pRb pathway (24, 25) Notably, mouse cells do not form robust SAHF, although they do display a marked increase in staining of certain components of SAHF such as macroH2A (26).

To date, a number of molecular markers of SAHF have been described [reviewed in (6, 11, 27)] including: macroH2A (9), a histone variant known to contribute to X chromosome inactivation and gene silencing (28); high mobility group A (HMGA) proteins, which coordinate with p16^{INK4a} to induce SAHF formation and are required for maintaining SAHF (15); and di- or tri-methylated lysine 9 histone H3 (H3K9Me2/3) and bound HP1 proteins (4, 7), two common markers of heterochromatin (29). Together with DAPI, co-staining for these markers is a simple and reliable method to determine the presence of SAHF in senescent cells. Here, using oncogenic-RAS (H-RAS^{G12V}) as an inducer of senescence and SAHF, we describe a method for the immunofluorescent detection of SAHF using DAPI and specific antibodies to components of SAHF such as macroH2A, H3K9Me2/3, and HP1 proteins.

2. Materials

2.1. Cell culture for expression of oncogenic RAS

1. pBABE-puro and pBABE-puro-H-RAS^{G12V} constructs (Addgene) (*see* Note 1)
2. 2.5 M $CaCl_2$

[1]As discussed in the introduction, other oncogenes (BRAF/MEK/ERK) and other cellular stressors can cause the formation of SAHF. We recommend using H-RAS^{G12V} as a positive control because activation of RAS induces distinct SAHF formation in a variety of cell types.

3. 2X BBS: 50 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95 (*see* Note 2)
4. Laemmli sample buffer [50 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue, pH 6.8]
5. Equipment and reagents for SDS-polyacrylamide gel electrophoresis (PAGE)
6. Bradford reagent (Bio-Rad) and 1 mg/mL bovine serum albumin (BSA, Pierce) as standard
7. PVDF transfer membrane
8. Towbin transfer buffer [170 mM glycine, 22 mM Tris-HCl, and 0.01% (w/v) SDS, pH 8.3]
9. Anti-RAS antibody (BD Transduction Laboratories)
10. 0.45 μm filter
11. Sterile-filtered ddH_2O
12. Phoenix cells (a gift from Gary Nolan) growing in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Clontech), 1% (w/v) penicillin-streptomycin, and 1% (w/v) L-glutamine in a humidified 37°C, 5% (v/v) CO_2 incubator (*see* Note 3).
13. IMR90 cells (ATCC) growing in DMEM supplemented with 20% (v/v) FBS, 1% (w/v) L-glutamine, 1% (v/v) Non-Essential Amino Acids Solution (Cellgro), 2% (v/v) Essential Amino Acids (Cellgro), 1% (v/v) Vitamins (Cellgro), and 1% (w/v) Penicillin-Streptomycin in a humidified 37°C, 5% (v/v) CO_2 incubator (*see* Notes 4–[5])
14. 0.25% (w/v) Trypsin + 1 mM EDTA
15. Sterile-filtered Dulbecco's phosphate-buffered saline (PBS), pH 7.3
16. Sterile-filtered, 1 mg/mL puromycin in PBS, pH 7.3 (Clontech)
17. Sterile-filtered, 8 mg/mL (w/v) Polybrene in ddH_2O (Sigma)
18. 10-cm cell culture dishes and 6-well cell culture plates
19. Clean and sterile glass coverslips

2.2. Fluorescent staining of SAHF

1. 4% (w/v) paraformaldehyde (Sigma) (*see*Note 6).

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[2]Correct pH of the 2X BBS solution is necessary for efficient plasmid transfection using this method.

[3]Never let cells reach confluence as this may decrease transfection efficiency. Passage Phoenix cells in 300 μg/mL hygromycin (Roche) and 1 μg/mL diptheria toxin (Sigma) for 1 week every month to maintain transfection efficiency. For more information, please visit Gary Nolan's website: (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html).

[4]For oncogene-induced SAHF formation (or to study other types of stressors), low population doubling (PD) IMR90 cells should be used (PD<35). However, if you wish to study replicative senescence-induced SAHF, high PD IMR90 cells (e.g., PD65) should be used instead.

[5]Other cell lines and cell types form SAHF (10); however, this must be determined on a cell type by cell type basis. It is important to note that mouse cells do not form distinct SAHF structures that are visible by DAPI staining alone. Instead, macroH2A may server as an marker for SAHF in senescent mouse cells (26).

[6]To make 4% paraformaldehyde, weigh 2 g of paraformaldehyde (Sigma) and add 25 mL of ddH2O, 25 mL 2X PBS, pH 7.3, and 100 μL 1 N NaOH. Vortex and incubate this mixture at 65°C for 10–15 min to dissolve the paraformaldehyde (vortex the tube every 5 min). Cool the solution to room temperature before use.

2. PBS, pH 7.3
3. 0.2% (v/v) and 1% (v/v) Triton-X in PBS, pH 7.3
4. 3% (w/v) bovine serum albumin (Sigma) in PBS, pH7.3 (*see* Note 7)
5. Primary antibodies to macroH2A, H3K9Me2, H3K9Me3, HP-1α, HP-1β, or HP-1γ (*see* Note 8 and Table 1)
6. Appropriate secondary antibodies (*see* Note 9 and Table 2)
7. 5 mg/mL 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) in ddH_2O
8. Clean microscope slides
9. Anti-fade fluorescence mounting media: 0.25 g p-phenylenediamine (Sigma) dissolved in 25 mL 1X PBS (pH 9.0) mixed with 225 mL glycerol (*see* Note 10)

2.3. Microscopic examination of SAHF

1. Fluorescent microscope with the ability to view blue, green, and red channels (e.g., Nikon 80*i*).

3. Methods

3.1. Cell culture for expression of oncogenic RAS

The infectious retrovirus is generated by transfecting the plasmid DNA into a packaging cell line, for example, Phoenix cells (http://www.standford.edu/group/nolan/protocols/pro_helper_dep.html). Transfection-quality DNA of the retrovirus plasmid is made using a Qiafilter Plasmid Maxi kit, according to the manufacturer's instructions. After transfection, the Phoenix cells reverse transcribe the plasmid DNA into an RNA that is packaged into infectious virus and expelled from the cells. Then, the tissue culture supernatant containing the virus is applied to the cells of interest to deliver the activated oncogenes (e.g., H-RAS^{G12V}) to the target cells. The infected cells can be selected in puromycin to enrich for cells that have been infected. To assess the efficiency of puromycin to kill uninfected cells, perform a mock virus infection. To assess the efficiency of infection, infect one plate with a virus known to have a high titer (e.g., pBABE-puro vector, Addgene).

1. Split the Phoenix cells to 5×10^6 cells per 10-cm dish in 10 mL medium 24 h before transfection (*see* Note 11). Culture in a 37°C, 5% (v/v) CO_2 incubator overnight.
2. Twenty-four hours later, and 4 h prior to transfection, change the medium to 9 mL of prewarmed, fresh growth media [DMEM supplemented with 10% FBS, 1% (w/v) penicillin-streptomycin, and 1% (w/v) L-glutamine].
3. Dilute the required amount of 2.5 M $CaCl_2$ to 250 mM in sterile ddH_20 and aliquot 0.5 mL per transfection to separate, sterile 15 mL polystyrene tubes.
4. Add 30 μg of the supercoiled plasmid DNA of the intended virus to each tube.

[7]Adding 0.02% (w/v) sodium azide to the 3% (w/v) BSA in PBS will decrease potential contamination and increase the shelf life.
[8]Any of these markers can be used in addition to DAPI staining to identify SAHF. See Table 1 for antibody catalog numbers, dilutions, and references.
[9]Secondary antibodies to the appropriate species can be purchased from Jackson Immunolabs. See Table 2 for details.
[10]Mounting media should aliquoted and stored at −80°C. Once the media changes to an orange color, replace with a fresh batch.
[11]Although the Phoenix cells are easy to remove from the plate, use 0.25% (w/v) trypsin + 1 mM EDTA to to fully separate the cells and avoid cell aggregation.

5. Add 0.5 mL of 2X BBS by dripping slowly from a 1 mL pipette vertically down the center of the tube (1–2 drops per second). Do not mix. Incubate the mixture for 15 min. At this time, the precipitate should be barely visible to the naked eye.
6. Use a 1 mL pipette to blow air bubbles through the solution to mix the precipitate. Evenly distribute the mixture drop-wise into the medium of the plate of Phoenix cells.
7. Rock the plates back and forth very gently to mix the calcium phosphate precipitate and then place in a humidified 37°C incubator with 5% (v/v) CO_2 overnight.
8. Change the medium to 6 mL fresh, prewarmed growth media approximately 24 h later (*see* Note 12).
9. On the same day as **Step 8**, prepare the target IMR90 primary human embryonic fibroblast cells for infection. A confluent 10 cm dish of cells should be split 1:4 in regular growth media so that cells are 50–60% confluent on the next day (i.e., the first day of infection).
10. Twenty-four hours later, collect the supernatant from the Phoenix cells after **step 8** and put through a 0.45 μm filter. Add 6 mL of fresh, prewarmed growth media to the Phoenix cells (*see* Note 12).
11. Infect the target cells. Aspirate the IMR90 media from the dish. For a 10 cm dish of target cells, add 3 mL fresh media and 3 mL of virus-containing supernatant harvested from the Phoenix cells drop wise onto the IMR90 cells.
12. Add 6 μL 8 mg/mL (w/v) polybrene to make a final concentration of 8 μg/mL, and swirl the dishes to mix. Put the cell dish back into a 5% (v/v) CO_2-containing incubator.
13. Culture the infected cells for 24 h. Repeat the infection (**Steps 10–12**) to increase efficiency.
14. Remove the medium from the IMR90 cells 24 h post-infection and replace with 10 mL of fresh, prewarmed IMR90 growth media that contains puromycin at a final concentration of 1 μg/mL.
15. Typically, 3 d after addition of puromycin, all of the non-infected cells should be dead. At this time, there should be no surviving cells left in the mock virus-infected dish.
16. At the desired time point (*see* Note 13), split the IMR90 cells into 6-well plates containing clean, sterile glass coverslips.
17. At the same time, harvest an aliquot of the infected cells for Western blot analysis (*see* Note 14).

[12]Be very gentle when adding fresh media as Phoenix cells do not adhere very well to the plate. It is typical that Phoenix cells change shape after changing them into fresh medium. They will fully recover after 6–8 h of culture in the newly changed medium.

[13]SAHF should start to appear approximately 3–4 d after starting selection and typically reach the highest percentage with ~50% RAS-infected cells positive for DAPI-stained SAHF around 7–9 d after staring drug selection.

[14]Protein lysates can be made by directly adding 1X Laemmli sample buffer followed by boiling for 5 min. Samples can be stored at −80°C. Ectopically expressed oncogenic-RAS should be confirmed by Western blotting. Cell extracts should be fractionated by SDS-PAGE, transferred to a PVDF membrane, and then Western blotted with antibody to RAS (BD Biosciences, 610001). An antibody to a house-keeping gene (e.g., β-actin) should be used as a loading control.

3.2. Fluorescent staining of SAHF

To visualize SAHF, DAPI is used to stain for DNA, while specific antibodies to components of SAHF (e.g., macroH2A, H3K9Me2/3, and HP1 proteins) should be used for indirect immunofluorescence staining. To assess the specificity of the antibody staining pattern, an isotype matched control primary antibody and/or omission of the primary antibody should be included as negative controls for immunofluorescence staining. In addition, IMR90 cells infected with vector control-encoding retrovirus should be included as a negative control.

1. Gently wash the coverslips 3 times with PBS, pH 7.3 to remove all culture media (*see* Note 15).
2. Fix cells in freshly prepared 4% (w/v) paraformaldehyde for 10 min at room temperature (*see* Note 16).
3. Wash the coverslips 3 times with PBS, pH 7.3 to remove fixing solution (*see* Note 17).
4. Permeabilize the cells by incubating the coverslips in 0.2% (v/v) Triton-X 100 in PBS (pH 7.3) for 5 min at room temperature.
5. Block the coverslips with 3% (w/v) BSA in PBS, pH 7.3 for 5 minutes at room temperature.
6. Incubate the coverslips with the primary antibodies diluted in 3% (w/v) BSA in PBS, pH 7.3 by inverting the coverslip onto 200 μL of diluted antibody spotted on parafilm. Incubate 1–2 h at room temperature.
7. Put the coverslips back into the 6-well plates with the cell side face up. Wash the coverslips 3 times with 1% (v/v) Triton X-100 in PBS, pH 7.3.
8. Dilute the appropriate secondary antibodies (Table 2) in 3% (w/v) BSA in PBS, pH 7.3. Incubate the coverslips with 2 mL secondary antibody solution in each well for 1 h at room temperature in the dark.
9. Stain SAHF by incubating the coverslips with 0.15 μg/mL final concentration of DAPI diluted in 3% (w/v) BSA in PBS, pH 7.3 for 3 min at room temperature (*see* Note 18).
10. Wash the slide 3 times with PBS, pH 7.3.
11. Mount the inverted coverslips (cells facing the microscope slide) into 1 drop of mounting media (approximately 20 μL). Aspirate the excess liquid, and seal the coverslips using nail polish (*see* Note 19).
12. After the slides have dried, observe SAHF using a fluorescent microscope (*see* Notes 20–[21]).

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[15]Senescent cells do not adhere very well to coverslips; therefore, rinsing coverslips with PBS before fixation should be done very carefully. In instances where rinsing coverslips with PBS may lead to loss of cells, this step can be skipped, and coverslips can be directly immersed in 4% (w/v) paraformaldehyde for 10 minutes.

[16]At this step, soluble proteins can be removed to only retain tightly-bound chromatin proteins. To do so, cells can be pre-extracted with 5 mM $MgCl_2$ in 0.2% (v/v) Triton-X in PBS for 5 min to preserve SAHF while removing the soluble proteins. Extra care should be take when performing pre-extraction and the following steps of washing and fixation as these pre-extracted cells can be easily washed off the coverslips.

[17]After fixation, coverslips can be washed 3X with PBS and stored at 4°C for up to 2 weeks before processing.

[18]Higher DAPI concentratins and longer incubation periods will make it harder to distinguish DAPI-stained SAHF.

[19]Avoid air bubbles when mounting coverslips.

[20]Slides can be stored at −20°C for up to a month. However, it is important to note that the fluroescent signal may fade over time.

[21]The same methods can be applied to other cell lines/cell types to determine SAHF formation. As not all cell lines or all causes of cellular senescence induce SAHF formation (see Introduction for details), we recommend the use of IMR90 cells expressing oncogenic H-RAS^{G12V} as a positive control.

3.3. Microscopic examination of SAHF

After immunofluorescence staining, the processed slides can be examined under a fluorescent microscope. DAPI staining should reveal punctate staining in oncogenic H-RAS^{G12V} infected cells, while vector control cells should display diffuse staining across the cell nuclei (Fig. 1). The number of cells with SAHF staining depends upon the duration of RAS expression. For example, ~50% of RAS-infected cells display SAHF by DAPI staining 7–9 d post-drug selection (4, 7). Components of SAHF such as macroH2A, HP1 proteins, and H3K9Me2/3 form foci that co-localize with DAPI-stained SAHF in RAS-infected cells. In vector control cells, HP1 proteins and H3K9Me2/3 staining is largely diffuse. MacroH2A staining marks the single foci in female mammalian cells (such as IMR90 and WI38 primary human fibroblast cells) that is the inactivated X chromosome (Fig. 1). As negative controls, staining using isotype matched IgG controls as the primary antibody or using the secondary antibody only (without the primary antibody) should not show foci that co-localize with DAPI-stained SAHF. The controls herein will obviously be specific to each component of SAHF. Importantly, regardless of the specific components, not all cells with DAPI-stained SAHF-positive cells will display foci for components of SAHF. This is because relocalization of components of SAHF into DAPI stained foci is typically a later event compared with formation of DAPI foci (9).

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References

1. Kuilman T, et al. The essence of senescence. Genes Dev. 2011; 24:2463–2479. [PubMed: 21078816]
2. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell. 2005; 120:513–522. [PubMed: 15734683]
3. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007; 8:729–740. [PubMed: 17667954]
4. Narita M, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell. 2003; 113:703–716. [PubMed: 12809602]
5. Ren B, et al. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev. 2002; 16:245–256. [PubMed: 11799067]
6. Zhang R, Adams PD. Heterochromatin and its relationship to cell senescence and cancer therapy. Cell Cycle. 2007; 6:784–789. [PubMed: 17377503]
7. Zhang R, et al. Molecular dissection of formation of senescence-associated heterochromatin foci. Mol Cell Biol. 2007; 27:2343–2358. [PubMed: 17242207]
8. Zhang R, et al. HP1 proteins are essential for a dynamic nuclear response that rescues the function of perturbed heterochromatin in primary human cells. Mol Cell Biol. 2007; 27:949–962. [PubMed: 17101789]
9. Zhang R, et al. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell. 2005; 8:19–30. [PubMed: 15621527]
10. Kosar M, et al. Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). Cell Cycle. 2011; 10:457–468. [PubMed: 21248468]
11. Adams PD. Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. Gene. 2007; 397:84–93. [PubMed: 17544228]
12. Di Micco R, et al. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol. 2011; 13:292–302. [PubMed: 21336312]

13. Funayama R, et al. Loss of linker histone H1 in cellular senescence. J Cell Biol. 2006; 175:869–880. [PubMed: 17158953]
14. Pagano M, et al. Cyclin A is required at two points in the human cell cycle. Embo J. 1992; 11:961–971. [PubMed: 1312467]
15. Narita M, et al. A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell. 2006; 126:503–514. [PubMed: 16901784]
16. Collado M, et al. Tumour biology: senescence in premalignant tumours. Nature. 2005; 436:642. [PubMed: 16079833]
17. Collado M, Serrano M. Senescence in tumours: evidence from mice and humans. Nat Rev Cancer. 2010; 10:51–57. [PubMed: 20029423]
18. Herbig U, et al. Cellular senescence in aging primates. Science. 2006; 311:1257. [PubMed: 16456035]
19. Kreiling JA, et al. Age-associated increase in heterochromatic marks in murine and primate tissues. Aging Cell. 2011; 10:292–304. [PubMed: 21176091]
20. Martin C, et al. Changed genome heterochromatinization upon prolonged activation of the Raf/ERK signaling pathway. PLoS One. 2010; 5:e13322. [PubMed: 20967285]
21. Michaloglou C, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature. 2005; 436:720–724. [PubMed: 16079850]
22. Kennedy AL, et al. Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis. Mol Cell. 2011; 42:36–49. [PubMed: 21474066]
23. Tu Z, et al. Oncogenic Ras regulates BRIP1 expression to induce dissociation of BRCA1 from chromatin, inhibit DNA repair, and promote senescence. Dev Cell. 2011; 21:1–15. [PubMed: 21763596]
24. Deng Q, et al. High intensity ras signaling induces premature senescence by activating p38 pathway in primary human fibroblasts. J Biol Chem. 2004; 279:1050–1059. [PubMed: 14593117]
25. Ye X, et al. Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Mol Cell Biol. 2007; 27:2452–2465. [PubMed: 17242198]
26. Kennedy AL, et al. Senescent mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. Cell Div. 2010; 5:16. [PubMed: 20569479]
27. Funayama R, Ishikawa F. Cellular senescence and chromatin structure. Chromosoma. 2007; 116:431–440. [PubMed: 17579878]
28. Costanzi C, Pehrson JR. Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. Nature. 1998; 393:599–601. [PubMed: 9634239]
29. Trojer P, Reinberg D. Facultative heterochromatin: is there a distinctive molecular signature? Mol Cell. 2007; 28:1–13. [PubMed: 17936700]

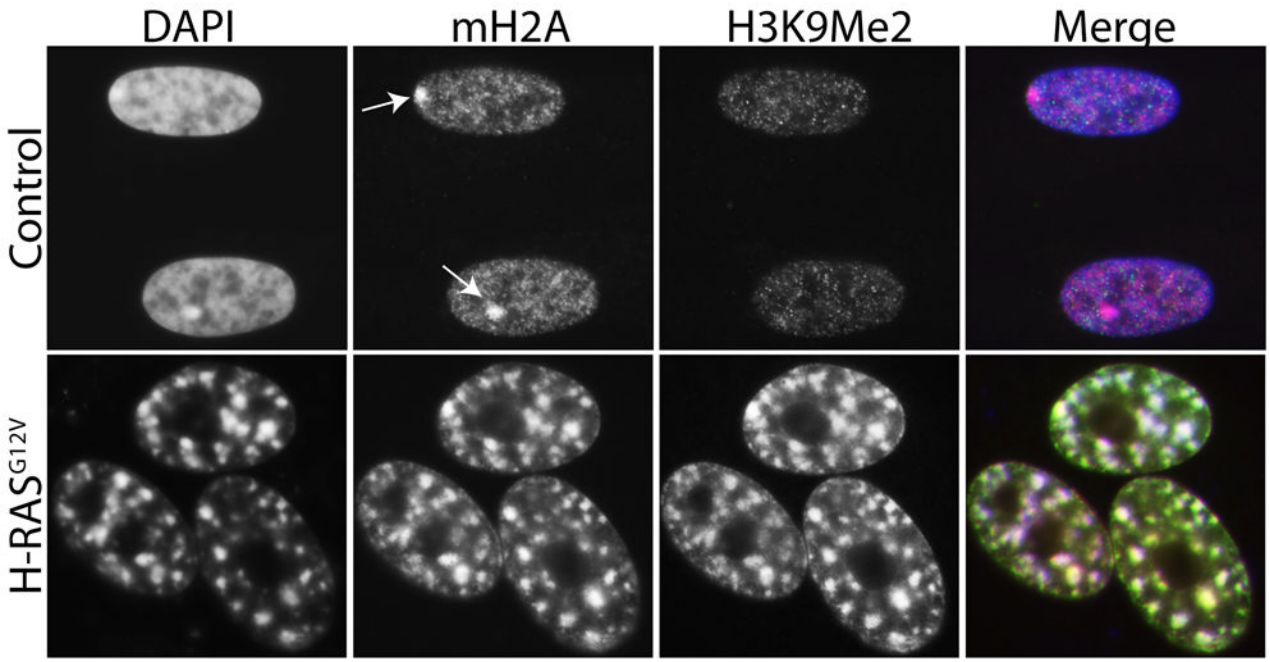

Fig. 1.

IMR90 cells were infected with a puromycin-resistant retrovirus encoding oncogenic H-RAS^{G12V} or vector control. Drug-selected cells were stained with antibodies to H3K9Me2 and histone H2A variant macroH2A. DAPI counterstaining was used to visualize SAHF. Note the robust punctate DAPI foci in the H-RAS^{G12V} infected cells, which co-localize with both macroH2A and H3K9Me2. Arrows point to inactivated X chromosome in control IMR90 female fibroblast cells. Chicken anti-macroH2A1.2 primary antibody was obtained from Dr. John R. Pehrson (University of Pennsylvania) (28). Rabbit anti-H3K9Me3 is from AbCam (ab8898) and was used at 1:500. Secondary antibodies were FITC-labeled goat anti-chicken (1:2500) and Cy3-labeled goat anti-rabbit (1:5000), which were both obtained from Jackson Immunolabs.

Table 1

Primary antibodies that can be used to identify SAHF by immunofluorescence.

Protein	Company or Lab	Dilution	Reference
macroH2A	Drs. Rugang Zhang (Fox Chase Cancer Center) and Peter D. Adams (University of Glasgow)	1:2000	(7)
HP-1α	Dr. William Earnshaw (University of Edinburgh)	1:200	(8)
HP-1β	Millipore (MAB3448)	1:20000	(9)
HP-1γ	Millipore (MAB3450)	1:2000	(7)
H3K9Me3	Abcam (ab8898)	1:500–1:1000	(9)
H3K9Me2	Abcam (ab7312)	1:500–1:1000	(9)

Table 2

Secondary antibodies from Jackson Immunolabs that can be used to fluorescently visualize SAHF components.

Antibody	Catalog Number	Dilution
Cy3 Goat anti-Mouse	115-165-003	1:5000
FITC Goat anti-Rabbit	111-095-003	1:1000