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Formation of MacroH2A-Containing Senescence-Associated Heterochromatin Foci and Senescence Driven by ASF1a and HIRA

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Summary

In senescent cells, specialized domains of transcriptionally silent senescence-associated heterochromatic foci (SAHF), containing heterochromatin proteins such as HP1, are thought to repress expression of proliferation-promoting genes. We have investigated the composition and mode of assembly of SAHF and its contribution to cell cycle exit. SAHF is enriched in a transcription-silencing histone H2A variant, macroH2A. As cells approach senescence, a known chromatin regulator, HIRA, enters PML nuclear bodies, where it transiently colocalizes with HP1 proteins, prior to incorporation of HP1 proteins into SAHF. A physical complex containing HIRA and another chromatin regulator, ASF1a, is rate limiting for formation of SAHF and onset of senescence, and ASF1a is required for formation of SAHF and efficient senescence-associated cell cycle exit. These data indicate that HIRA and ASF1a drive formation of macroH2A-containing SAHF and senescence-associated cell cycle exit, via a pathway that appears to depend on flux of heterochromatic proteins through PML bodies.

Introduction

Most normal human cells undergo a limited number of cell divisions, eventually entering an irreversibly arrested state, through either terminal differentiation or senescence. Both senescence and differentiation are accompanied by reorganization of chromatin structure. This reorganization is well documented in differentiated cells and is thought to directly contribute to the altered cell phenotype (Kosak and Groudine, 2004). Chromatin structure is also extensively remodeled in senescent cells (Howard, 1996; Narita et al., 2003). Specifically, Lowe and coworkers showed that in senescent cells, proliferation-promoting genes are incorporated into transcriptionally silent heterochromatin (senescence-associated heterochromatin foci, SAHF) (Narita et al., 2003). Accordingly, it was proposed that formation of SAHF contributes to exit from the cell cycle in senescent cells. In an attempt to understand the principles that govern such chromatin changes, we have investigated the mechanisms responsible for formation of heterochromatin in senescent cells.

Heterochromatin is condensed and modified, transcriptionally silent chromatin, and inclusion of genes into such chromatin promotes their stable repression (Richards and Elgin, 2002). Constitutive heterochromatin is condensed throughout the cell cycle and is found at repetitive DNA sequences, such as pericentromeres. It is typically characterized by histone hypoacetylation, methylation of lysine[9] of histone H3 (Me-K[9]-H3), and binding of heterochromatin proteins 1 (HP1α, β, γ) (Maison and Almouzni, 2004). Facultative heterochromatin is inducible heterochromatin and is most prominent at the single X chromosome of female cells that is silenced during embryogenesis (X inactivation). Facultative heterochromatin of the inactive X chromosome contains a histone H2A variant, macroH2A, that is actually a family of three related proteins (macroH2A1.1, 1.2, and 2, where 1.1 and 1.2 are splice variants) (see Figure 4B; Costanzi and Pehrson, 1998, 2001). Family members have an N-terminal histone H2A-like domain and a C-terminal "macro domain" of $\sim$200 residues that is unrelated to other histones. MacroH2A-containing chromatin, assembled in vitro, is resistant to ATP-dependent remodeling proteins and binding of transcription factors (Angelov et al., 2003), thus supporting a direct role in gene silencing.

SAHF is facultative heterochromatin, because it is induced in senescent cells. Formation of SAHF depends on the pRB tumor suppressor pathway and is thought to repress expression of proliferation-promoting E2F target genes, such as cyclin A, DHFR, and Mcm3 (Narita et al., 2003). Apart from inclusion of common heterochromatin markers such as Me-K[9]-H3 and HP1 proteins, the molecular constituents of SAHF are poorly characterized. Moreover, we do not know the factors that mediate SAHF formation. Finally, the contribution of SAHF to the cell cycle exit associated with senescence has not been tested.

The yeast Hir1p, Hir2p, and Asf1p proteins, and/or their evolutionarily conserved orthologs, exhibit chromatin assembly activity in vitro and copurify from cell extracts with newly synthesized histones (Krawitz et al., 2002; Ray-Gallet et al., 2002; Sharp et al., 2001; Tagami et al., 2004; Tyler et al., 1999). In addition, they contribute to formation of heterochromatin and silencing of telomeres, pericentromeres, and mating loci in vivo (Kaufman et al., 1998; Le et al., 1997; Moshkin et al., 2002; Sharp et al., 2002; Singer et al., 1998). The three proteins physically associate and this interaction is necessary for telomeric silencing by Asf1p (Daganzo et al., 2003;

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Sharp et al., 2001; Sutton et al., 2001). Interestingly, they are also repressors of histone genes normally expressed in S-phase (Nelson et al., 2002; Spector et al., 1997; Sutton et al., 2001). Taken together, these data suggest that these three proteins contribute to formation of transcriptionally silent heterochromatin structures through chromatin assembly and/or compaction and also repress expression of at least one class of proliferation-linked genes, the replication-dependent histones.

Human cells contain two orthologs of Asf1p, ASF1a and ASF1b, as well as a single protein, HIRA, which is a fusion of yeast Hir1p and Hir2p. In light of data summarized above, we wondered whether human HIRA, ASF1a, and ASF1b promote formation of SAHF and cell cycle exit in senescent cells. Consequently, we investigated the structure of SAHF, its mode of assembly, and contribution to cell cycle arrest. Here we present evidence for a HIRA/ASF1a-dependent pathway that drives formation of macroH2A- and HP1-containing SAHF, thus contributing to cell cycle exit in senescent cells.

Results

SAHF Are Enriched in the Histone Variant macroH2A

To better characterize SAHF (defined by DAPI foci) at the molecular level, we analyzed SAHF in primary WI38 fibroblasts for markers of heterochromatin. As reported previously (Narita et al., 2003), SAHF contained Me-K^9-H3 and HP1α, β, and γ (Figures 1A–1E). Also, we observed that SAHF are enriched in all three members of the variant histone macroH2A family (Figure 1 and data not shown). However, not all SAHF contained macroH2A foci (Figure 1F, cell marked with asterisk). In fact, time course analyses showed that macroH2A was incorporated into SAHF after their appearance by DAPI staining. This was true regardless of whether senescence was induced by an activated oncogene (Figure 1G) or extended growth in culture. After 50 and 55 population doublings (PD) in culture, approximately 50% and 97%, respectively, of cells with DAPI foci contained colocalizing macroH2A foci (data not shown; see also Figure 3C). Thus, concentration of macroH2A in SAHF is not simply a result of increased chromatin density at these sites. Consistent with macroH2A playing key role in stable formation of SAHF, we found that its knockdown by a targeted short-hairpin (sh) RNA greatly decreased accumulation of SAHF (data not shown). However, this treatment also caused severe defects in cell cycle progression, making it difficult to assess the specificity of this phenotype. Regardless, our data show that macroH2A is specifically enriched in SAHF, as a relatively late event in their formation. Notably, incorporation of macroH2A into the inactive X chromosome is also a relatively late event (Okamoto et al., 2004).

HP1 Proteins Are Transiently Recruited to PML Bodies prior to Incorporation into SAHF

Close inspection of HP1α, β, and γ staining patterns, in cells approaching senescence, revealed that not all HP1 foci colocalized with SAHF. Frequently, we also observed 10–30 bright, round nuclear foci that failed to colocalize with SAHF and were reminiscent of PML nuclear bodies (Figure 2A, 4 days postinfection with Ras). PML bodies are 0.2–1.0 μM diameter structures that contain the PML protein and numerous other proteins and are implicated in induction of senescence. Specifically, PML bodies become larger and more numerous as cells enter senescence, ectopic expression of PML induces senescence, and shRNA-mediated knockdown of PML impairs senescence (de Stanchina et al., 2004; Ferbeyre et al., 2000; Pearson et al., 2000). Significantly, HP1 proteins have been previously reported to localize to PML bodies, although no link to senescence was defined (Everett et al., 1999; Seeler et al., 1998). To test whether the observed HP1 proteins are contained in PML bodies, young and senescent WI38 cells were co-stained with antibodies to HP1β and PML. No specific colocalization between HP1β and PML bodies was observed in young, nonsenescent WI38 cells (Figure 2, 1 day postinfection and control infected cells, cells with no DAPI foci). However, as cells approached senescence, there was marked colocalization between HP1β and PML foci (Figure 2, 4 days postinfection, cells with intermediate DAPI foci). Finally, in late senescent cells, the extent of colocalization between HP1β and PML declined (Figure 2, 10 days after infection, cells with well-formed DAPI foci). Strikingly, incorporation of HP1β into SAHF occurred after its localization to PML bodies, and, at the same time as localization to PML bodies declined, incorporation into chromatin reached a maximum (Figure 2B). Each of the HP1 subtypes behaved the same, except HP1α showed a higher level of localization to PML bodies in young, growing cells (data not shown). In sum, these data show that HP1 proteins transiently localize to PML bodies in the early stages of senescence, prior to their stable incorporation into SAHF.

HIRA also Enters PML Bodies prior to Formation of SAHF

The first clue that HIRA contributes to SAHF came from its localization in primary and transformed human cells. In asynchronously growing transformed cell lines of various tissue origins, e.g., U20S, HCT116, WI38-VA13, SAOS2, MDAMB435, MCF7, HeLa, and HL60, HIRA was diffused throughout the nucleus. In contrast, in a proportion of primary human cells, e.g., WI38, IMR90, and MRC5, HIRA was concentrated in 10–30 discrete nuclear foci per cell, as well as being diffused throughout the nucleus (Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/8/1/19/DC1/ and data not shown).

One difference between primary and transformed cells is that the former, but not the latter, become senescent. Therefore, we hypothesized that the HIRA foci are linked to onset of senescence. To test whether HIRA foci formed during senescence after extended cell culture, we grew WI38 fibroblasts from PD#29 to senescence at PD#55 and analyzed the cells for HIRA foci and markers of senescence, senescence-associated β-galactosidase (SA β-gal; Dimri et al., 1995), SAHF (DAPI foci), and colocalizing macroH2A foci. The number of cells with SA β-gal, SAHF, and macroH2A foci increased exponentially as the cells approached senescence (Figures 3A–3C). The number of cells with HIRA

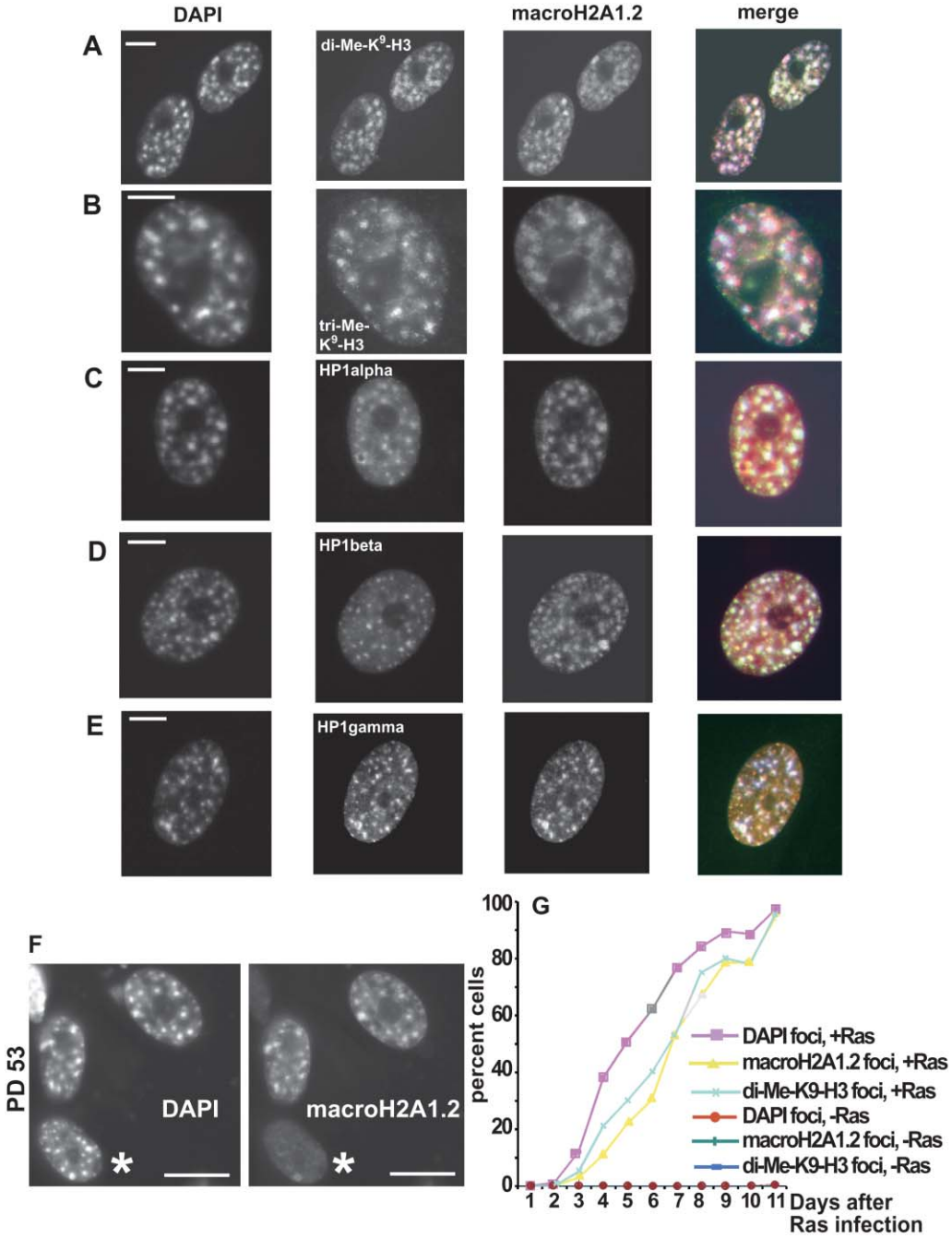


Figure 1. SAHF Is Enriched in macroH2A

(A–E) Senescent WI38 cells stained with DAPI and antibodies to macroH2A1.2 and (A) di-Me-K^9-H3, (B) tri-Me-K^9-H3, (C) HP1α, (D) HP1β, and (E) HP1γ as indicated. Images obtained by epifluorescence with a cooled CCD camera.

(F) WI38 cells (PD#53) stained with antibodies to macroH2A1.2 and with DAPI. Asterisk marks a cell that contains DAPI foci without macroH2A1.2.

(G) Growing WI38 cells were infected with a retrovirus encoding activated Ras or a control virus. Infected cells were selected in puromycin and then fixed and stained with DAPI and antibodies to macroH2A1.2 and di-Me-K^9-H3. Results were quantitated by scoring 100 cells on each slide, from two independent experiments.

foci also increased, although these appeared earlier than other markers of senescence and increased linearly over time (Figures 3B and 3C). Despite the correlated appearance of HIRA foci and SAHF in senescent cells, in individual cells there was no specific colocalization between the two (Figure 3B). To test whether HIRA foci are induced by activated oncogenes, we infected WI38 cells with a retrovirus encoding oncogenic RasV12. This treatment induced HIRA foci in nearly 100% of the cells (Figure 3D). To further test whether HIRA foci are linked to senescence-associated exit from the cell cycle, asynchronously growing WI38 cells were labeled with 5′-BrdU for 1, 18, or 24 hr immediately prior to harvesting. In middle-aged cells (PD#40), approximately 20% of the

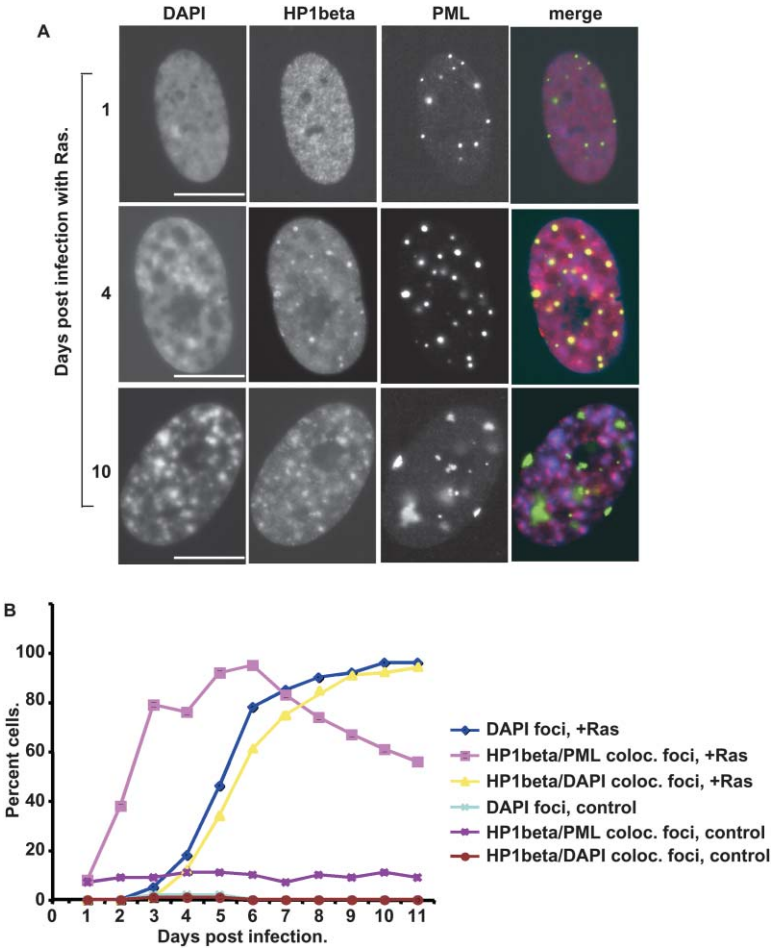


Figure 2. HP1β Is Transiently Recruited to PML Bodies prior to Formation of SAHF

(A) WI38 cells were infected with a retrovirus encoding an activated Ras oncogene. The infected cells were selected in puromycin and fixed and stained with DAPI and antibodies to HP1β and PML 1, 4, or 10 days later.

(B) As (A), except the percent of cells with DAPI foci, colocalizing HP1β/PML foci, and colocalizing HP1β/DAPI foci in Ras and control infected cells were quantitated by scoring 100 cells from each slide.

total cells were in S-phase at any time, as determined by a 1 hr pulse with 5′-BrdU (Supplemental Figure S1B). Over a 24 hr pulse period, most cells entered S-phase and labeled with 5′-BrdU. The cells containing HIRA foci also incorporated 5′-BrdU, but much less efficiently than the bulk population of cells. Thus, cells with HIRA foci pass through S-phase less frequently than those without HIRA foci. Taken together, these results suggest that in cells approaching senescence and exit from the cell cycle, a proportion of HIRA is recruited to nuclear foci.

These HIRA foci are, like the HP1 foci, reminiscent of PML nuclear bodies (Borden, 2002). Therefore, we asked whether HIRA foci are also in PML bodies. Indeed, by confocal and epifluorescence microscopy, HIRA foci colocalized perfectly with PML and another marker of PML bodies, SP100 (Figure 3E and Supplemental Figure S1C). When WI38 cells were infected with a retrovirus encoding wild-type HA-tagged HIRA (or a fragment spanning residues 421–729, HA-HIRA[421-729]), the staining observed with anti-HA antibodies also colocalized with PML (Supplemental Figure S1D and data not shown). PML bodies were observed in all cells as reported previously, but HIRA only colocalized with PML in a subset of, presumably, presenescent or senescent cells (Supplemental Figure S1E). In sum, these data show that HIRA, like HP1 proteins, is recruited to PML bodies as cells enter senescence. Indeed, a comparison of the two proteins showed that HIRA and HP1β colocalize in PML bodies (Figure 3F) and the two enter PML bodies virtually simultaneously (data not shown), prior to formation of SAHF (Figures 2B and 3C). Eventually, however, the steady-state abundance of HP1 proteins decreases in PML bodies and, instead, they accumulate in SAHF (Figure 2B).

In light of these data, and since HIRA and ASF1a orthologs contribute to heterochromatin formation in yeast and flies (see Introduction), we hypothesized that HIRA and ASF1a promote formation of HP1- and macroH2A-containing SAHF in senescent human cells. Alternatively, relocalization of the proteins in senescent cells could reflect a function unrelated to chromatin structure. To test whether relocalization of HIRA was specifically linked to changes in chromatin structure, we asked whether other treatments that directly affect chromatin structure also cause relocalization of HIRA. WI38 cells were treated with various drugs and genotoxic stresses. Only those known to directly perturb chromatin structure, namely trichostatin A (TSA) and sodium butyrate (NaBu), recruited HIRA to PML bodies (Supplemental Figures S2A–S2C). TSA and NaBu are histone deacetylase (HDAC) inhibitors that promote histone acetylation and relaxation of chromatin structure (Johnstone, 2002). Genotoxic stresses (hydroxyurea [HU], ultraviolet light [UV], and ionizing radiation [IR]) and phosphatase inhibitors (sodium orthovanadate [NaV] and okadaic acid [OA]) had no effect over a range of doses and times (Supplemental Figure S2A and data not shown). Formation of HIRA foci was not due to cell cycle perturbation

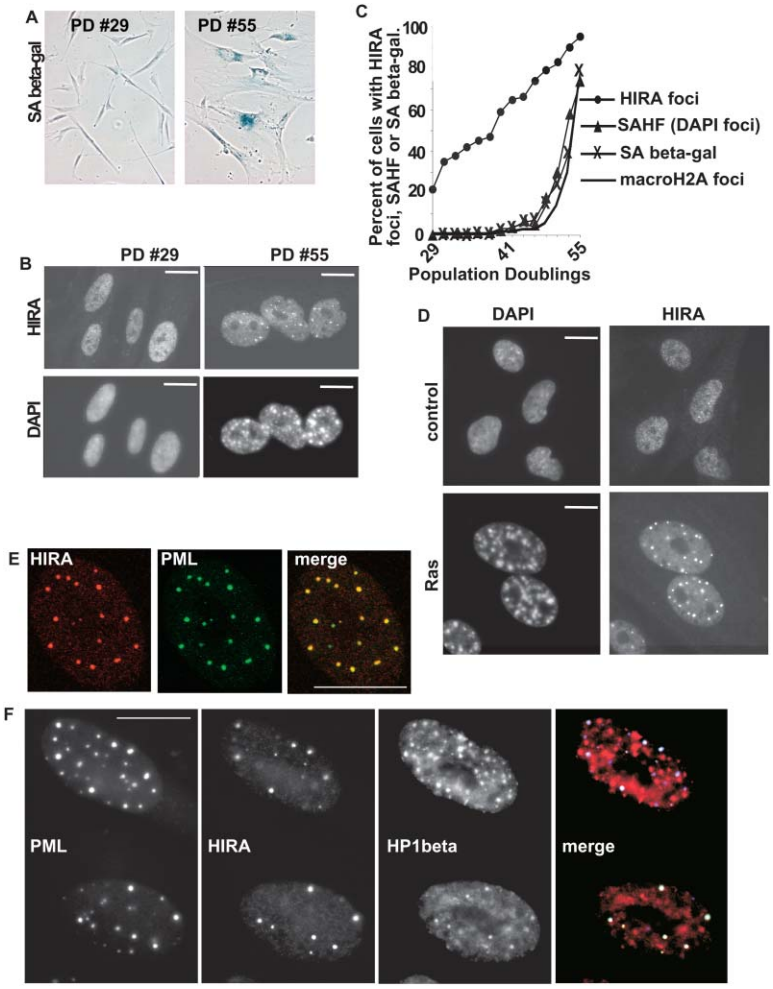


Figure 3. HIRA Is Localized to PML Bodies and Colocalizes with HP1β as Cells Enter Senescence

(A) Growing and senescent WI38 cells (PD#29 and #55, respectively) fixed and stained for expression of SA β-gal.

(B) As (A), but cells stained with DAPI and antibodies to HIRA.

(C) WI38 cells were passaged from PD#29 to senescence at PD#55. At every other PD, the cells were stained for SA β-gal, with antibodies to HIRA and macroH2A1.2 and with DAPI to visualize SAHF.

(D) WI38 cells were infected with a control or RasV12-expressing retroviruses, selected in puromycin for 8 days, and stained with DAPI and antibodies to HIRA.

(E) WI38 cells stained with antibodies to HIRA and PML and visualized by confocal microscopy.

(F) WI38 cells were infected with a retrovirus encoding activated Ras, drug selected, and then stained with antibodies to HIRA, HP1β, and PML.

by TSA and NaBu, because TSA, NaBu, HU, UV, and IR had comparable effects on the cell cycle (data not shown). Consistent with the idea that recruitment of HIRA to PML bodies and insertion of macroH2A into chromatin are in the same pathway, cells treated with TSA and NaBu (but not HU, UV, IR, OA, or NaV) also accumulated nuclear foci of macroH2A that did not colocalize with HIRA foci (Supplemental Figure S2D and data not shown). In sum, recruitment of HIRA to PML bodies correlates tightly with altered chromatin structure and deposition of macroH2A into chromatin. In turn, this supports the notion that the redistribution of HIRA in presenescence is linked to construction of macroH2A-containing SAHF.

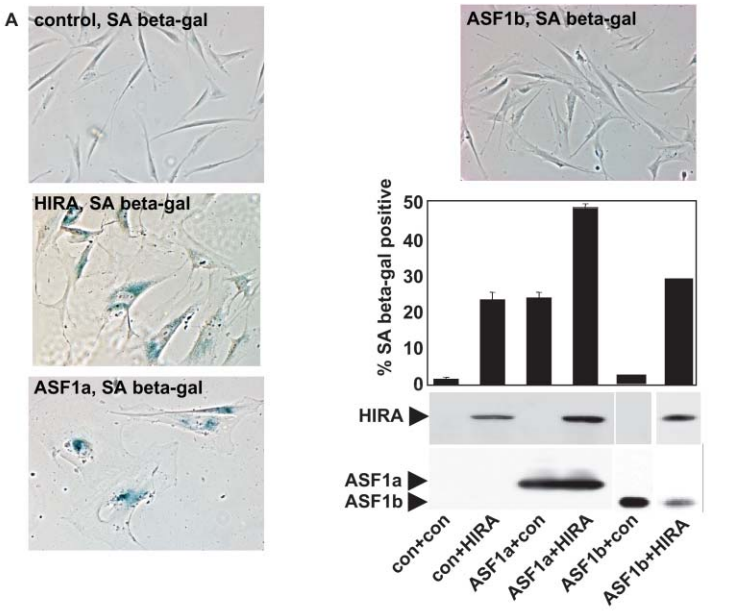
HIRA and ASF1a, but Not ASF1b, Are Rate Limiting for Formation of SAHF

To test directly whether HIRA and ASF1a promote formation of SAHF, primary human fibroblasts were infected with retroviruses encoding HIRA, ASF1a, or both. HIRA and ASF1a each caused cells to assume markers of senescence, including a large flat morphology, expression of SA β-gal activity, and macroH2A-containing SAHF. The effect was more pronounced when both proteins were coexpressed (Figures 4A and 4B). Strikingly, ASF1b was inactive in all assays (Figures 4A and 4B). These results are consistent with a role for HIRA and ASF1a in induction of SAHF and senescence.

Induction of SAHF by HIRA and ASF1a Requires Their Physical Interaction

The yeast orthologs of HIRA and ASF1a physically interact, and human HIRA and ASF1a copurify from cells, suggesting that they are in the same complex (Sharp et al., 2001; Sutton et al., 2001; Tagami et al., 2004). Indeed, endogenous HIRA and ASF1a coimmunoprecipitated from asynchronously growing WI38 cells, confirming that they directly or indirectly associate in cells (Figures 5A and 5B). Significantly, human HIRA did not interact with ASF1b at endogenous levels in primary human cells, or in vitro (Figures 5B and 5C). Similarly, Tagami et al. (2004) previously found that HIRA copurified with ASF1a but not ASF1b. The finding that ASF1a binds to HIRA and induces SAHF, whereas ASF1b does neither, is consistent with the idea that induction of SAHF depends upon the interaction between ASF1a and HIRA.

To test this directly, we compared a panel of ASF1a mutants for binding to HIRA and induction of macroH2A- and HP1-containing SAHF, as well as SA β-gal. This panel included alanine substitution mutants ASF1a (ED36-37AA) and ASF1a(VGP62-64AAA), which we showed previously do not bind to HIRA (Daganzo et al.,



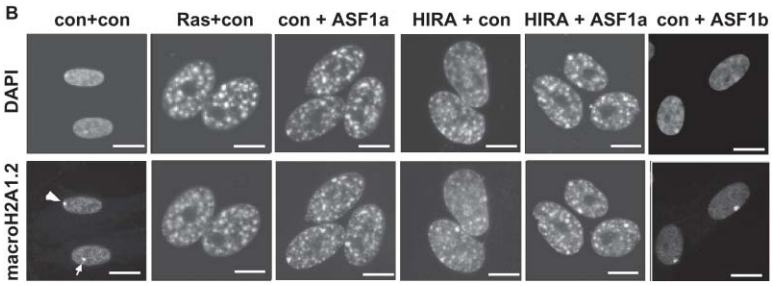


Figure 4. Ectopic Expression of HIRA and ASF1a Induces Senescence and macroH2A-Containing SAHF

(A) WI38 cells were infected with control, HA-ASF1a, HA-ASF1b, or Myc-HIRA encoding retroviruses, as indicated. The cells were selected in puromycin and neomycin and stained for SA β-gal expression after 8 days. The histogram shows results from 100 cells of each infection. Results are means of three independent experiments. Extracts were Western blotted with anti-HA and anti-myc.

(B) Cells infected as in (A) were stained with antibodies to macroH2A1.2 and DAPI. White arrows mark inactive X chromosome.

2003), and three additional substitution mutants that retain HIRA binding, ASF1a(E,E121,124A,A), ASF1a(P,-V144,146A,A), and ASF1a(V45A) (Figure 5D and Supplemental Figures S3A and S4). There was an excellent correlation between ASF1a's ability to bind to HIRA, both in vitro and in vivo, and its ability to induce macroH2A- and HP1-containing SAHF and SA β-gal (Figures 5D and 5E and Supplemental Figures S3A, S4, S5A, and S5B). This supports the notion that induction of SAHF by ASF1a depends on its physical interaction with HIRA.

If so, ASF1b might fail to induce SAHF because it does not bind to HIRA. To test this, we designed a variant of ASF1b that binds more efficiently to HIRA. ASF1a and ASF1b are 71% identical over their entire length. However, two regions are quite poorly conserved, perhaps accounting for their different HIRA binding properties (Figure 5F). First, the C-terminal 50 residues of the two proteins show low sequence conservation. Second, residues 31–37 of ASF1a (IEDLSED) are quite poorly conserved in ASF1b. Supporting the significance of this N-terminal sequence, we already showed that mutation of residues 36 and 37 to alanine inactivated HIRA binding (ASF1a(ED36-37AA)) (Figure 5D and Supplemental Figure S3A). Moreover, substitution of another three residues that, in a model of ASF1a based on the crystal structure of yeast Asf1p, are folded adjacent to this N-terminal sequence also inactivated HIRA binding (ASF1a(VGP62-64AAA)) (Figure 5D; Supplemental Figures S3A and S4; Daganzo et al., 2003). Based on these results, we substituted residues 31–37 and the C terminus of ASF1a in place of the corresponding ASF1b sequences, to create ASF1b-a. This protein bound more efficiently to HIRA in vitro and in vivo, confirming that the swapped sequences are key determinants of HIRA binding (Figure 5D and Supplemental Figure S3A; note that when ectopically overexpressed in vivo, ASF1b wild-type did bind to HIRA. However, since endogenous ASF1b does not bind to endogenous HIRA [Figure 5B; Tagami et al., 2004], this is likely to reflect nonspecific binding due to high-level overexpression). In addition, ASF1b-a was a more potent inducer of macroH2A-containing SAHF and SA-β gal, compared to ASF1b wild-type (Figure 5E). This supports the notion that the failure of ASF1b to induce SAHF stems, at least in part, from its failure to bind HIRA. Taken together, the results in Figure 5 strongly argue that the ability of ASF1a to induce SAHF depends upon its ability to bind HIRA.

We then asked whether the ability of HIRA to induce SAHF depends on its binding to ASF1a. To do this, we tested a panel of HIRA deletion mutants for their ability to bind to ASF1a and induce SAHF. There was a strong correlation between binding to ASF1a, *both in vitro and in vivo*, and induction of macroH2A-containing SAHF

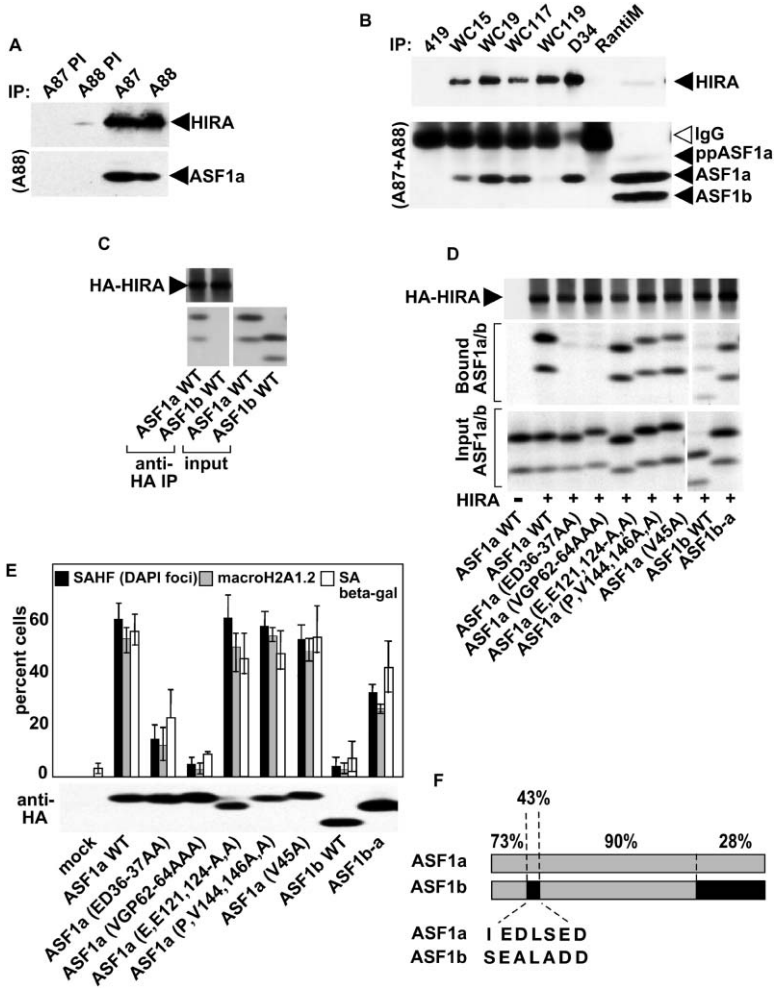


Figure 5. Induction of macroH2A-Containing SAHF by ASF1a Requires Its Interaction with HIRA

(A) Extracts from WI38 cells were immunoprecipitated with anti-ASF1a (A87, A88) or control (A87 preimmune serum [PI], A88 PI) and Western blotted with anti-HIRA (WC119) or anti-ASF1a (A88).

(B) As (A), except extracts were immunoprecipitated with anti-HIRA (WC15, WC19, WC117, WC119, D34) or control (419, RαM). Anti-ASF1a/b Western blot was with A87 and A88.

(C) In vitro translated ^{35}S-labeled Myc-ASF1a WT and Myc-ASF1b WT were incubated with ^{35}S-labeled HA-HIRA and immunoprecipitated with anti-HA antibodies.

(D) In vitro translated ^{35}S-labeled HA-HIRA, Myc-ASF1a WT, Myc-ASF1b WT, mutants of Myc-ASF1a, and Myc-ASF1b-a were incubated and immunoprecipitated with antibodies to HA.

(E) WI38 cells were infected with retroviruses encoding HA-tagged versions of ASF1 proteins from (D), the cells were selected in puromycin for 12 days and then scored for SA β-gal, SAHF (DAPI foci), and macroH2A1.2 foci. Results are means of three independent experiments. Cell extracts were Western blotted with anti-HA antibodies to detect ectopic proteins.

(F) A schematic comparing ASF1a and ASF1b primary sequences. The percent identity in each region is indicated.

(Figure 6 and Supplemental Figure S3B). Of particular note, a 37 amino acid deletion of the evolutionarily conserved B domain (HIRA(delB)) (Kirov et al., 1998; Nelson et al., 2002) inactivated HIRA in all assays, whereas all active mutants contained this entire domain (Figure 6C). We conclude that the ability of HIRA to induce macroH2A-containing SAHF depends on its ability to bind ASF1a through the evolutionarily conserved B-domain. Together, Figures 5 and 6 and Supplemental Figures S3, S4, S5A, and S5B show that induction of macroH2A/HP1-containing SAHF by HIRA and ASF1a depends on a physical interaction of the two proteins.

ASF1a Is Required for Formation of SAHF

To test whether the HIRA/ASF1a complex is required to reshape chromatin in senescent cells, we infected primary human fibroblasts with a retrovirus encoding a RasV12 oncogene, together with a retrovirus encoding an shRNA that knocks down ASF1a, or two different control shRNAs to luciferase (both knocked down luciferase activity [data not shown]). The shRNA to ASF1a, but not control shRNAs, dramatically decreased the number of cells with macroH2A- and HP1-containing SAHF (Figures 7A and 7B and Supplemental Figures S5C and S5D). Thus, ASF1a is required for efficient formation of SAHF.

ASF1a Is Required for Senescence-Associated Cell Cycle Exit

To test whether ASF1a is also required for cell cycle exit, the cells from Figures 7A and 7B were pulse-labeled with 5′-BrdU. Up until 6 days after infection, knockdown of ASF1a resulted in a 4- to 5-fold increase in 5′-BrdU-positive cells, compared to cells expressing activated Ras plus a control shRNA (Figure 7C). Importantly, knockdown of ASF1a did not affect the cell cycle distribution of cells growing in the absence of an activated Ras oncogene (Figure 7C). We conclude that ASF1a does not detectably inhibit the cell cycle in normal growing cells, but does contribute to efficient senescence-associated cell cycle exit and formation of SAHF.

Together, these results are consistent with a model whereby ASF1a is required for cell cycle exit due to its contribution to SAHF. In line with this idea, wild-type HIRA, ASF1a, and all mutants that promoted SAHF also forced cell cycle exit, as judged by 5′-BrdU incorporation and cell proliferation assays (Figures 7D, 7E, 5E, and 6B). Of special note, wild-type ASF1b that did not induce SAHF did not efficiently block cell proliferation, whereas the ASF1b variant that induced SAHF (ASF1b-a) blocked proliferation very efficiently (Figure 7E). This is consistent with SAHF contributing to cell cycle exit.

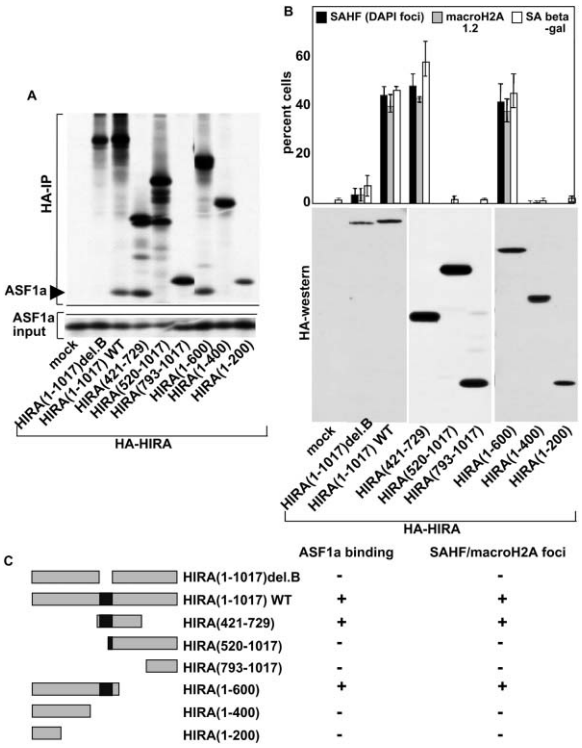


Figure 6. Induction of macroH2A-Containing SAHF by HIRA Requires Its Interaction with ASF1a

(A) Wild-type in vitro translated ^{35}S-labeled HA-HIRA WT and deletion mutants were incubated with ^{35}S-labeled Myc-ASF1a and immunoprecipitated with antibodies to HA.

(B) WI38 cells were infected with retroviruses encoding HA-HIRA WT or deletion mutants and selected for 10 days in puromycin, and 100 cells were scored for SAHF (DAPI foci), macroH2A1.2 foci, and SA β-gal. Results are means of three independent experiments. Cell extracts were Western blotted with anti-HA antibodies to detect expression of HA-HIRA proteins (the middle three lanes are underexposed relative to the others).

(C) A schematic of the HIRA mutants, their binding to ASF1a, and induction of SAHF. The B-domain is shaded black.

These results, together with those obtained from ASF1a knockdown, show that forced assembly or disruption of SAHF is tightly linked to induction or abrogation of cell cycle arrest, respectively. However, formation of SAHF is not a consequence of cell cycle exit, because several mutants—HIRAdelB, HIRA(520-1017), ASF1a(ED36-37AA), and ASF1a(VGP62-64AAA)—arrested the cell cycle but did not induce SAHF (Figures 7D, 7E, 5E, and 6B). In addition, abolition of SAHF by shASF1a was not due to cell cycle re-entry, because eventually cells lacking ASF1a exited the cell cycle, but, even at this time, they were still deficient in SAHF (Supplemental Figure S6). These results show that ASF1a is required for efficient senescence-associated cell cycle exit and suggest that this is, at least in part, due to its requirement for SAHF formation.

Discussion

We have identified the histone H2A variant, macroH2A, as a molecular component of SAHF. In addition, we have demonstrated a role for two known regulators of chromatin structure, HIRA and ASF1a, in formation of SAHF. Finally, we have shown that ASF1a is required for efficient senescence-associated cell cycle exit and have presented evidence that this is, at least in part, through its formation of SAHF.

HIRA and ASF1a Are Rate Limiting and Necessary for Formation of SAHF

Three lines of evidence show that HIRA and ASF1a play a key role in formation of SAHF in senescent cells. First, HIRA and HP1 proteins transiently colocalize in PML bodies, prior to deposition of HP1 proteins in SAHF (Figures 2 and 3). Second, ectopic expression of HIRA and ASF1a in primary cells induces SAHF (Figure 4), and the interaction between HIRA and ASF1a is necessary to form SAHF (Figures 5 and 6). Third, ASF1a is required for formation of SAHF (Figure 7).

Several lines of evidence indicate that HIRA and ASF1a directly affect chromatin structure, instead of as a secondary consequence of effects on cell proliferation and senescence. First, recruitment of HIRA to PML bodies also occurs in response to other perturbations of chromatin structure that do not acutely induce other markers of senescence (Supplemental Figure S2). Specifically, HIRA relocalization occurred in response to HDAC inhibitors, TSA and NaBu. TSA and NaBu should increase demand for replication-independent chromatin assembly and/or compaction in the cell (Johnstone, 2002; Lomvardas and Thanos, 2002). Thus, activation of the HIRA/ASF1a pathway is tightly linked to direct changes in chromatin structure. Second, four mutants that failed to induce SAHF did arrest cell proliferation (Figures 7D, 7E, 5E, and 6B). Third, shRNA-mediated knockdown of ASF1a impaired, but did not abolish, senescence-associated cell cycle exit. Eventually, cell cycle arrest occurred, but did so without normal assembly of SAHF (Figures 7B and 7C and Supplemental Figure S6). Thus, the ability of ASF1a knockdown to abrogate SAHF does not depend on forced re-entry into the cell cycle. Fourth, HIRA, ASF1a, and orthologs are well-documented regulators of chromatin structure (see Introduction). Most notably, telomeric silencing by Asf1p in yeast requires binding to Hir1p (Daganzo et al., 2003). Likewise, induction of SAHF by HIRA and ASF1a requires their physical interaction (Figures 5 and 6 and Supplemental Figure S3). In sum, we have shown that human HIRA and ASF1a are mechanistically related to their yeast orthologs in terms of their ability to generate heterochromatin, and we have specifically linked this activity to creation of SAHF in senescent human cells.

How Do HIRA and ASF1a Promote Deposition of macroH2A?

De novo deposition of macroH2A into chromatin is tightly linked to apparent activation of the HIRA/ASF1a chromatin-remodeling pathway, as reflected by recruitment of HIRA to PML bodies. Specifically, recruitment of HIRA to PML bodies during senescence or after treatment of cells with HDAC inhibitors, TSA and NaBu, correlates with incorporation of macroH2A into chromatin

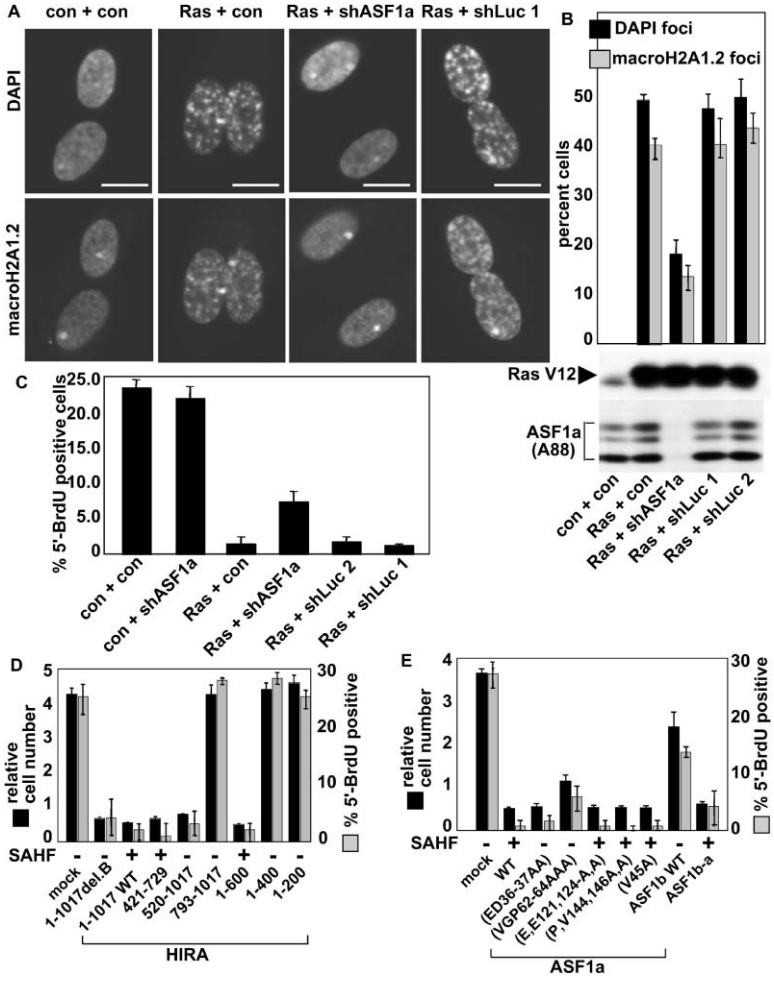


Figure 7. ASF1a Is Required for Formation of SAHF, which Drives Cell Cycle Exit

(A) WI38 cells were infected with retroviruses encoding activated Ras (and resistance to neomycin) together with shRNAs to ASF1a or luciferase. Viruses encoding shRNAs also encoded GFP. Cells were drug selected for 6 days and then stained with antibodies to macroH2A1.2 and DAPI. Only GFP$^+$ cells are shown.

(B) 100 GFP$^+$ cells from (A) were scored for SAHF (DAPI foci) and macroH2A foci. Results are means of three independent experiments. Cell extracts were Western blotted to detect RasV12 and ASF1a (with A88). Cells were lysed by boiling in Laemmli buffer, which better preserves the phosphorylated forms of ASF1a, compared to the nondenaturing lysis buffer used for IPs in Figures 5A and 5B.

(C) The cells from (A) were pulse labeled with 5′-BrdU for 1 hr, and 100 GFP$^+$ cells were scored for incorporation of 5′-BrdU. Results are means of three independent experiments.

(D) Cells were infected with retroviruses expressing HA-tagged HIRA-derived proteins. Relative cell proliferation rates and incorporation of 5′-BrdU were determined as described in Experimental Procedures. Results are means of three independent experiments. See Figure 6B for expression analysis.

(E) As (D) but with the indicated HA-tagged ASF1a, ASF1b, and mutants. See Figure 5E for expression analysis.

(Figures 1 and 3 and Supplemental Figure S2). In addition, HIRA and ASF1a are both rate limiting and ASF1a is necessary for deposition of macroH2A (Figures 5 and 6 and Supplemental Figure S6). Since HIRA directs replication-independent chromatin assembly in vitro and deposits the histone H3.3 variant into chromatin, it is tempting to speculate that HIRA and ASF1a directly deposit macroH2A into chromatin (Ray-Gallet et al., 2002; Tagami et al., 2004). However, two lines of evidence argue against this model. First, we have not detected a direct physical interaction between HIRA or ASF1a and macroH2A in vivo, as was shown for HIRA and histone H3.3 (Ray-Gallet et al., 2002; Tagami et al., 2004). Second, based on our mutation analyses, Asf1p/ASF1a-mediated telomeric silencing and formation of SAHF are mechanistically related (Daganzo et al., 2003). However, yeast does not contain macroH2A. In sum, our data show that HIRA/ASF1a activity promotes deposition of macroH2A, but the HIRA/ASF1a complex does not seem likely to directly deposit macroH2A. In line with this, Jessica Tyler and coworkers recently showed that yeast Asf1p has nucleosome disassembly activity (Adkins et al., 2004), suggesting that human ASF1a could contribute to incorporation of macroH2A by disassembling chromatin, prior to insertion of macroH2A by other factors.

HIRA, ASF1a, and SAHF Drive Cell Cycle Exit

All ASF1a and HIRA mutants that form SAHF also drive cell cycle exit (Figures 7D, 7E, 5E, and 6B). Importantly, not all HIRA and ASF1a mutants that forced exit from the cell cycle induced SAHF, showing that cell cycle arrest is not the cause of SAHF. Conversely, ASF1a is required for formation of SAHF and efficient cell cycle exit during senescence (Figures 7A–7C). Eventually, cells lacking ASF1a do exit the cell cycle, but they are still deficient in SAHF (Supplemental Figure S6), showing that abrogation of SAHF is not a consequence of forced re-entry into the cell cycle. Together, these observations show that ASF1a promotes and is required for efficient senescence-associated cell cycle exit and support the notion that HIRA/ASF1a-mediated formation of SAHF directly contributes to cell cycle exit during senescence. Significantly, mouse embryos lacking HIRA die between days 7.5 and 11 of embryonic development, depending on the genetic background (Roberts et al., 2002). In some embryos, the primitive streak was reported to have many more cells than usual, and the worst affected embryos consisted of small, relatively undifferentiated balls of cells. This phenotype is consistent with a requirement for HIRA in cell cycle exit associated with the morphogenetic movements of gastrulation. Thus, HIRA and ASF1a might also contribute to some of the changes

in chromatin structure associated with morphogenesis and cell differentiation (Kosak and Groudine, 2004). Future studies will test this idea.

The Role of PML Bodies

Our data linking PML bodies to formation of SAHF are consistent with previous studies implicating PML bodies in induction of senescence (de Stanchina et al., 2004; Ferbeyre et al., 2000; Pearson et al., 2000). However, there was no significant colocalization between HIRA-containing PML bodies and SAHF (Figures 3B and 3D). Still, even in senescent cells, a proportion of HIRA is diffused throughout the entire nucleus. In addition, localization of HP1 proteins to PML bodies occurs transiently, prior to their deposition in SAHF (Figure 2). One model, consistent with these observations, is that the detectable enrichment of HIRA and HP1 proteins in PML bodies, based on steady-state measurements, reflects a dynamic process in which HP1 proteins exit PML bodies and translocate to SAHF. Whether HIRA shuttles between PML bodies and nascent SAHF or whether HIRA somehow promotes SAHF while localized to PML bodies is not yet clear. Either way, according to this model, PML bodies and HIRA play a catalytic role in formation of SAHF. This model is consistent with real-time imaging studies of other nuclear bodies, such as nuclear speckles (Phair and Misteli, 2000) and Cajal bodies (Dundr et al., 2004) and with the known role of PML bodies in assembly/modification of macromolecular regulatory complexes, such as those containing p53 and Mad proteins (Fogal et al., 2000; Guo et al., 2000; Khan et al., 2001; Pearson et al., 2000).

Intact PML bodies possess tumor suppressor activity. Specifically, disruption of PML bodies promotes cell transformation and their reconstitution correlates with transformation suppression (Dyck et al., 1994; Kastner et al., 1992; Koken et al., 1994; Wang et al., 1998; Weis et al., 1994). Disruption of PML bodies and inactivation of the linked HIRA/ASF1a-mediated SAHF assembly pathway might result in failure to exit the cell cycle and cell transformation. If so, in some cases, genetic alterations of HIRA and ASF1a might also contribute to cell transformation. This possibility will be addressed in future studies of the HIRA/ASF1a SAHF assembly pathway defined here.

Experimental Procedures

Cell Culture and Plasmids

Cells were cultured as described previously and according to the ATCC (http://www.atcc.org/) (Adams et al., 1996; Bartholomew et al., 1976; Nelson et al., 2002). All plasmids have been published previously (Hall et al., 2001; Nelson et al., 2002) or were generated using standard molecular biology procedures; details are available on request.

Relative Cell Growth Rates and 5′-BrdU Labeling

To determine relative growth rates, 3 days after retrovirus infection, 1×10^4 drug-selected cells were plated and grown for 11 days. The number of viable cells was determined by trypan blue staining, and this is directly proportional to the relative growth rate. To perform 5′-BrdU labeling, 12 days after infection, the cells were pulse-labeled with 10 μM 5′-BrdU for 1 hr and the percent 5′-BrdU-positive cells was determined by immunofluorescence (IF).

Immunological and IF Techniques

Anti-HP1α were gifts of Dr. William Earnshaw and Dr. David Schultz. Anti-HA (12CA5) (Roche), anti-myc (9E10) (Santa-Cruz), anti-PML antibodies (AB1370 and N19) (Chemicon and Santa-Cruz, respectively), anti-5′-BrdU-FITC (Becton-Dickinson), anti-HP1β (Chemicon), anti-HP1γ (Chemicon), anti-di-Me-K^9-H3 (Abcam), and anti-tri-Me-K^9-H3 (Abcam) were from the indicated suppliers. Anti-HIRA antibodies (mouse monoclonals WC15, 19, 117, and 119 and rabbit polyclonal D34) have been described previously (Hall et al., 2001). Anti-ASF1a antibodies (A87 and A88) were raised in rabbits against GST-ASF1a.

IP, IF, and Western blots were performed as described previously (Adams et al., 1996; Hall et al., 2001). 2- and 3-color IF was performed as described previously (Hall et al., 2001; Nelson et al., 2002; Ye et al., 2003). Image collection was by epifluorescence microscopy recorded digitally using a cooled CCD camera or, where stated, optical sections obtained with a confocal microscope (Figure 3E). SAHF (DAPI foci) were detected by staining with 0.13 μg/ml DAPI for 2 min at room temperature (as opposed to standard conditions of 1 μg/ml for 5 min). Detailed IF methods are available on request. All scale bars are 10 μM. Results of cell counting experiments are expressed as means with error bars showing SD.

Retroviruses and shRNAs

The following plasmids were used for generating retroviruses: pBABE-puro or neo H-Ras V12 (gift of Dr. Robert A. Weinberg); pQCXIP(Clontech)-HA-ASF1a, HA-ASF1b, HA-HIRA, and derivatives; pQCXIN(Clontech)-Myc-HIRA; and pQCXIP-GFP with the U6 promoter shASF1a/sh-luciferase cassette subcloned into the 3′ LTR. The shRNA to ASF1a (sense strand GCGTAACTGTTGTGCTAAT TACTTGTACC) was designed using the approach described by Dr. Greg Hannon.

Retroviral-mediated gene transfer was performed using the Phoenix packaging cells (Dr. Gary Nolan, Stanford University). Briefly, Phoenix cells were transfected by the calcium-phosphate method with retroviral plasmid DNA and a plasmid encoding vesicular stomatitis virus glycoprotein (VSV-G). Virus-containing medium was collected, supplemented with 8 μg/ml of polybrene (Sigma), and incubated with target WI38 cells at 37°C for 24 hr. Frequently, a second round of infection was performed on the same target cells. Infected cells were purified by drug selection (3 μg/ml puromycin or 500 μg/ml G418).

Other Techniques

In vitro transcription and translation of proteins and Western blotting was described previously (Adams et al., 1996; Nelson et al., 2002). SA β-gal in senescent cells was assayed as described previously (Dimri et al., 1995).

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