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Award Number: DAMD17-02-1-0726

TITLE: Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

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REPORT DATE: October 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2005	Final	30 Sep 2002 –29 Sep 2005

4. TITLE AND SUBTITLE
Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0726

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Peter D. Adams, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, PA 19111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We showed that defects in chromatin assembly trigger DNA damage, a potent source of genome instability and cause of human cancers. Although we failed to make a specific link between defects in chromatin assembly and breast cancer, our survey was not exhaustive and leaves open the possibility that future studies might reveal such a link. Second, we showed that the histone chaperones HIRA and ASF1a drive formation of specialized domains of facultative heterochromatin in senescent cells and senescence-associated cell cycle exit. Although the funding period has ended, my lab is continuing to investigate whether genetic alterations of HIRA and ASF1a and other factors in this pathway contribute to cancers of breast and other tissues.

15. SUBJECT TERMS
chromatin assembly checkpoint

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	34	19b. TELEPHONE NUMBER *(include area code)*

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Introduction. Although the intial hypothesis proposed in the application was not confirmed, this period of DOD funding has been very successful - leading to publications in *Molecular Cell* and *Developmental Cell.* DOD funding generated important new insights into the links between chromatin structure and both genome stability and cell proliferation. These areas remain of intense interest to my lab and the funding provided by DOD during the first 5 years of starting my lab has been crucial.

Body. The initial goal of this application was to investigate whether defective chromatin assembly in S-phase of the cell cycle contributes to breast cancer. Specifically, in the original application, we proposed that defects in chromatin assembly in S-phase activate a checkpoint that blocks on-going DNA synthesis - a so-called "chromatin assembly checkpoint". We proposed that defects in this checkpoint might disrupt chromatin structure and contribute to cell transformation. Subsequent studies failed to support the notion of a "chromatin assembly checkpoint", but did demonstrate that defects in chromatin assembly cause DNA damage in S-phase *(1, 2)*. Since DNA damage is known to drive neoplastic transformation, we asked whether human tumors harbor mutations in chromatin assembly factors that might contribute to DNA damage, genome instability and cancer *(3)*. Despite encouraging preliminary data from cell lines, no such mutations were found in human breast cancers.

Subsequent efforts shifted away from the impact of chromatin structure on genome stability, and towards the impact of chromatin structure on cell proliferation. In human cells, expression of proliferation-promoting genes drives cell growth. For example, histone genes and other genes required for progression through S-phase are expressed in a periodic fashion in growing cells, but repressed in cells that have irreversibly exited the cell cycle, through senescence or differentiation. These changes in gene expression are thought to depend on changes in long-range nuclear organization and chromatin structure *(4-6)*. For example, in senescent cells, specialized domains of transcriptionally silent heterochromatin, called senescence associated heterochromatin foci (SAHF), are thought to repress expression of proliferation-linked genes *(5)*. SAHF contains well-characterized markers of heterochromatin.

To understand the changes in chromatin structure that contribute to changes in gene expression and senescence-associated cell cycle exit, we investigated the structure and formation of SAHF. We defined a set of players that interact to drive formation of SAHF. These are: known heterochromatin proteins, HP1 proteins *(7)*; a histone variant, macroH2A *(8)*; a subnuclear organelle, the PML nuclear body *(9)*; and two regulators of chromatin structure, human HIRA and ASF1a *(10-19)*.

MacroH2A is similar to canonical histone H2A, but it contains a large C-terminal extension of ~200 residues that is unrelated to other histones *(8)*. This histone variant has been previously linked to gene silencing through *in vitro* studies and its specific enrichment in the inactive X-chromosome *(20-22)*. PML bodies are subnuclear structures, about 0.1-1 m in diameter, that contain numerous proteins, in addition to PML itself. PML bodies have been previously implicated in tumor suppression and initiation of cell senescence *(9, 23-25)*. At the molecular level, their function is unclear, but they have been suggested to serve as assembly sites of macromolecular complexes *(25-28)*. The orthologs of human HIRA and ASF1a, in yeast, flies and *Xenopus*, are required for formation of heterochromatin *in vivo*, they serve as chromatin assembly factors *in vitro* and they repress expression of some proliferation-promoting genes *in vivo*, such as the replication-dependent histones *(10-19, 29)*. Consistent with a role for human HIRA in control of replication-dependent histone gene expression, we showed previously that

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human HIRA is an *in vivo* substrate of the proliferation and cell cycle-promoting kinases, cyclin A and E/cdk2, and its ectopic expression in cells represses histone gene transcription *(30, 31)*.

Our most recent data linked the ability of HIRA and ASF1a to promote heterochromatin formation and repress expression of proliferation-linked genes, such as histones, to a specific cell context, namely cell senescence *(32)*. We showed that: i) SAHF is enriched in macroH2A; ii) as cells approach senescence, HIRA and HP1 proteins are recruited to PML bodies where they transiently colocalize, prior to deposition of HP1 proteins into SAHF; iii) ectopic expression of HIRA and/or ASF1a drives formation of SAHF, and this depends upon a physical complex between HIRA and ASF1a. This was shown, in part, by using a set of HIRA and ASF1a mutants, some of which were rationally designed based on a crystal structure of the yeast ASF1a ortholog, that was obtained by our collaborator Dr. Paul Kaufman; iv) shRNA-mediated knock down of ASF1a prevents efficient formation of SAHF and senescence-associated cell cycle exit. Taken together, these data suggest that a HIRA/ASF1a complex drives formation of macroH2A and HP1 containing SAHF, through a pathway that appears to depend on flux of HP1 proteins from PML bodies to SAHF *(32)*.

Key Research Acccomplishments.

- The first demonstration that completion of S-phase in mammalian cells depends upon on concurrent replication-coupled chromatin assembly. Uncoupling DNA synthesis and chromatin assembly causes DNA damage.

- Definition of a novel cell-signaling pathway that drives formation of facultative heterochromatin in senescent human cells, and senescence-associated cell cycle exit.

Reportable Outcomes including Bibliography of Publications.

Ye, X., Franco, A.A., Santos, H., Nelson, D.M., Kaufman, P.D., and Adams, P.D., (2003) Defective S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. *Molecular Cell*, 11, 341-351.

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Zhang, R., et al., (2005) Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Developmental Cell*, 8(1), 19-30.

Conclusions. First, we conclude that defects in chromatin assembly can trigger DNA damage, a potent source of genome instability and cause of human cancers. Although we failed to make a specific link between defects in chromatin assembly and breast cancer, our survey was not exhaustive and leaves open the possibility that future studies might reveal such a link. Second, we conclude that the histone chaperones HIRA and ASF1a drive formation of specialized domains of facultative heterochromatin in non-proliferating senescent cells. Although the funding period has ended, my lab is continuing to investigate whether genetic alterations of HIRA and ASF1a and other factors in this pathway contribute to cancers of breast and other tissues.

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Appendices.

Ye, X., Franco, A.A., Santos, H., Nelson, D.M., Kaufman, P.D., and Adams, P.D., (2003) Defective S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. *Molecular Cell*, 11, 341-351.

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Molecular Cell, Vol. 11, 341–351, February, 2003, Copyright ©2003 by Cell Press

Defective S Phase Chromatin Assembly Causes DNA Damage, Activation of the S Phase Checkpoint, and S Phase Arrest

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Summary

The S phase checkpoint protects the genome from spontaneous damage during DNA replication, although the cause of damage has been unknown. We used a dominant-negative mutant of a subunit of CAF-I, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S phase chromatin assembly and found that this induced S phase arrest. Arrest was accompanied by DNA damage and S phase checkpoint activation and required ATR or ATM kinase activity. These results show that in human cells CAF-I activity is required for completion of S phase and that a defect in chromatin assembly can itself induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability.

Introduction

Cancer cells characteristically have a high frequency of genome rearrangements (Lengauer et al., 1998), although the cause of rearrangements is poorly understood. Genome integrity during S phase of the cell cycle depends on the S phase checkpoint. This checkpoint is activated by DNA damage or stalled replication forks and inhibits ongoing DNA synthesis (Abraham, 2001; Osborn et al., 2002), thus giving time for DNA repair. DNA double-strand breaks caused by ionizing radiation (IR) activate ATM kinase, whereas stalled replication forks caused by hydroxyurea (HU) and lesions caused by ultraviolet (UV) light activate the related kinase, ATR. Downstream effectors of ATM and ATR include BRCA1, NBS1, Mre11, FANCD2, Chk1 and Chk2 kinases, the histone H2A variant, H2AX, and p53 (Abraham, 2001; Taniguchi et al., 2002; Redon et al., 2002). Underscoring the importance of the S phase checkpoint, many S phase checkpoint genes, such as ATM, NBS1, Mre11, BRCA1 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000), and FANCD2 (Taniguchi et al., 2002), are mutated in human cancers.

The S phase checkpoint also maintains genome stability in the absence of external genotoxic stress. Inactivation of ATR (Brown and Baltimore, 2000; de Klein et al., 2000), Chk1 (Liu et al., 2000; Takai et al., 2000), Hus1 (Weiss et al., 2000), BRCA1 (Hakem et al., 1996; Liu et al., 1996), NBS1 (Zhu et al., 2001), or Mre11 (Xiao and Weaver, 1997) in normal somatic cells is lethal, and mouse cells lacking ATR exhibit high levels of chromosome abnormalities (Brown and Baltimore, 2000; de Klein et al., 2000). In addition, it was recently shown that deficiency of ATR in mammalian cells causes expression of "fragile sites," characterized by formation of gaps and breaks on metaphase chromosomes (Casper et al., 2002). Depletion of xMre11 from *X. laevis* cell-free extracts causes the accumulation of double-strand breaks in S phase (Costanzo et al., 2001). In yeast, mutant alleles, such as *mec1*, *mre11*, *chk1*, and *rad53* (inactivated yeast homologs of human ATR/ATM, Mre11, Chk1, and Chk2, respectively), cause spontaneous "gross chromosomal rearrangements" (GCRs) (Kolodner et al., 2002). In sum, the S phase checkpoint protects against spontaneous DNA damage that arises in a normal S phase.

One likely source of spontaneous damage is stalled replication forks that are processed to Holliday junctions and double-strand breaks (Osborn et al., 2002). A cell's response to stalled forks depends on the S phase checkpoint. In yeast, the checkpoint is required to reinitiate DNA replication after transient HU-mediated arrest (Desany et al., 1998), to maintain stable replication forks in the presence of an HU-mediated arrest (Lopes et al., 2001), and to prevent collapse of replication forks in response to methyl methanesulphonate (MMS)-mediated DNA damage (Tercero and Diffley, 2001). In the absence of exogenous DNA-damaging agents, Mec1 promotes fork progression through "replication slow zones," where forks have a tendency to stall (Cha and Kleckner, 2002)

Electron microscopy studies showed that in wild-type yeast, stalled forks retain a bifurcated, Y-shaped appearance. In cells lacking the S phase checkpoint, however, stalled forks frequently reversed to form Holliday junction-like "chickenfoot" structures that, by inappropriate processing, could give rise to double-strand breaks (Sogo et al., 2002). Therefore, the S phase checkpoint acts to prevent stalling and collapse of replication forks and, consequently, DNA damage and genome instability. The factors that influence the frequency of stalled forks are poorly understood.

In S phase, nucleosomes are assembled onto newly synthesized DNA within a few hundred base pairs of the fork by chromatin assembly factors, including CAF-I and ASF1 (Tyler, 2002). CAF-I is a heterotrimeric complex consisting of p150CAF-I, p60CAF-I, and p48CAF-I (Smith and Stillman, 1989). Direct binding of p150CAF-I to the replication processivity protein, PCNA, targets CAF-I to sites of DNA synthesis and contributes to coupling of DNA synthesis and chromatin assembly (Krawitz et al., 2002; Marheineke and Krude, 1998; Martini et al., 1998; Moggs et al., 2000; Shibahara and Stillman, 1999). We showed recently that repression of histone synthesis triggers S phase arrest in human cells, suggesting that

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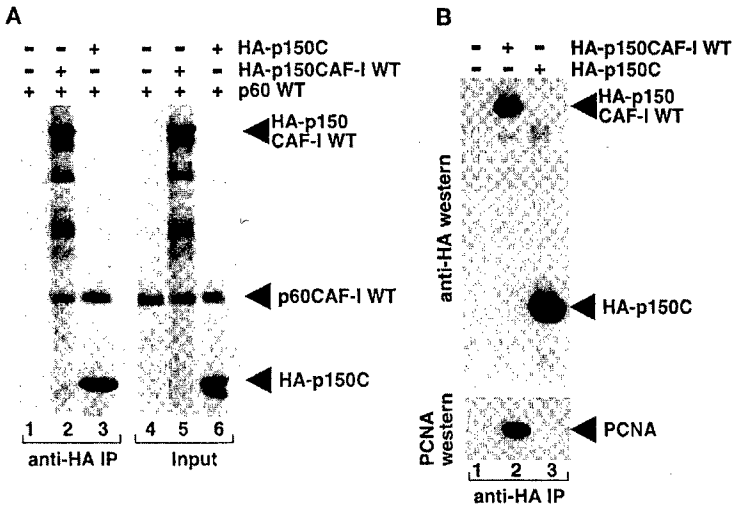

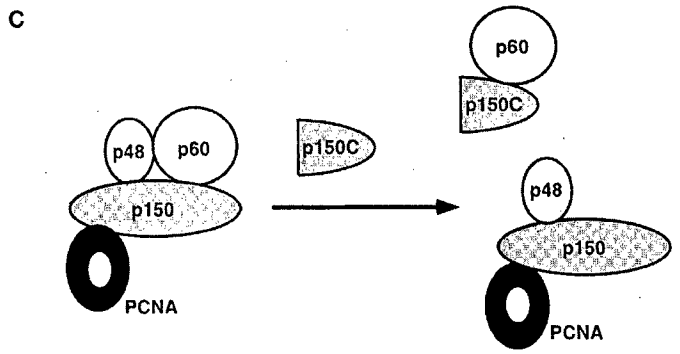

Figure 1. HA-p150C Is a Putative Dominant-Negative Inhibitor of CAF-I

(A) ^{35}S-labeled p60CAF-I was in vitro translated with or without cotranslation of HA-p150CAF-IWT or HA-p150C as indicated. Reactions were immunoprecipitated with an anti-HA antibody (12CA5, lanes 1–3) and fractionated by SDS-PAGE. Lanes 4–6 contain 20% of input proteins.

(B) U2OS cells were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150C as indicated. Cell lysates were immunoprecipitated with an anti-HA antibody (Y11) and Western blotted with antibodies to HA (12CA5) or PCNA (PC10) as indicated.

(C) A model for the proposed mode of action of HA-p150C (see text for details).

DNA synthesis and chromatin assembly are obligatorily coupled (Nelson et al., 2002). Here, we directly tested whether disruption of S phase chromatin assembly affected DNA synthesis by inhibition of CAF-I. Indeed, inhibition of CAF-I blocked DNA synthesis, induced DNA damage, and activated the S phase checkpoint. These results suggest that errors in chromatin assembly, either spontaneous or resulting from genetic mutations or environmental agents, are likely to increase the rate of DNA mutation and genome instability.

Results

A Dominant-Negative Mutant of p150CAF-I

As described previously, both HA-p150CAF-IWT and HA-p150C bound stably to p60CAF-I (Figure 1A), whereas only HA-p150CAF-IWT bound stably to PCNA (Figure 1B) (Kaufman et al., 1995; Moggs et al., 2000). We reasoned that HA-p150C could behave as a dominant-negative inhibitor of chromatin assembly by CAF-I via titration of p60CAF-I into nonfunctional complexes (Figure 1C).

During replication-coupled, CAF-I-dependent chromatin assembly assays in vitro, incorporation of newly replicated plasmid DNA into nucleosomes causes the DNA to become negatively supercoiled (Smith and Stillman, 1989). In this assay, HA-p150C inhibited CAF-I-dependent chromatin assembly (Figures 2A and 2B). There was no effect on DNA synthesis (production of ^{32}P-labeled plasmid), indicating that it does not perturb the progression of replication forks directly. Importantly, inhibition of nucleosome formation by HA-p150C was abolished by excess purified, recombinant human CAF-I (Figure 2B), confirming that HA-p150C acts as a specific inhibitor of CAF-I.

We predicted that overexpression of HA-p150C in human cells would disrupt the interaction between endogenous p60CAF-I and p150CAF-I (Figure 1C). Indeed, endogenous p150CAF-I coprecipitated with endogenous p60CAF-I in the absence but not the presence of ectopically expressed HA-p150C (Figure 2C). Additionally, expression of HA-p150C in cells resulted in a dramatic reduction in the total amount of p150CAF-I (Figure 2C, lanes 5–8), suggesting that p150CAF-I is degraded when not incorporated into the CAF-I complex. Expression of p60CAF-I was unaffected by HA-p150C (Figure 2D). However, p60CAF-I was stably bound to chromatin in punctate DNA replication foci in 45% of control cells but only 15% of the cells expressing HA-p150C (Figure 2E; see note in Experimental Procedures). These data indicate that HA-p150C disrupts the interaction between endogenous p150CAF-I and p60CAF-I and prevents tight association of p60CAF-I with chromatin and sites of DNA synthesis.

Inhibition of DNA Synthesis

We next tested whether HA-p150C affected DNA synthesis in vivo. As shown in Figure 3A, mock-transfected cells released synchronously into S phase progressed normally through S phase. In contrast, cells transiently

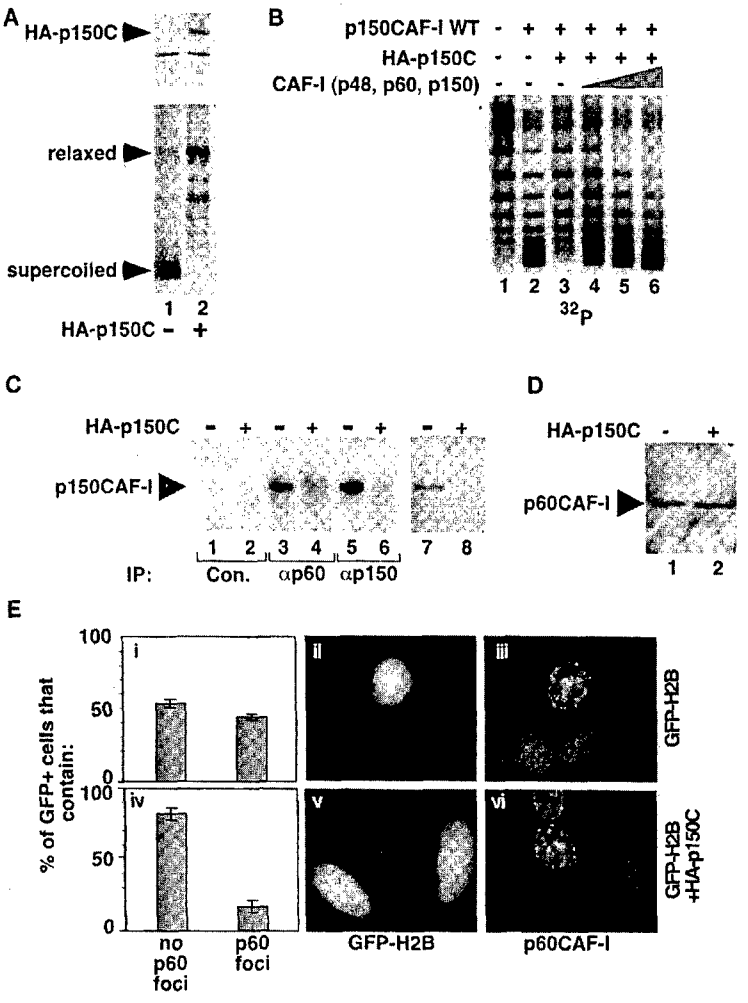

Figure 2. HA-p150C Inhibits CAF-I-Dependent Chromatin Assembly In Vitro and Disrupts the Endogenous CAF-I Complex In Vivo

(A) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed with purified p150CAF-I and an in vitro translation reaction that was unprogrammed (lane 1) or expressed HA-p150C (lane 2). HA-p150C was detected by Western blotting with anti-HA (12CA5, upper panel). Purified ^{32}P-labeled replicated plasmid DNA was fractionated by agarose gel electrophoresis (lower panel) to resolve relaxed (nonnucleosomal) and supercoiled (nucleosomal) plasmids.

(B) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed in the absence or presence of ~4 ng p150CAF-IWT, in vitro-translated HA-p150C, and purified recombinant trimeric CAF-I complex (5.5, 11, or 22 ng as indicated by the triangle).

(C) U2OS cells were transiently transfected with pCMV-CD19 together with pcDNA3 HA-p150C or pcDNA3 as indicated. Transfected cells were immunopurified with anti-CD19 coated magnetic beads, and lysates were prepared and immunoprecipitated with antibodies to p60CAF-I (SS24), p150CAF-I (SS1), or control (419) as indicated, fractionated by SDS-PAGE, and Western blotted with an antibody to p150CAF-I (SS1). Lanes 7 and 8 contain 150 μg of whole-cell lysate derived from immunopurified transfected cells.

(D) Lysates from (C) were Western blotted with antibodies to p60CAF-I (a cocktail of SS3, SS53, SS60, and SS96).

(E) U2OS cells were transiently transfected with pBOS-GFP-H2B in the absence (Ei–Eiii) or presence (Eiv–Evi) of pCDNA3 HA-p150C. The cells were stained with antibodies to p60CAF-I (SS75) and visualized by immunofluorescence microscopy to detect GFP-H2B and p60CAF-I. 100 cells were counted and scored as p60CAF-I positive or negative. The results of two independent experiments are plotted in (Ei) and (Eiv).

transfected with a plasmid encoding HA-p150C had a profound defect in S phase progression. Many failed to detectably exit G1 phase, and most of those that did arrested within S phase. We also measured DNA synthesis by pulse labeling with 5′-BrdU at a time when the cells had accumulated in S phase. Most of the HA-p150C-expressing cells failed to incorporate 5′-BrdU and thus were not actively synthesizing DNA (Figure 3B). (Thirteen percent of HA-p150C-expressing cells were 5′-BrdU positive compared to 56% of the untransfected cells on the same coverslip; these results are representative of more than five similar experiments.) Therefore, both FACS analysis and 5′-BrdU labeling demonstrated that HA-p150C inhibited DNA synthesis. In contrast, full-length HA-p150CAF-IWT failed to inhibit DNA synthesis (Figures 3D and 3E), and coexpression of HA-p150CAF-IWT with HA-p150C abolished the arrest (Figures 3F and 3G). These data confirm that the effect of HA-p150C on DNA synthesis depends on its ability to perturb the endogenous CAF-I complex. Significantly, the cell cycle arrest induced by HA-p150C was indistinguishable from the arrest induced by ectopic expression of human HA-HIRA (Figure 3C), a protein whose ectopic expression represses histone gene expression, thus indirectly inhibiting chromatin assembly (Hall et al., 2001; Nelson et al., 2002).

The model in Figure 1C predicts that overproduction of any fragment of p150CAF-I that binds p60CAF-I but not PCNA will inhibit DNA synthesis. Consistent with this idea, HA-p150CAF-I(451-938), which did not bind PCNA, efficiently induced arrest, but HA-p150CAF-I(250-938), which did bind to PCNA, failed to induce arrest (Figures 4A and 4C). These data also indicated that the PCNA binding domain of HA-p150CAF-IWT is between residues 250 and 451, consistent with previous sequence analysis which identified a partial consensus PCNA binding domain at residues 421–431 (Krawitz et al., 2002). As anticipated, deletion of residues 421–431 produced a polypeptide (HA-p150CAF-IΔPCNA) that bound to p60CAF-I but not PCNA and inhibited DNA synthesis (Figures 4A–4C). Expression of HA-p150CAF-IΔPCNA did not affect localization of PCNA to DNA replication foci (Figure 4D; see note in Experimental Procedures) (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985). Thus, perturbation of CAF-I inhibits DNA synthesis but, as far as we can tell from Figure 4D, does not affect assembly of DNA replication foci.

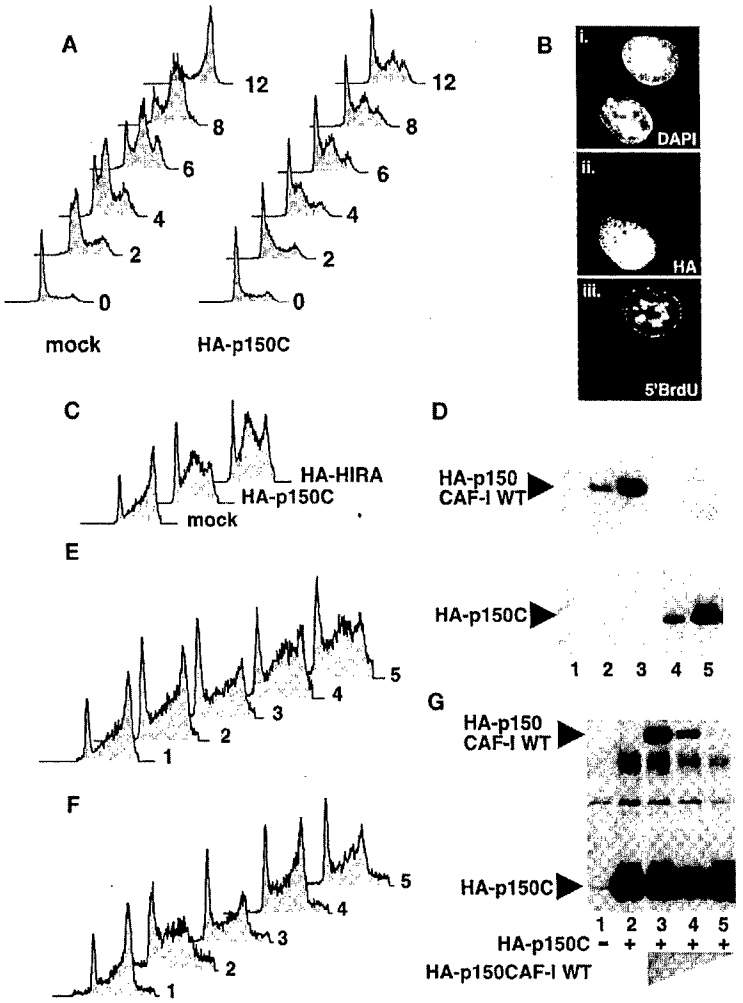

Figure 3. HA-p150C Blocks DNA Synthesis and Progression through S Phase

(A) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3 or pcDNA3 HA-p150C as indicated. 16 hr later, the cells were arrested in mimosine for 20 hr and released into S phase, and at time intervals afterwards, the cell cycle distribution of the $CD19^+$ cells was determined by FACS.

(B) U2OS cells were transiently transfected with pcDNA3 HA-p150C, and 36 hr later were pulse labeled for 30 min with 10 μM 5′-BrdU and stained with DAPI to visualize the DNA (Bi), anti-HA (Y11) (Bii), and anti-5′-BrdU-FITC (Biii).

(C) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3, pcDNA3 HA-p150C, or pcDNA3 HA-HIRA, as indicated, and processed as in (A).

(D) U2OS cells were transfected with pCMV-CD19 together with pcDNA3 (lane 1), 14 and 28 μg of pcDNA3 HA-150CAF-IWT (lanes 2 and 3, respectively), or 1 and 3 μg of pcDNA3 HA-p150C (lanes 4 and 5, respectively). 5 μg of whole-cell lysate was fractionated by SDS-PAGE and Western blotted with anti-HA (12CA5). Lanes 1–3 and 4–5 of are nonadjacent lanes from the same exposure of the same gel.

(E) The same transfections as (D) processed as in (A).

(F) U2OS cells were transfected with pCMV CD19 together with pcDNA3 HA-p150C or pDNA3, as indicated, and decreasing amounts of pcDNA3 HA-p150CAF-IWT (as indicated by the shaded triangle in [G]). The cells were processed as in (A).

(G) 5 μg of whole-cell lysate from cells in (F) was fractionated by SDS-PAGE and Western blotted with anti-HA (12CA5).

Additionally, we predicted that fragments of HA-p150CAF-I that fail to bind to both PCNA and p60CAF-I should fail to induce S phase arrest. As anticipated, deletion of the C-terminal p60CAF-I binding site (Kaufman et al., 1995) from HA-p150CAF-IΔPCNA protein resulted in a polypeptide, HA-p150CAF-IΔPCNA(1-547), that failed to bind to either p60CAF-I or PCNA and did not induce S phase arrest (Figures 5A and 5B).

We expected that the cells arrested in S phase would have defective chromatin structure. Digestion with micrococcal nuclease (MNase) was used to probe the chromatin structure of cells in S phase (Nelson et al., 2002). Chromatin from HA-p150CAF-I(451-938)-expressing cells was indeed more sensitive to digestion than chromatin from mock-transfected control cells (Figures 5C and 5D). We conclude that disruption of the endogenous CAF-I complex induces chromatin abnormalities.

Induction of DNA Damage

One cause of S phase arrest is DNA damage (Abraham, 2001). An early response to DNA damage, particularly double-strand breaks, is phosphorylation of histone H2AX in chromatin surrounding the lesion (Redon et al., 2002). To determine whether the DNA in arrested cells contained double-strand breaks, we tested whether H2AX was phosphorylated. Phosphorylated H2AX (γH2AX) staining was enriched in HA-p150C- and HA-p150CAF-IΔPCNA-expressing cells relative to the untransfected cells or cells expressing HA-p150CAF-IWT (Figures 6A and 6B). Significantly, nuclei that had the brightest γH2AX staining often contained visibly abnormal DAPI-stained nuclear structures, consistent with a defect in chromatin assembly and/or extensive DNA fragmentation (Figure 6A).

In addition, even without MNase treatment, the DNA extracted from HA-p150CAF-I(451-938)-expressing cells reproducibly migrated faster on agarose gels than DNA from mock-transfected cells or cells transfected with HA-p150CAF-I (Figures 5C and 6C; data not shown). However, at the same time after transfection (36 hr), the dominant-negative fragments of HA-p150CAF-I did not detectably induce three characteristic markers of apoptosis: chromatin condensation, cleavage of genomic DNA into a "nucleosomal ladder," and cells with less than 2n DNA content (Loo and Rillema, 1998) (Figures 6 and 3C; data not shown). These data suggest that

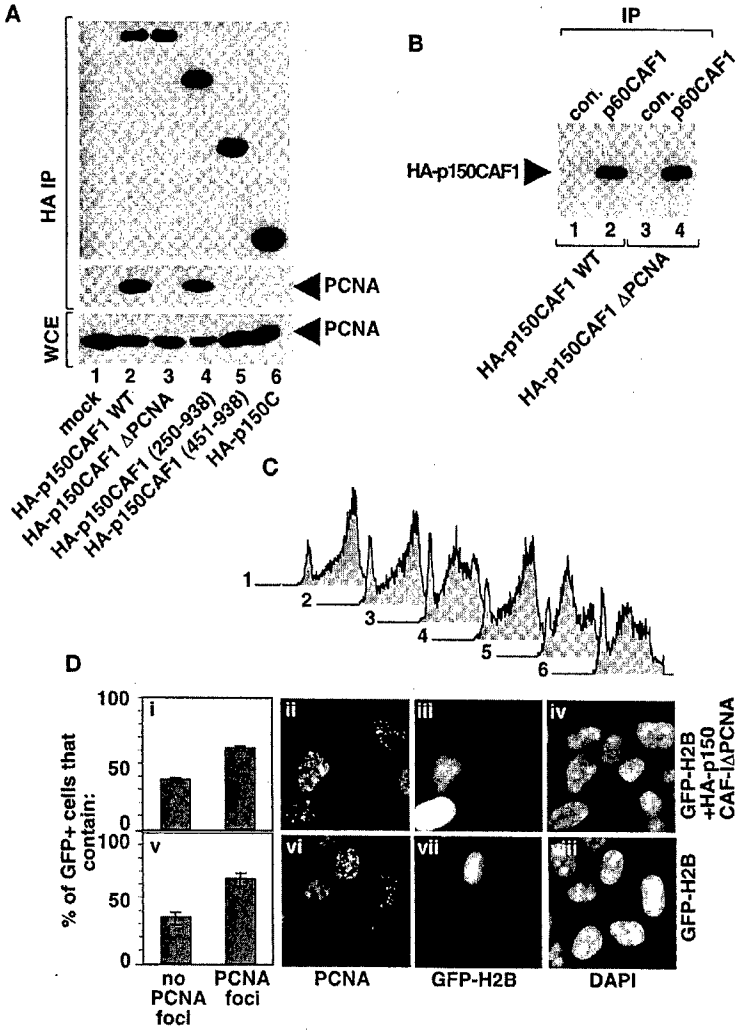

Figure 4. S Phase Arrest Correlates with Failure of HA-p150CAF-I Mutants to Bind to PCNA

(A) U2OS cells were transiently transfected with pcDNA3, pcDNA3 HA-p150CAF-I WT, or the mutants derived from HA-p150CAF-I, as indicated. Cell lysates were immunoprecipitated with anti-HA antibodies (Y11) and then Western blotted with anti-HA (12CA5, top panel) or anti-PCNA (PC10, middle panel). The bottom panel (WCE) shows an anti-PCNA (PC10) Western blot of 10 μg of the whole-cell lysate.

(B) U2OS cells were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150CAF-IΔPCNA as indicated. Cell lysates were immunoprecipitated with antibodies to p60CAF-I (SS24) or a control antibody of the same subclass ("con.," 419, IgG_1) and Western blotted with anti-HA (12CA5).

(C) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3, pcDNA3 HA-p150CAF-I WT, or the same set of mutants used in (A) and indicated by the same numbers 1–6. The cells were processed as in Figure 3A.

(D) U2OS cells grown on coverslips were transiently transfected with pBOS GFP-H2B in the absence (Dv–Dviii) or presence (Di–Div) of pcDNA3 HA-p150CAF-IΔPCNA. The cells were stained with antibodies to PCNA, and expression of GFP-H2B (Diii and Dvii) and localization of PCNA (Dii and Dvi) were visualized by immunofluoresence microscopy. 100 GFP-H2B-positive cells were scored as PCNA positive or negative, and the results were plotted (Di and Dv). The results shown are the means of two independent experiments.

inhibition of CAF-I results in DNA double-strand breaks but not a general apoptotic program.

Activation of the S Phase Checkpoint

Phosphorylation of H2AX in response to external genotoxic stresses is mediated by ATR and/or ATM kinases (Redon et al., 2002), suggesting that one or both of these kinases is activated in cells expressing HA-p150C. Activation of ATR is accompanied by its relocalization to discrete nuclear foci (Tibbetts et al., 2000). Expression of HA-p150C induced nuclear ATR foci in 23.2% of cells (standard deviation [SD] of 6.6% from four independent experiments) compared to less than 1% of HA-p150CAF-IWT-expressing or untransfected cells (Figure 7). Similarly, HA-HIRA also activated ATR, as judged by induction of nuclear ATR foci (data not shown).

If the ATR foci are linked to S phase arrest, they should exist in S phase cells. Under the conditions used to optimally detect both ATR foci and PCNA in U2OS cells, the S phase cells were readily identified by their "granular" PCNA staining pattern (Figures 7Aiv–7Avi). Seventy-three percent of the HA-p150C-expressing cells that contained ATR foci were in S phase (asterisks in Figures 7Ai–7Aiii) compared to 51% of the untransfected cells, suggesting that the ATR foci formed preferentially in S phase. However, very few (less than 1%) of the HA-p150C-expressing cells that contained ATR foci were actively synthesizing DNA, as measured by pulse labeling with 5′-BrdU (Figures 7Avii–7Aix). Taken together, these results are consistent with the idea that ATR foci are present in cells arrested in S phase. In addition to H2AX, other downstream effectors of ATR and ATM include the p53 tumor suppressor protein and BRCA1. Phosphorylation of p53 on serine 15 is catalyzed by ATR and ATM (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999) and contributes to stabilization of the protein (Vousden, 2002). Both HA-p150C and HA-HIRA stabilized p53 (Figures 7B and 7C) and induced phosphorylation of p53 on serine 15. In the case of BRCA1, activation by genotoxic stress results in dispersal of small nuclear foci normally present in S phase (Scully et al., 1997). Only 13% (SD = 1.2%, three separate experiments) of the HA-p150C-expressing cells that contained ATR foci contained detectable BRCA1 foci (Figures 7Ax–7Axii), compared to 50.7% (SD = 13.4%, three separate experiments) of the untransfected cells

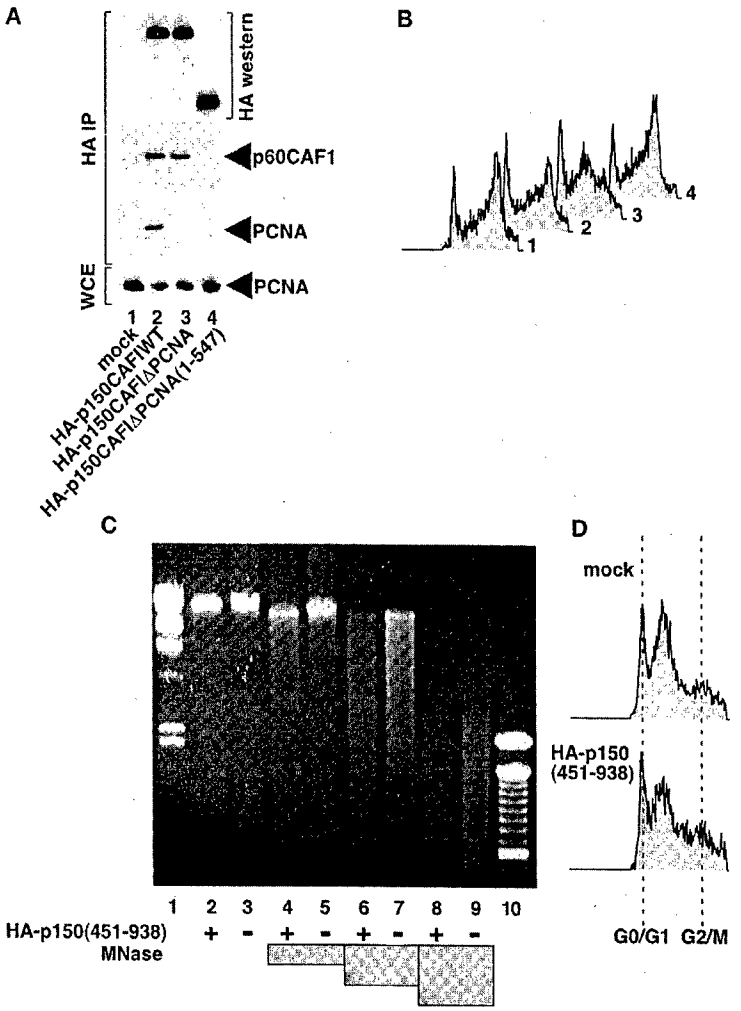

Figure 5. S Phase Arrest Correlates with Sequestration of p60CAF-I and Is Accompanied by Defects in Chromatin Structure

(A) U2OS cells were transiently transfected with pcDNA3 (lane 1), pcDNA3-HAp150CAF-IWT (lane 2), pcDNA3HAp150CAF-IΔPCNA (lane 3), or pcDNA3HAp150CAF-IΔPCNA(1-547) together with pCMV CD19. Lysates were immunoprecipitated with antibodies to anti-HA (Y11) and then Western blotted with antibodies to HA (12CA5), p60CAF-I, and PCNA. The bottom panel is a Western blot of PCNA in the crude lysate.

(B) As in (A), except that the cell cycle distribution of $CD19^+$ cells was determined as in Figure 3A.

(C) U2OS cells were transiently transfected with pcDNA3 (lanes 3, 5, 7, and 9) or pcDNA3 HAp150CAF-I(451-938) (lanes 2, 4, 6, and 8) together with pCMV CD19. The cells were arrested in mimosine for 20 hr and then washed to release them into S phase for 6 (pcDNA3) or 8 hr (pcDNA3 HAp150CAF-I[451-938]) to give equal proportions of cells in S phase. $CD19^+$ cells were immunopurified, nuclei prepared, and treated with 0, 0.5, 1.5, and 5 Worthington Units/ml of MNase as indicated by the shaded boxes. Genomic DNA was purified and fractionated by agarose gel electrophoresis. Lane 1 contains 1 μg of phage λ DNA digested with HindIII, and lane 10 contains 1 μg of a 100 bp ladder.

(D) The same transfection as in (C), but the cell cycle distribution of the $CD19^+$ cells was determined as in Figure 3A. Top, pcDNA3; bottom, pcDNA3 HAp150CAF-I(451-938).

on the same coverslip. BRCA1 activation is also frequently reflected by hyperphosphorylation and relocalization of BRCA1 to morphologically distinct nuclear foci that colocalize with PCNA, ATR, and DNA repair proteins (Gatei et al., 2001; Scully et al., 1997; Tibbetts et al., 2000; Khanna and Jackson, 2001), but neither of these responses was observed in HA-p150C-expressing cells. Significantly, IR has not always been found to induce BRCA1 foci, and complete phosphorylation is not essential for relocalization of BRCA1 to damage-induced foci (Scully et al., 1997; Cortez et al., 1999; Gatei et al., 2000). Conceivably, BRCA1 phosphorylation and detectable DNA damage-induced foci depend upon recruitment of BRCA1 to a specific chromatin structure that is absent from cells in which chromatin assembly is perturbed. Whatever the reason, we conclude that disruption of chromatin assembly results in ATR-/ATM-mediated phosphorylation and stabilization of p53 and dispersal of BRCA1 foci, consistent with activation of S phase checkpoint signaling.

In response to exogenous genotoxic stress, activated ATR and ATM trigger cell cycle arrest (Abraham, 2001). Caffeine, an inhibitor of ATR and ATM (Blasina et al., 1999; Sarkaria et al., 1999), abolished the HA-p150C-induced S phase accumulation (Figure 7D), indicating that one or both kinases are required for the arrest. Caffeine abolished the arrest regardless of whether it was added before or after onset of arrest, indicating that it does act simply by blocking S phase entry (data not shown). Significantly, when cells were harvested 60 hr after transfection, HA-p150CAF-IΔPCNA decreased the viability of the cells, and this was potentiated by caffeine (Figure 7E). In contrast, expression of a dominant-negative mutant of ATR (ATRkd) (Cliby et al., 1998) had no effect on the arrest induced by HA-p150C in U2OS cells (data not shown), suggesting that ATM also contributes. Indeed, coexpression of ATRkd with HA-p150CAF-I ΔPCNA or HA-p150C in $ATM^{-/-}$ cells completely abolished the inhibition of DNA synthesis (Figure 7F; data not shown). This shows that simultaneous inactivation of both ATR and ATM is sufficient to prevent a viable cell cycle arrest by HA-p150CAF-IΔPCNA and HA-p150C.

Discussion

Our data demonstrate that disruption of CAF-I inhibits S phase progression and causes DNA damage in human

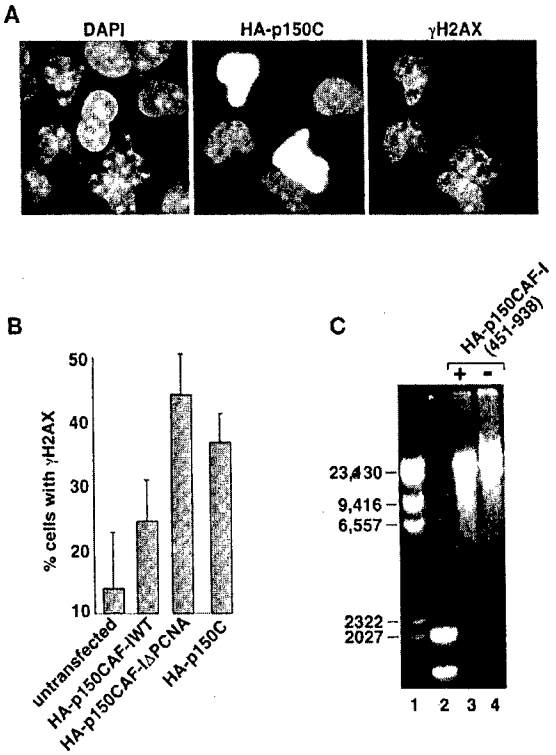

Figure 6. Dominant-Negative HA-p150CAF-I Mutants Induce DNA Damage

(A) U2OS cells on coverslips were transiently transfected with pcDNA3 HAp150C. The cells were stained with DAPI, anti-γH2AX antibodies, or anti-HA antibodies, as indicated, and visualized by immunofluorescence microscopy.

(B) 100 transfected HA^+ cells or 100 untransfected HA^- cells from the same coverslip were scored as positive or negative for γH2AX. The results are the mean of 12 (untransfected), 8 (HA-p150CAF-IWT and HA-p150C), or 2 (HA-p150CAF-IΔPCNA) independent experiments.

(C) U2OS cells were transiently transfected with pcDNA3 (lane 4) or pcDNA3 HAp150CAF-I(451-938) (lane 3) together with pCMV CD19. The cells were processed, and genomic DNA was purified as in lanes 2 and 3 of Figure 5C (without MNase). Lane 1 contains 1 μg of phage λ DNA digested with HindIII (MW markers indicated on left of gel), and lane 2 contains 1 μg of a 100 bp ladder.

cells. We propose that inhibition of S phase chromatin assembly causes stalled replication forks, which are inappropriately processed to DNA double-strand breaks. Most likely, stalled forks and double-strand breaks are responsible for ATR- and ATM-dependent checkpoint activation and cell cycle arrest.

CAF-I Is Required for S Phase Progression

Several lines of evidence indicate that inhibition of DNA synthesis by HA-p150C and HA-p150CAF-IΔPCNA results from inhibition of CAF-I-dependent chromatin assembly. First, HA-p150C inhibits chromatin assembly but not DNA synthesis in vitro, disrupts the interaction between endogenous p150CAF-I and p60CAF-I in vivo, and blocks stable association of p60CAF-I with replication foci in vivo (Figure 2). Second, HA-p150C-induced inhibition of DNA synthesis is abolished by coexpression of HA-p150CAF-IWT, just as excess trimeric CAF-I complex restores nucleosome assembly to HA-p150C-inhibited reactions in vitro (Figures 2 and 3). Third, the ability of HA-p150CAF-IWT and HA-p150CAF-I fragments to inhibit cell cycle progression correlates inversely with their ability to bind PCNA (Figure 4). Fourth, inhibition of DNA synthesis is not due to total disruption of DNA replication foci, because in arrested cells, PCNA was still localized in a punctate pattern characteristic of normal replication foci (Figure 4D). Fifth, the ability of HA-p150CAF-I fragments to inhibit cell cycle progression appears to depend upon their ability to sequester endogenous p60CAF-I away from the endogenous p150CAF-I/PCNA complex, because a mutant that fails to bind to both PCNA and p60CAF-I is inert (Figure 5). Finally, chromatin from S phase cells expressing a dominant-negative HA-p150CAF-I fragment was more sensitive to MNase digestion than chromatin from control S phase cells, showing directly that inhibition of CAF-I in S phase induces defects in chromatin structure (Figure 5C). Together, these data strongly suggest that disruption of CAF-I-dependent chromatin assembly is responsible for inhibition of DNA synthesis.

Cell cycle arrest induced by HA-p150C and HA-p150CAF-IΔPCNA is similar to that induced by human HIRA (Hall et al., 2001; Nelson et al., 2002) (Figure 3C). Previously, we showed that repression of histone synthesis was the direct cause of HIRA-induced S phase arrest (Nelson et al., 2002). Therefore, direct inhibition of chromatin assembly by HA-p150C and HA-p150CAF-IΔPCNA or, presumably, indirect inhibition of chromatin assembly by HIRA-mediated repression of histone synthesis both block S phase DNA synthesis in human cells. Importantly, the fact that HA-p150C and repression of histone synthesis have identical effects on S phase very strongly suggests that chromatin assembly, rather than DNA synthesis per se, is the target of HA-p150C.

Previous investigations suggested that inhibition of chromatin assembly would not block DNA synthesis in S phase. First, in yeast none of the likely DNA synthesis-linked chromatin assembly factors identified to date, such as CAF-I, Asf1, or the Hir proteins, is essential for viability either alone or in combination (Kaufman et al., 1998; Sharp et al., 2001; Tyler et al., 1999). Second, in vitro replication of plasmid DNA in mammalian cell extracts or *D. melanogaster* embryo extracts does not require chromatin assembly (Bulger et al., 1995; Stillman, 1986). Third, yeast expressing histones from conditional promoters replicate their entire genome when new histone synthesis and chromatin assembly are blocked (Han et al., 1987; Kim et al., 1988). Fourth, in *C. elegans* and *D. melanogaster* embryos, the reduced histone synthesis caused by mutant alleles of SLBP have no obvious effect on DNA synthesis (Sullivan et al., 2001; Kodama et al., 2002). Fifth, although in *X. laevis* perturbation of CAF-I blocks development past the mid-blastula transition, it has no detectable effect on a somatic cell line (Quivy et al., 2001). In contrast, our data demonstrate that in intact human somatic cells, inhibition of chromatin assembly blocks DNA synthesis in S phase. Thus, DNA synthesis and chromatin assembly appear to be more tightly coupled in intact human cells than in other model organisms.

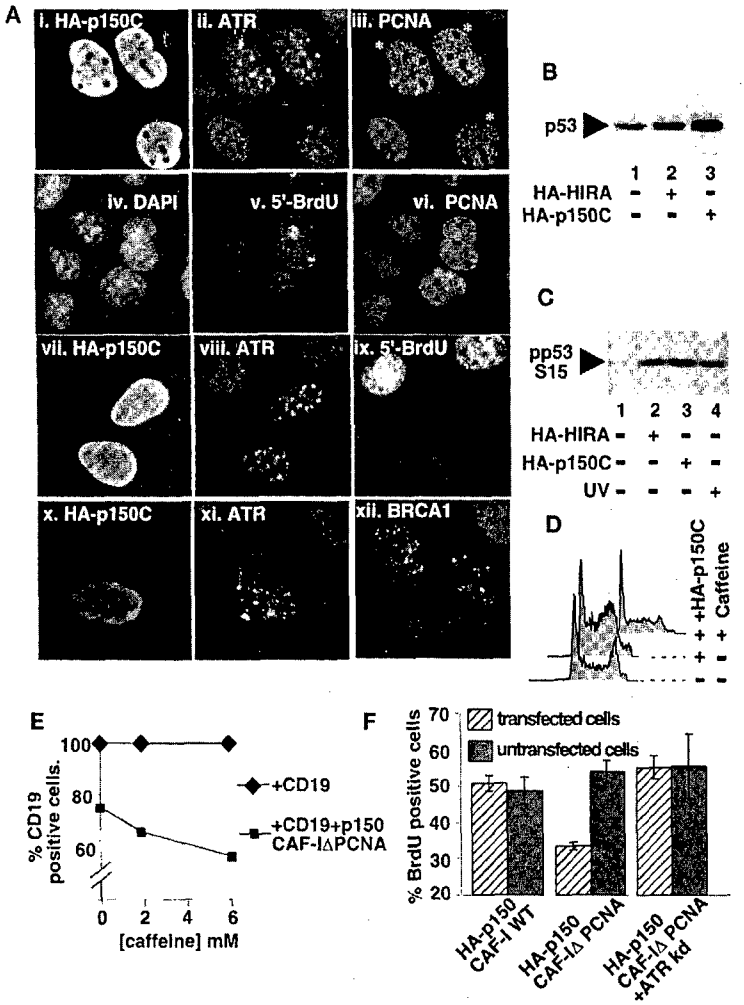

Figure 7. S Phase Arrest Depends upon Activation of the S Phase Checkpoint

(A) U2OS cells on coverslips were transiently transfected with pcDNA3 HA-p150C. In (Aiv)–(Aix), cells were pulse labeled for 15 min with 5′-BrdU prior to harvesting. Cells were stained with antibodies to HA, ATR, and PCNA (Ai–Aiii); 5′-BrdU and PCNA (Aiv–Avi); HA, ATR, and 5′-BrdU (Avii–Aix); and HA, ATR, and BRCA1 (Ax–Axii).

(B) U2OS cells were transiently transfected with pcDNA3 (lane 1), pcDNA3 HA-HIRA(421-729) (lane 2), or pcDNA3 HAp150C (lane 3) together with pCMV CD19. $CD19^+$ cells were immunopurified with magnetic beads, and cell lysates were Western blotted with an antibody to p53.

(C) Cell lysates from (B) were Western blotted with an antibody to p53pSer15. Lane 4 contains lysate from cells irradiated with UV light.

(D) U2OS cells were transiently transfected with a plasmid, pCMV CD19, in the absence or presence of pcDNA3 HA-p150C. 16 hr later, the cells were treated with 1.7 mM caffeine or PBS, and 24 hr later, the cell cycle distribution of the $CD19^+$ cells was determined.

(E) U2OS cells were transiently transfected with pCMV CD19 in the absence or presence of pcDNA3 HA-p150CAF-IΔPCNA. 36 hr later, the cells were treated with 2 or 6 mM caffeine or PBS, and 24 hr later, the percentage of the $CD19^+$ cells was determined by FACS. The number of $CD19^+$ HA-p150CAF-IΔPCNA-expressing cells is expressed as a percentage of the control (CD19 alone) at each dose of caffeine.

(F) $ATM^{-/-}$ human fibroblasts were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150CAF-I ΔPCNA in the absence or presence of a pcDNA3 ATRkd as indicated. 36 hr later, the cells were pulse-labeled for 1 hr with 10 μM 5′-BrdU and stained with anti-HA(Y11) and anti-5′-BrdU and DAPI. 100 untransfected cells (HA-negative) and 100 transfected cells (HA-positive) were scored as 5′-BrdU positive or negative and expressed as the percentage of 5′-BrdU positive. Each bar with standard deviation is the mean of four separate experiments.

Defects in Chromatin Assembly Cause DNA Damage

Inhibition of chromatin assembly induces DNA double-strand breaks as measured by accumulation of γH2AX and fragmentation of genomic DNA (Figure 6). These breaks are likely to result from inappropriate processing of stalled replication forks (Osborn et al., 2002). Several scenarios that result in damage are possible. First, PCNA binds to both replication and chromatin assembly proteins, such as DNA polymerase δ and p150CAF-I, respectively (Warbrick, 2000), suggesting that replication and chromatin assembly machineries function as an integrated complex in which disruption of one affects the other. Second, failure to package newly replicated DNA into chromatin might result in steric constraints that impede fork progression. Third, inhibition of chromatin assembly might not increase the rate of fork stalling but the frequency with which stalled forks are processed to double-strand breaks. Failure to incorporate the newly synthesized DNA into nucleosomes might increase the frequency of fork reversal, formation of a "chickenfoot," and resolution of this structure to give a double-strand break (Osborn et al., 2002; Sogo et al., 2002).

Checkpoint Activation

Several lines of evidence indicate that inhibition of chromatin assembly activates the S phase checkpoint. First, H2AX, a known substrate of ATR and ATM kinases, is phosphorylated (Figures 6A and 6B) (Redon et al., 2002). Second, ATR is recruited into nuclear foci, an indicator of ATR activation (Figure 7A) (Tibbetts et al., 2000). Third, p53 is stabilized and phosphorylated on serine 15, a known ATR and ATM phosphorylation site (Figures 7B and 7C) (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Fourth, the S phase BRCA1 foci in HA-p150C-expressing cells that contain ATR foci are dispersed, a marker of BRCA1 activation (Figure 7A) (Scully et al., 1997). Fifth, inactivation of the S phase checkpoint abolishes the S phase arrest and decreases cell viability (Figure 7D–7F), suggesting that a failure to arrest DNA synthesis is lethal to the cell. Taken together, these results show that inhibition of chromatin assembly activates the S phase checkpoint.

Conceivably, the defect in chromatin assembly might be directly responsible for checkpoint activation. Perhaps consistent with this idea, it was recently shown

that treatments that perturb chromatin structure without apparently inducing DNA damage, such as hypotonic buffer, chloroquine or trichostatin A, activate ATM kinase (Bakkenist and Kastan, 2003). However, the simplest model to explain checkpoint activation is that defects in chromatin assembly cause stalling of replication forks and double-strand breaks (Figure 6) and that these structures activate the ATR- and ATM-dependent checkpoints, similar to HU and IR (Abraham, 2001).

Chromatin Assembly and Genome Stability

Cancer cells are characterized by a high frequency of genome abnormalities (Lengauer et al., 1998), including "gross chromosomal rearrangements" (GCRs), such as translocations and large deletions. In the absence of an S phase checkpoint, progression though a normal S phase is associated with a high frequency of GCRs (Kolodner et al., 2002). This suggests that S phase is an inherently mutagenic process and that one function of the checkpoint is to suppress formation of GCRs. In addition, the S phase checkpoint prevents stalling of replication forks and stabilizes them after they have stalled (Cha and Kleckner, 2002; Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001). Since stalled replication forks can be processed to double-strand breaks, which are a potent source of GCRs (Osborn et al., 2002), it seems likely that the S phase checkpoint suppresses GCRs, at least in part, by protecting the integrity of replication forks and preventing conversion of stalled forks to double-strand breaks (Kolodner et al., 2002). However, the processes that influence replication fork stalling and formation of double-strand breaks in a normal S phase are largely unknown.

We propose that defects in S phase chromatin assembly cause double-strand breaks due to stalling and inappropriate processing of replication forks and that the S phase checkpoint limits the damage caused by defective chromatin assembly by stabilizing the stalled replication forks, inhibiting further DNA synthesis, and promoting DNA repair. If so, defects in chromatin assembly and inactivation of the S phase checkpoint should act synergistically to increase DNA damage. Indeed, Kolodner and coworkers have observed a synergistic effect in yeast on accumulation of GCRs due to mutations in the S phase checkpoint and chromatin assembly factors, such as *cac1* and *asf1* (K. Myung et al., personal communication).

In human cells, errors in chromatin assembly combined with inactivation of the S phase checkpoint might promote genome instability and neoplastic transformation. Several components of the S phase checkpoint, such as ATM, BRCA1, NBS1, Mre11 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000), and FANCD2 (Taniguchi et al., 2002), are known to be mutated in human cancer. Errors in chromatin assembly might occur spontaneously or result from genetic mutations or environmental agents that inhibit chromatin assembly factors. Admittedly, the phenotype reported here may represent an extreme case that results from near-total inactivation of CAF-I, and such a profound phenotype is, presumably, lethal in most cell contexts. However, more subtle defects in chromatin assembly that result from haploinsufficiency of chromatin assembly factors or from point mutations within the PCNA binding site of p150CAF-I (that weaken, but do not completely disrupt, the p150CAF-I/PCNA interaction) might be expected to be nonlethal but increase the error rate associated with DNA replication. Consistent with this idea, some genes encoding chromatin assembly factors, such as p150CAF1, p48CAF1, ASF1a, and ASF1b, are located in regions of chromosomes reported to be deleted in some cancers (19p13, 1p34, 6q22, and 19p13, respectively; Couch and Weber, 2000; Mertens et al., 1997; Oesterreich et al., 2001; Sheng et al., 1996). We are currently testing whether these genes are the targets of mutations in human cancers.

Experimental Procedures

Cell Culture and Transfections

U2OS cells were cultured, transfected, and synchronized as described previously (Adams et al., 1996; Nelson et al., 2002).

Plasmids

pcDNA3 Flag-ATRkd was a gift of Drs. Robert Abraham and Kathy Brumbaugh. pBOS-GFP-H2B was purchased from Becton-Dickinson. All other plasmids were generated using standard molecular biology procedures, and details are available on request.

CAF-I-Dependent In Vitro Chromatin Assembly Assays

In vitro SV40 DNA replication/nucleosome assembly assays were performed as described (Kaufman et al., 1995). The three-subunit CAF-I complex and the p150CAF-I subunit were produced in insect cells and purified as described (Kaufman et al. 1995). HA-p150C was expressed by in vitro translation using TnT T7 Quick for PCR DNA (Promega).

Immunological Techniques

Anti-HA (12CA5, mouse monoclonal) was purchased from Roche. Anti-HA (Y11, rabbit polyclonal), anti-PCNA (PC10 and FL261), and anti-ATR (C19) were purchased from Santa Cruz Biotech. Anti-p53 (Ab6) and anti-BRCA1 (Ab1) were purchased from Oncogene Research Products, and anti-p53pS15 was purchased from Cell Signaling. Anti-5′-BrdU-FITC was purchased from Becton-Dickinson. Anti-γH2AX was purchased from UBI. Anti-CD19-FITC was purchased from Caltag. Anti-p150CAF-I and p60CAF-I have been described previously (Smith and Stillman, 1991). Immunoprecipitation and Western blots were performed as described previously (Adams et al., 1996). When performing anti-HA immunoprecipitations followed by anti-HA Western blots, mouse (12CA5) and rabbit (Y11) anti-HA antibodies were used so as to avoid detection of antibody heavy chain in the Western blot.

Immunofluorescence was performed as described previously by us or others (Hall et al., 2001; Tibbetts et al., 2000). Detailed methods of two- and three-color immunofluorescence are available upon request. To optimize the detection of p60CAF-I and PCNA by immunofluorescence, it was necessary to preextract the cells with EBC (Adams et al., 1996). Under these conditions, HA-p150C and p60CAF-I not stably bound to chromatin were washed out of the cells. GFP-H2B served as an NP40-resistant marker of transfected cells (Figures 2E and 4D). GFP-H2B did not affect cell cycle- nor HA-p150C-induced S phase arrest (data not shown) (Kanda et al., 1998).

Collection and FACS of CD19$^+$-Transfected Cells

Collection using magnetic beads and FACS was described previously (Adams et al., 1996; Nelson et al., 2002).

Digestion with MNase and Purification of Genomic DNA

Transiently transfected (CD19$^+$) cells were collected with anti-CD19 coated beads. Permeabilized nuclei were prepared and treated with Mnase, and genomic DNA was purified as described previously (Hall et al., 2001; Nelson et al., 2002).

Acknowledgments

We thank Dr. Richard Kolodner for communication of unpublished results and anonymous reviewers for insightful comments. We thank Dr. Bruce Stillman for p150CAF-I and p60CAF-I antibodies, Dr. Tim Yen for ATM$^{-/-}$ fibroblasts, Drs. Robert Abraham and Kathy Brumbaugh for ATRkd, Dr. Bin-bing Zhou for advice, and Drs. Randy Strich and Song-Tao Liu for critical reading of the manuscript. The work of P.D.A. is supported by NIH RO1-GM62281. A.A.F. was supported by an NSF predoctoral training grant, an NIH predoctoral training grant, and NIH grant 1R01 GM55712 to P.D.K. P.D.K. was supported by the California Breast Cancer Research Program grant number 4JB-0089, Department of Energy Field Work Proposal KP110301, and by Department of Energy funds administered through the Lawrence Berkeley National Laboratory.

Received: August 5, 2002
Revised: December 5, 2002

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[Cell Cycle 2:3, 2-187, May/June 2003]; © 2003 Landes Bioscience

Extra Views

Coordination of S-Phase Events and Genome Stability

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Received 04/14/03; Accepted 04/15/03

Previously published online as a Cell Cycle Paper in Press at: http://www.landesbioscience.com/journals/cc/toc.php?volume=2&issue=3

KEY WORDS

Chromatin assembly, S-phase, Checkpoint, DNA damage, Histone synthesis

The lab of PDA is funded by grants from the NIH (GM62281), the DOD (DAMD17-02-1-0726) and a Scholar Award from the Leukemia and Lymphoma Society to PDA.

S-phase is defined as the time in the cell cycle when DNA synthesis occurs. However, it is also the time when histones are synthesized and when DNA, histones and non-histone chromatin proteins are assembled into mature chromatin. Chromatin is the physiological substrate for virtually all DNA-based transactions, such as transcription, repair, recombination and DNA synthesis itself. Moreover, a large amount of epigenetic information is stored in chromatin structure, as reflected by phenomena such as X chromosome inactivation and genetic imprinting.[1] In light of this, it seems likely that the S-phase events that build mature chromatin, namely DNA synthesis, histone synthesis and chromatin assembly, are very tightly controlled and coordinated with respect to each other.

Indeed, there is good evidence that S-phase events are tightly coordinated during S-phase. The pool of free histones in S-phase of somatic cells is thought to be very small and newly synthesized DNA is incorporated into nucleosomes within a few hundred base-pairs of the replication fork.[2,3] This indicates that the rates of DNA synthesis, histone synthesis and chromatin assembly are closely matched. Confirming this, it has long been known that inhibition of DNA synthesis with drugs such as hydroxyurea and aphidicolin causes a concerted inhibition of histone synthesis. In yeast this is due to repression of transcription but in mammalian cells it is largely due to rapid destabilization of histone mRNAs.[4]

However, until recently, there was little evidence to support the idea that DNA synthesis, histone synthesis and chromatin assembly are coupled in the reverse direction. In other words, that completion of DNA synthesis depends upon on-going histone synthesis and chromatin assembly. In fact, several lines of evidence from various model systems suggest that DNA synthesis in S-phase can occur in the absence of histone synthesis and chromatin assembly. First, in yeast none of the likely DNA synthesis-linked chromatin assembly factors identified to date, such as CAF-I, ASF1 or the Hir proteins, is essential for viability either alone or in combination.[5-7] Second, in vitro replication of plasmid DNA in mammalian cell extracts or *D. melanogaster* embryo extracts does not require chromatin assembly.[8,9] Third, yeast expressing histones from conditional promoters apparently replicate their entire genome when new histone synthesis and chromatin assembly are blocked.[10,11] Fourth, in *C. elegans* and *D. melanogaster* embryos the reduced histone synthesis caused by mutant alleles of SLBP has no obvious effect on DNA synthesis.[12] Fifth, although in *X. laevis* perturbation of CAF-I blocks development past the mid-blastula transition, it has no detectable effect on a somatic cell line.[13]

In contrast to these observations, Nelson et al. recently obtained evidence to indicate that in intact human cells on-going DNA synthesis is dependent upon continued histone synthesis and/or chromatin assembly.[14] Specifically, it was shown that repression of histone synthesis in human cells by ectopic expression of human HIRA, the human ortholog of two yeast repressors of histone transcription (Hir1p and Hir2p), inhibits DNA synthesis and causes arrest in S-phase.[14,15] One possible explanation of this result is that repression of histone synthesis inhibits chromatin assembly which, in turn, inhibits DNA synthesis. To test this idea Ye et al. inhibited the heterotrimeric Chromatin Assembly Factor-I (CAF-I) complex that is responsible for assembling newly synthesized DNA into nucleosomes. To do this the authors made use of a fragment of the 150kDa subunit of CAF-I that seemed to be a good candidate for a dominant negative inhibitor of CAF-I, since it was known to bind to one essential subunit of the complex, p60CAF-I, but not to PCNA which targets CAF-I to replication foci.[16,17] Indeed, Ye et al. showed that, as expected, this fragment, p150C, inhibits CAF-I dependent chromatin assembly in vitro, disrupts the heterotrimeric CAF-I complex and displaces p60CAF-I from the sites of DNA synthesis in vivo.[18] However, p150C did not affect the characteristic punctate S-phase distribution of PCNA throughout the nucleus, indicating that it does not completely disrupt nuclear replication foci. Most significantly, ectopic expression of p150C blocked progression of cells into and through S-phase, in a manner that was virtually indistinguishable from

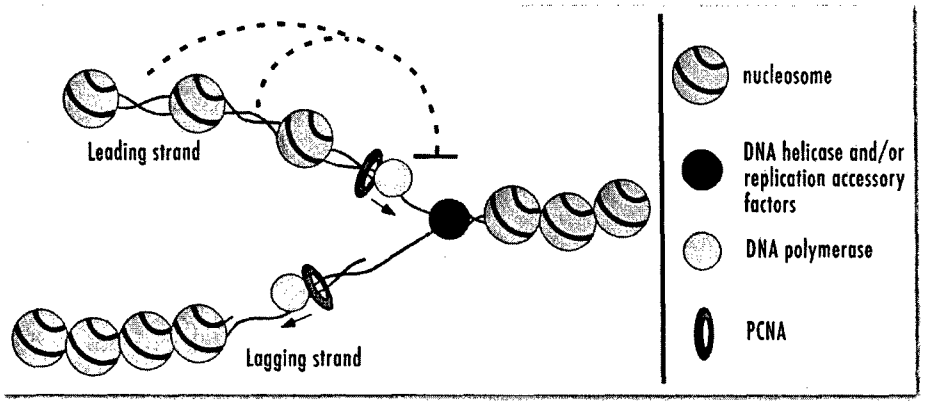

Figure 1. A putative chromatin assembly checkpoint. The figure shows a replication fork where nucleosome assembly has occurred normally on the lagging strand. In contrast, nucleosome assembly has been impaired on the leading strand, resulting in newly synthesized DNA with less than the normal complement of nucleosomes. This activates a checkpoint that prevents replication fork progression. Such a checkpoint can account for the experimental observation that chromatin assembly occurs within a few hundred base-pairs of fork passage and will facilitate the prompt and orderly assembly of chromatin behind the replication fork, thereby helping to preserve locus specific chromatin structures and epigenetic control of gene expression through S-phase.

ectopically expressed HIRA.[14,15,18] Taken together, the data suggest that inhibition of chromatin assembly, either due to repression of histone synthesis or direct inhibition of the chromatin assembly process itself, inhibits DNA synthesis in S-phase. Thus, three major processes in S-phase—DNA synthesis, histone synthesis and chromatin assembly—appear to be mutually dependent upon each other in intact mammalian cells.

What is the mechanism by which these three processes are linked in S-phase? In human cells inhibition of DNA synthesis triggers a rapid destabilization of histone mRNAs that is dependent upon a stem-loop structure within the 3'-UTR of the mRNA.[19,20] This stem-loop and 3'-UTR bind to a number of proteins involved in regulation of histone mRNA stability and processing, such as SLBP, the U7 snRNP and a zinc-finger protein, ZFP100 (see ref. 21 and refs. therein). However, the signal transduction pathway that presumably emanates from a stalled replication fork and ultimately triggers degradation of the mRNA is unknown. Interestingly, we found that hydroxyurea-induced destabilization of histone mRNAs is abolished by treatment of the cells with caffeine, an inhibitor of checkpoint activated ATR and ATM kinases.[22] This suggests that the well known genotoxic stress activated checkpoint pathways, centered on ATR and ATM, might trigger mRNA destabilization.[23]

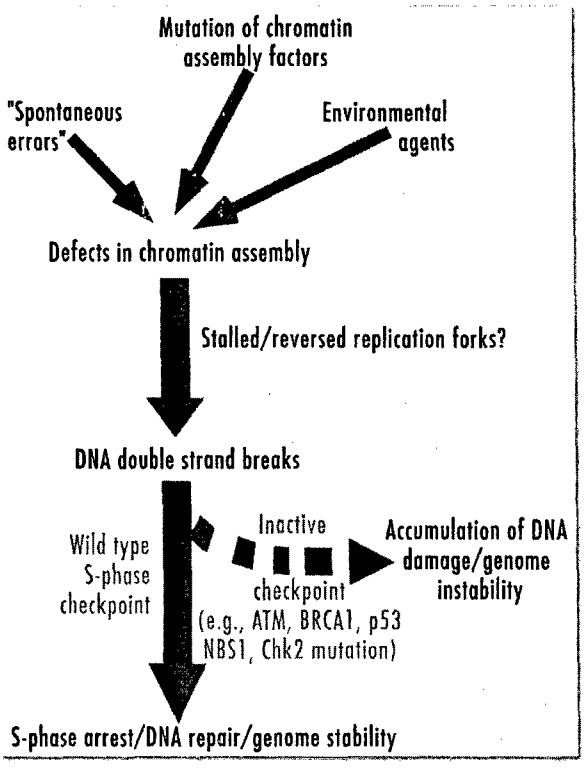

Figure 2. Defects in chromatin assembly cause DNA damage. Defects in chromatin assembly, arising spontaneously or due to mutation of chromatin assembly factors or the presence of environmental agents, cause DNA double strand breaks, likely due to inappropriate processing of stalled replication forks. In the presence of a wild type S-phase checkpoint, S-phase is arrested and DNA damage repaired, thus preserving genome integrity. In the absence of an S-phase checkpoint, due, for example, to any of several inactivating mutations found in human cancers, DNA damage persists and contributes to genome instability and, potentially, cancer.

One model to explain the converse coupling process, whereby inhibition of histone synthesis and/or chromatin assembly blocks DNA synthesis, invokes a putative "chromatin assembly checkpoint" (Fig. 1). According to this model, a failure to promptly incorporate newly synthesized DNA into chromatin results in activation of a checkpoint that prevents continued DNA synthesis. This checkpoint might be triggered by stretches of newly synthesized DNA with less then the normal complement of nucleosomes and would, presumably, facilitate the ordered assembly of chromatin structures behind the replication fork. Consistent with this idea, Kastan and coworkers showed recently that treatments which perturb chromatin structure without inducing DNA damage activate the ATM kinase.[24] This suggests that ATM and related kinases, such as ATR, are able to sense defects in chromatin structure. Consistent with p150C activating such a chromatin assembly checkpoint, the p150C-induced S-phase arrest was accompanied by checkpoint activation, as reflected by formation of nuclear ATR foci, stabilization and phosphorylation of p53 and dispersal of BRCA1 S-phase foci.[18] Moreover, efficient S-phase arrest induced by p150C was abolished in cells lacking ATR and ATM kinases, suggesting that the arrest requires activation of the S-phase checkpoint.

Although such a chromatin assembly checkpoint is an attractive idea which might help preserve chromatin-based epigenetic control of gene expression through S-phase, an alternative explanation for p150C-induced S-phase arrest was suggested by the observation that the p150C-induced S-phase arrest was accompanied by DNA double strand breaks. Such breaks are already well known to activate the S-phase checkpoint and cause cell cycle arrest and undoubtedly contribute to the arrest induced by p150C[23] (Fig. 2). But how do defects in chromatin assembly cause DNA double strand breaks? Most likely, the breaks result from inappropriate processing of stalled replication forks. Stalled replication forks can under-go fork reversal to form so-called "chicken-feet" that can, in turn, be resolved to form double strand breaks.[25,26] Defects in S-phase chromatin assembly

might cause stalling of replication forks due to obligate coupling of the chromatin assembly and DNA replication machineries, or because of steric problems caused by newly synthesized DNA that is not packaged into chromatin. Alternatively, inefficient chromatin assembly behind the replication fork might not increase stalling of replication forks but, instead, increase the frequency with which stalled forks form chicken-feet, the precursors of double strand breaks, or it might expose the newly synthesized DNA to nucleases.

Although this model perhaps sheds less light than the chromatin assembly checkpoint on the mechanism by which a cell coordinates DNA synthesis and chromatin assembly in a normal S-phase, it has significant implications for maintenance of genome stability. Kolodner and coworkers, in a series of elegant studies, have shown that the S-phase checkpoint is required in a normal S-phase, in the absence of exogenous DNA damaging agents, to prevent accumulation of genome instability.[27] In addition, numerous lines of evidence indicate that one role of the S-phase checkpoint is to stabilize replication forks, thus preventing their stalling and conversion to double strand breaks.[25,26,28] Thus, it seems likely that stalled replication forks are a potent source of genome instability in S-phase and one job of the S-phase checkpoint it to protect against this form of DNA damage. However, the causes of stalled replication forks are poorly understood. The results of Ye et al. suggest that defects in chromatin assembly, occurring spontaneously or as a result of genetic mutations or environmental toxins, are one source of stalled forks and double strand breaks.[18] One prediction of this model is that mutations that impair the function of chromatin assembly factors should cooperate with mutations that inactivate the S-phase checkpoint to promote genome instability and associated diseases, such as cancer. The S-phase checkpoint is known to be a frequent target of mutations in human cancer.[29-32] We are currently testing whether genes encoding chromatin assembly factors are also mutated in human cancers.

These two models, in which defects in chromatin assembly activate a chromatin assembly checkpoint or act as a source of DNA damage and genome instability, are not mutually exclusive. Indeed, one advantage of a chromatin assembly checkpoint might be that it would prevent a gross defect in chromatin structure from accumulating behind a replication fork, to a point where it causes fork stalling and a double strand break. Future experiments will determine whether a chromatin assembly checkpoint contributes to coordination of DNA synthesis and chromatin assembly in S-phase, and whether defects in S-phase chromatin assembly impact upon genome stability and human disease.

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Developmental Cell, Vol. 8, 19–30, January, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2004.10.019

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 ~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

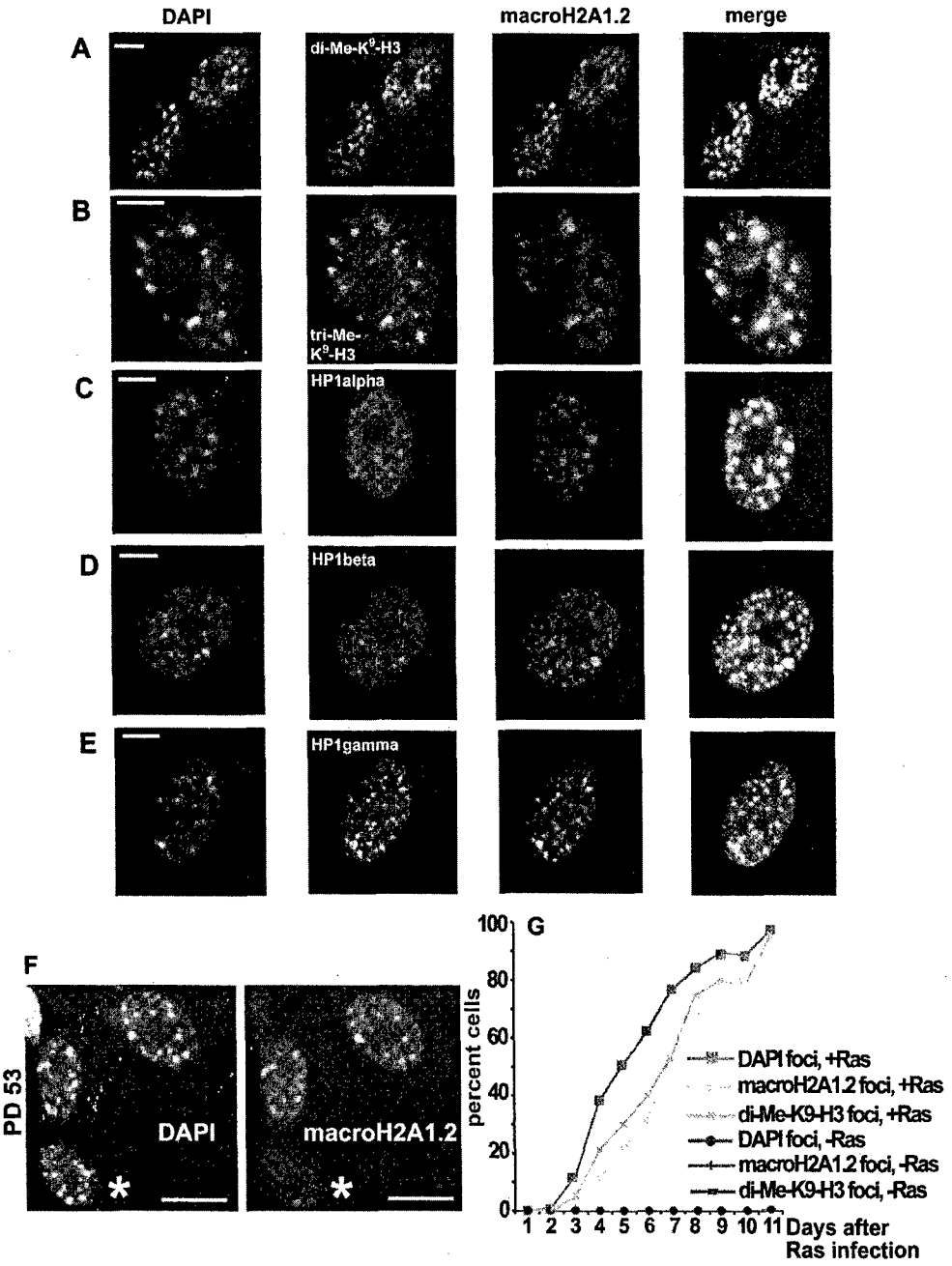

Figure 1. SAHF Is Enriched in macroH2A

(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

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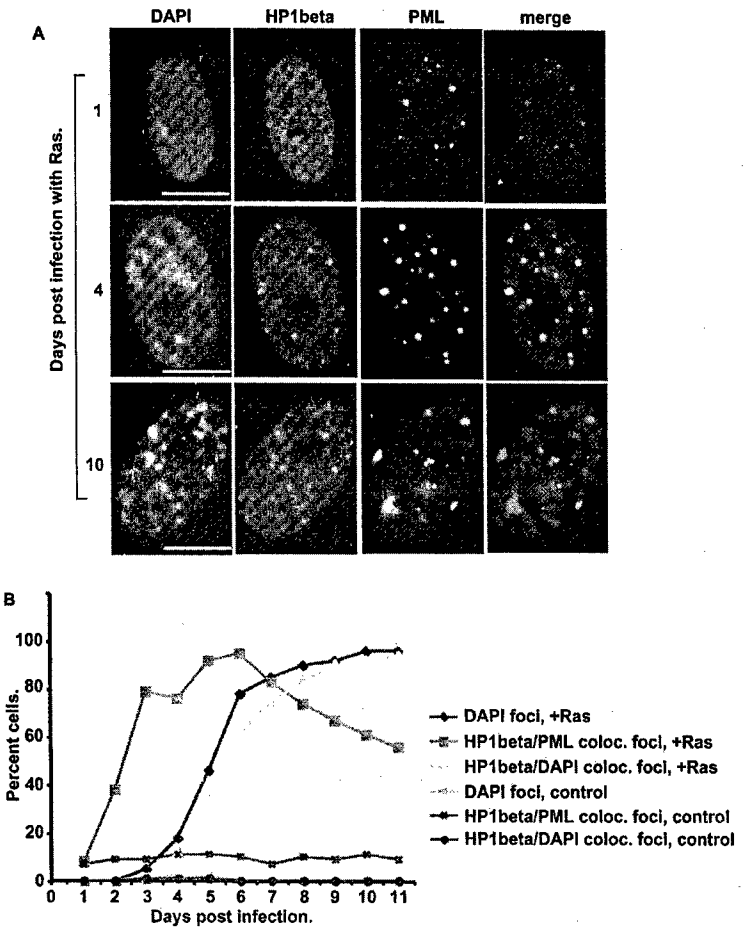


Figure 2. HP1β Is Transiently Recruited to PML Bodies prior to Formation of SAHF

(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

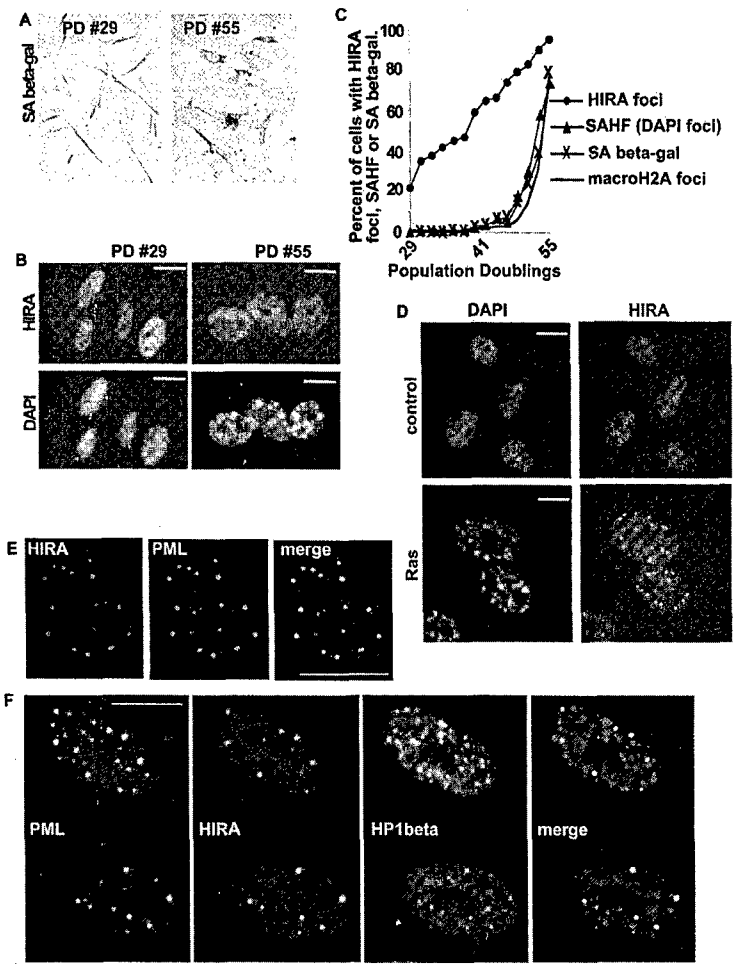


Figure 3. HIRA Is Localized to PML Bodies and Colocalizes with HP1β as Cells Enter Senescence

(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.

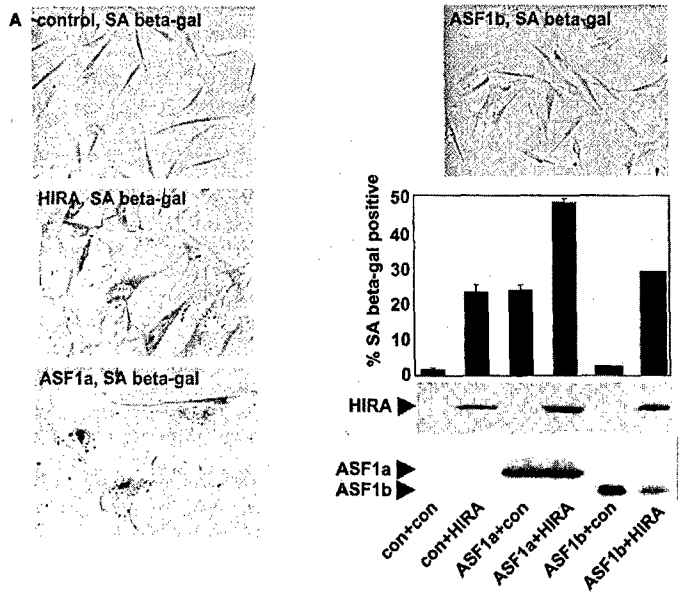
HIRA and ASF1a, but Not ASF1b, Are Rate Limiting for Formation of SAHF

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.,



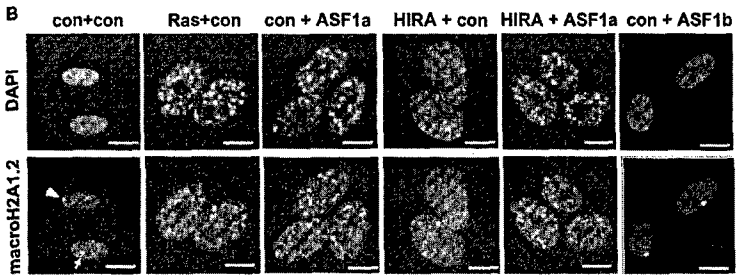


Figure 4. Ectopic Expression of HIRA and ASF1a Induces Senescence and macroH2A-Containing SAHF

(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

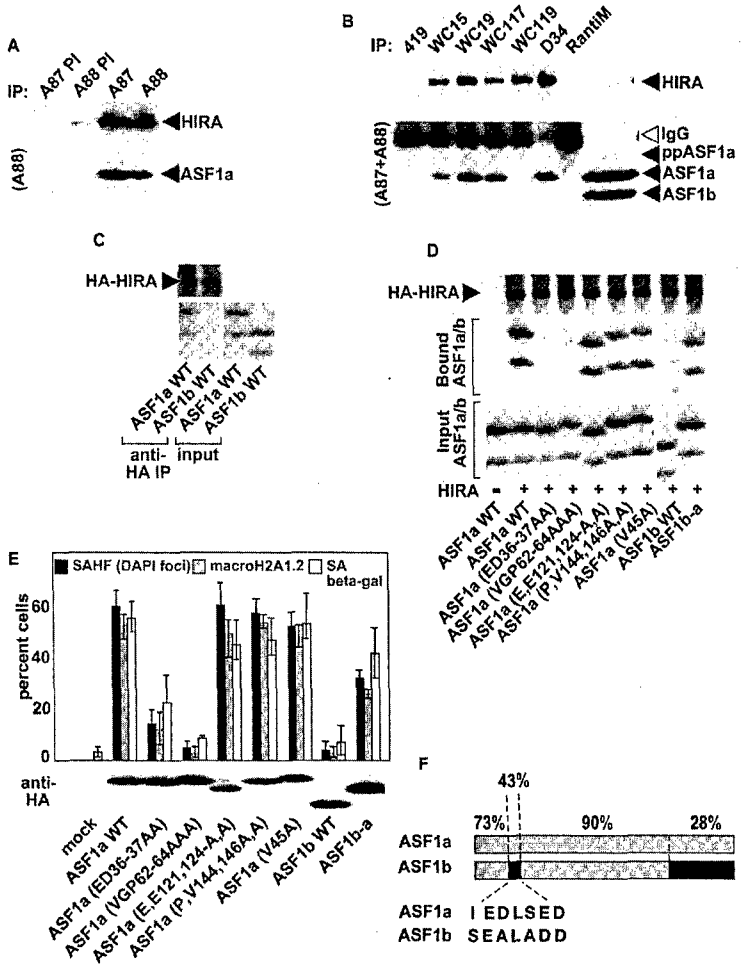


Figure 5. Induction of macroH2A-Containing SAHF by ASF1a Requires Its Interaction with HIRA

(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.

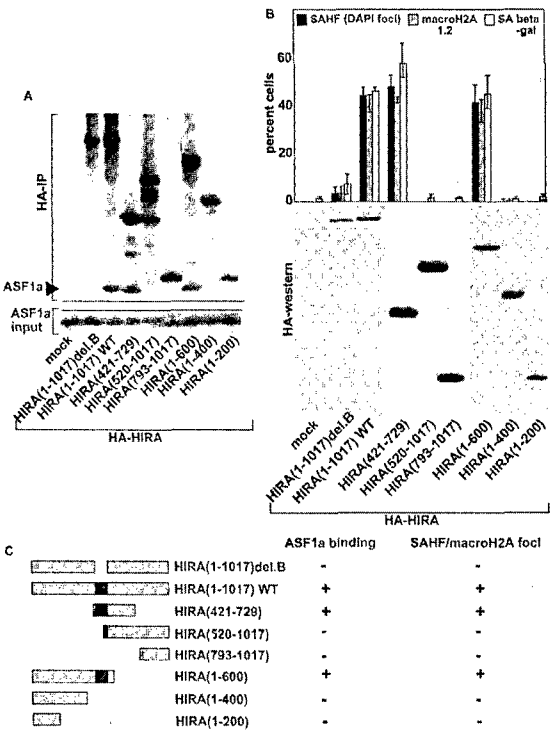


Figure 6. Induction of macroH2A-Containing SAHF by HIRA Requires Its Interaction with ASF1a

(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

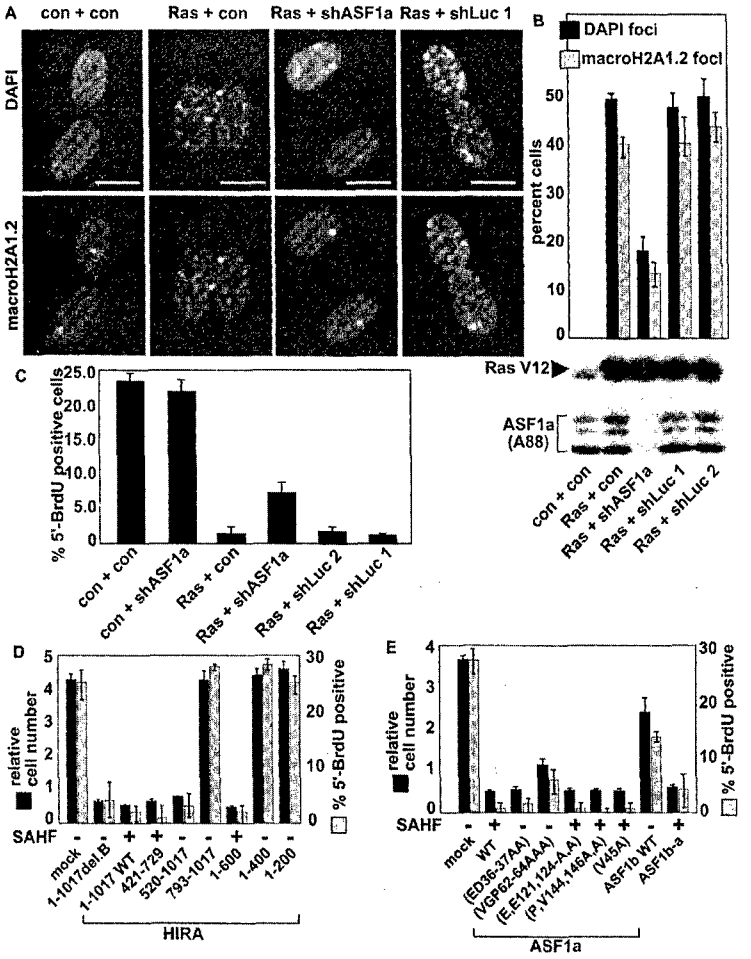


Figure 7. ASF1a Is Required for Formation of SAHF, which Drives Cell Cycle Exit

(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).

Acknowledgments

The authors thank William Earnshaw and David Schultz for antibodies, Ken Zaret for critical reading of the manuscript, Erica Golemis for technical guidance, and Robert Weinberg for pBABE-RasV12. P.D.K. is supported by the DOE (FWP KP110301) and NSF (MCB-0234014). This research was conducted while R.Z. was a Glenn/AFAR postdoctoral fellow; R.Z. was also supported by a W.J. Avery Fellowship. J.R.P. is supported by NIH (GM-49351). J.M.B. is supported by the G. Harold and Leila Y. Mathers Foundation. P.D.A. is a Leukemia and Lymphoma Society Scholar and is also supported by the DOD (DAMD17-02-1-0726) and the NIH (R01 GM062281).

Received: April 26, 2004
Revised: July 23, 2004
Accepted: October 12, 2004
Published: January 3, 2005

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