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A Novel Role for High-Mobility Group A Proteins in Cellular Senescence and Heterochromatin Formation

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SUMMARY

Cellular senescence is a stable state of proliferative arrest that provides a barrier to malignant transformation and contributes to the antitumor activity of certain chemotherapies. Senescent cells can accumulate senescence-associated heterochromatic foci (SAHFs), which may provide a chromatin buffer that prevents activation of proliferation-associated genes by mitogenic transcription factors. Surprisingly, we show that the High-Mobility Group A (HMGA) proteins, which can promote tumorigenesis, accumulate on the chromatin of senescent fibroblasts and are essential structural components of SAHFs. HMGA proteins cooperate with the $p16^{INK4a}$ tumor suppressor to promote SAHF formation and proliferative arrest and stabilize senescence by contributing to the repression of proliferation-associated genes. These antiproliferative activities are canceled by coexpression of the *HDM2* and *CDK4* oncogenes, which are often coamplified with *HMGA2* in human cancers. Our results identify a component of the senescence machinery that contributes to heterochromatin formation and imply that HMGA proteins also act in tumor suppressor networks.

INTRODUCTION

Cellular senescence was originally defined as the state of proliferative arrest that accompanies the replicative exhaustion of cultured human cells (Hayflick, 1965). Whereas "replicative" senescence is triggered by telomere erosion, an indistinguishable phenotype can be induced in "young" cells by activated oncogenes, chemotherapeutic drugs, oxidative stress, and suboptimal culture conditions (Campisi, 2005). Once considered an in vitro phenomenon, recent studies underscore the role of senescence in limiting oncogene-initiated tumorigenesis and enhancing the antitumor effects of some cancer drugs (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005; Roninson, 2003; Schmitt et al., 2002). Some genes linked to senescence in vitro also influence organismal life span in vivo, raising the possibility that the senescence program affects the balance between cancer and aging (Campisi, 2005).

The senescence program is best understood in normal diploid human fibroblasts, where cells undergo a series of events leading to marked morphological changes, the expression of a senescence-associated (SA) β-galactosidase, and diverse changes in gene expression, including upregulation of the *INK4a* cyclin-dependent kinase inhibitor (Dimri et al., 1994; Shelton et al., 1999). Most importantly, senescent cells remain permanently insensitive to mitogenic signals, owing to the combined action of the p53 and $p16^{INK4a}$-Rb tumor suppressor networks, which are frequently disabled in cancer cells (Ben-Porath and Weinberg, 2004). Thus, much like apoptosis, cellular senescence appears to involve an intricate program that actively suppresses tumorigenesis.

Although the stability of senescence presumably contributes to its antineoplastic activities, the molecular machinery that drives the cell into a permanent state of proliferative arrest is poorly understood. In some cell types, senescence is associated with global changes in chromatin structure, leading to the accumulation of heterochromatin protein 1 (HP1) and histone H3 trimethylated on lysine 9 (me-K9H3) in senescence-associated heterochromatic foci (SAHFs) and on the promoters of certain cell-cycle genes (Narita et al., 2003; Zhang et al., 2005). Importantly, changes in heterochromatin organization are observed in apparently senescent cells present in premalignant tumors (Braig et al., 2005; Chan et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005), and molecules that are required for these changes can contribute to senescence in vitro and in vivo (Braig et al., 2005; Narita et al., 2003). Such changes could promote senescence by producing a repressive chromatin environment that

prevents transcription of growth-promoting genes, thereby producing an extreme insensitivity to mitogenic stimulation.

The HMGA1 (formerly HMGI-Y) and HMGA2 (formerly HMGI-C) proteins are abundant nonhistone chromatin proteins encoded by two distinct genes and have three AT-hook domains that bind the minor groove of AT-rich DNA sequences (Reeves, 2001; Sgarra et al., 2004). In contrast to HP1, HMGA proteins are typically associated with gene activation and have been linked to proliferation (Reeves et al., 2001). Thus, both HMGA genes are highly expressed in the embryo and downregulated during differentiation (Zhou et al., 1995) and both are induced by mitogenic stimuli (Lanahan et al., 1992). In fact, HMGA proteins can promote tumorigenesis (Takaha et al., 2004) and are often overexpressed in tumors, particularly those of mesenchymal origin (Reeves, 2001). *HMGA* transgenic mice develop tumors after a long latency (Fedele et al., 2002, 2005), and gene amplifications and translocations of *HMGA* genes occur in human cancers (Reeves, 2001; Sgarra et al., 2004).

Here we take a biochemical approach to characterize the chromatin composition of senescent cells. Surprisingly, we identify HMGA1 and HMGA2 as proteins that specifically accumulate on senescent cell chromatin, and we show that they act as structural components of SAHFs and contribute to the stable repression of proliferation-associated genes. Consequently, HMGA proteins act with other senescence regulators to stabilize the senescent state. These studies identify a new activity for HMGA proteins and suggest that they can have both oncogenic and antioncogenic properties.

RESULTS

Senescent Cells Have a Distinct Pattern of Chromatin-Associated Proteins

To further characterize chromatin changes that contribute to cellular senescence, we examined the protein composition of chromatin preparations from quiescent (reversibly arrested) and senescent normal diploid IMR90 fibroblasts. Senescent IMR90 populations were produced following retroviral delivery of oncogenic *ras*, which synchronously induces senescence in vitro and may recapitulate relevant senescence-inducing signals in vivo (Braig et al., 2005; Collado et al., 2005). Nuclei were isolated at various times and subjected to a no-salt extraction procedure that enriches for chromatin-associated proteins (Mendez and Stillman, 2000). As expected, *ras*-senescent cells displayed SAHFs that were enriched for HP1γ and excluded histone H3 acetylated on Lys9/14 (Ac-K9/14H3), a euchromatic marker (Figure 1A). Furthermore, these morphological changes were accompanied by a corresponding increase in HP1γ bound to chromatin and a decrease in Ac-K9/14H3 (Figure 1B). In contrast, quiescent cells displayed a nuclear morphology and pattern of chromatin-associated proteins that, at this level of resolution, were indistinguishable from growing cells (Figure 1B; data not shown).

Using Coomassie staining to visualize the protein composition of each chromatin preparation, we also noticed that several unknown proteins were either enriched or depleted from chromatin preparations in senescent cells. Among these were two low-molecular-weight polypeptides, designated bands 1 and 2, that accumulated on senescent cell chromatin, with band 1 appearing early and band 2 coinciding with the appearance of SAHFs. This pattern was not unique to IMR90 cells since senescent WI38 cells also displayed prominent SAHFs and accumulated proteins corresponding to both both band 1 and band 2 (Figure 1C). However, senescent BJ fibroblasts, which poorly express $p16^{INK4a}$ and are less stably arrested (Beausejour et al., 2003), displayed fewer SAHFs and only accumulated band 1 on chromatin (Figure 1C). These data confirm that gross changes in chromatin composition accompany senescence.

The High-Mobility Group A Proteins Accumulate on Chromatin during Senescence

The experiments described above identify new proteins that might influence chromatin structure during senescence. We therefore subjected bands 1 and 2 to microsequencing by mass spectrometry. Multiple peptides corresponding to HMGA1 (band 1) and HMGA2 (band 2) were recovered from two independent chromatin preparations (Figure 1D), and immunoblotting using HMGA1- or HMGA2-specific antibodies confirmed that each protein was enriched on senescent cell chromatin (Figure 1E, compare lane 3 to lanes 1 and 2). Furthermore, bands 1 and 2 were selectively depleted from chromatin preparations isolated from cells coexpressing *ras* and short-hairpin RNAs (shRNAs) capable of suppressing HMGA1 and HMGA2, respectively (Figure 1F). Thus, both HMGA1 and HMGA2 specifically accumulate on chromatin in senescent IMR90 cells.

These results were unexpected as HMGA proteins levels often increase in tumor cells (Reeves, 2001). To examine the relationship between HMGA expression and proliferation in our system, we measured the total levels of HMGA1 and HMGA2 in growing, quiescent, and senescent IMR90 cells. Relative to growing cells, *HMGA* mRNA and protein levels were increased in cells triggered to undergo senescence by oncogenic *ras*, etoposide, or replicative exhaustion, but not during quiescence produced by confluence or serum starvation (Figures 1G and S1; data not shown). Consistent with previous reports (Lanahan et al., 1992), serum-starved cells showed a reduction in HMGA expression, although confluent cells did not (Figure 1G, compare lane 1 to lanes 2 and 3). Adenovirus E1A, which prevents SAHF formation, cyclin A downregulation, and senescence, abolished the increase in HMGA protein levels and in fact repressed HMGA2 (Figure 1G, compare lane 4 to 5). Hence, *HMGA* can be induced by either mitogenic or prosenescence signals.

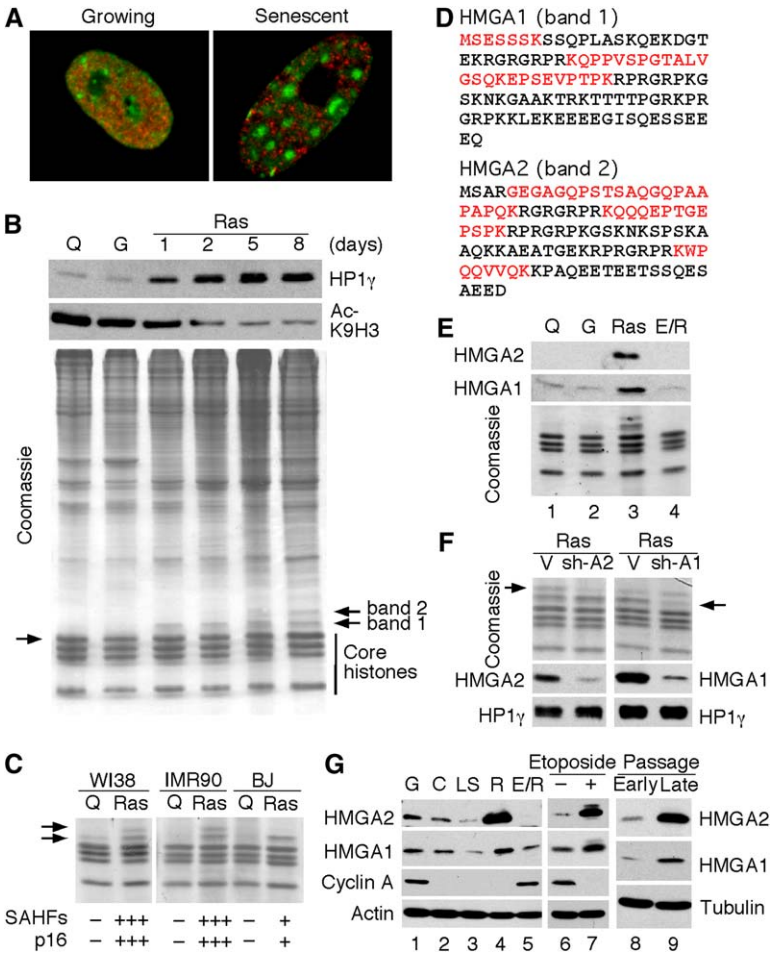

Figure 1. HMGA Proteins Accumulate on Chromatin during Cellular Senescence

(A) Merged confocal images for indirect immunofluorescence of HP1γ (green) and Ac-K9/14H3 (red) in normal growing (Growing) and *ras*-induced senescent (Senescent) IMR90 cells.

(B) Chromatin protein profile in quiescent (Q), normal growing (G), and *ras*-induced senescent IMR90 (Ras) cells at different times after selection for *ras*-expressing cells. SDS-PAGE was followed by immunoblotting for HP1γ and Ac-K9/14H3 and Coomassie blue staining. Arrows show bands 1 and 2; unlabeled arrow shows histone H3.

(C) Chromatin fractions from quiescent (Q) and *ras*-induced senescent (Ras) IMR90, WI38, and BJ cells were resolved in SDS-PAGE followed by Coomassie blue staining. Arrows show bands 1 and 2.

(D) Recovered peptide sequences (red) from those two bands (B, arrows) by mass-spectrometry analysis correspond to HMGA1 and HMGA2.

(E) Western blotting for HMGA proteins in chromatin fractions from the indicated cells. Coomassie staining visualizes core histones and serves as a loading control. E/R; E1A/ras-transformed cells.

(F) Chromatin fractions from IMR90 cells expressing both *ras* and either a control vector (V) or an shRNA for *HMGA* (sh-A1 or sh-A2) were resolved in SDS-PAGE followed by Coomassie blue staining or immunoblotting for HMGA and HP1γ.

(G) Immunoblotting of the indicated cell lysates for HMGA proteins and cyclin A, with actin or tubulin serving as a loading control. Shown are the following: G, growing; C, confluent; LS, low serum; R, ras; E/R, E1A/ras-transformed; Etoposide+, DNA damage-induced senescent cells.

HMGA Proteins Colocalize with SAHFs

We next examined the subcellular localization of each HMGA protein using indirect immunofluorescence. In growing and quiescent cells, HMGA1 and HMGA2 were expressed diffusely throughout the nucleus except for pronounced foci that corresponded to the inactive X chromosome (Figure S2; see also Chadwick and Willard, 2003 and Harrer et al., 2004), a well-characterized heterochromatic structure that is prominent in IMR90 cells (Heard et al., 2001). In contrast, in senescent cells, HMGA1 and HMGA2 were predominantly localized to DAPI-dense SAHFs that also contained the heterochromatin protein HP1γ (Figure 2A; data not shown) and excluded the euchromatic markers Ac-K9/14H3 and K4 trimethyl histone H3 (me-K4H3) (Figure S3). Electron microscopy confirmed these results, where the immunogold staining for both HMGA proteins colocalized with the electron-dense regions that correspond to SAHFs (Narita et al., 2003) (Figure 2B). These results were surprising, as most studies link HMGA proteins with open chromatin configurations (Reeves, 2001).

HMGA proteins enhance transcription, in part through their ability to associate with the minor groove of AT-rich DNA sequences (Reeves, 2001). To determine whether the association of HMGA with SAHFs requires this activity, we treated SAHF-positive cells with Hoechst 33258, a membrane-permeable DNA dye that binds the minor groove of AT-rich DNA and displaces HMGA proteins from chromatin (Radic et al., 1992). Senescent IMR90 cells were treated with Hoechst 33258 for 2 days and then subjected to subcellular fractionation to assess the specificity of competition or examined for SAHFs. Whereas Hoechst 33258 had no impact on the distribution of the HP1 proteins, it was able to displace both HMGA1 and HMGA2 from the chromatin fraction (Figure 2C, compare lanes 3 and 6). Hoechst-treated cultures also displayed fewer SAHF-positive cells (Figures 2D and 2E), suggesting that HMGA proteins are integral SAHF

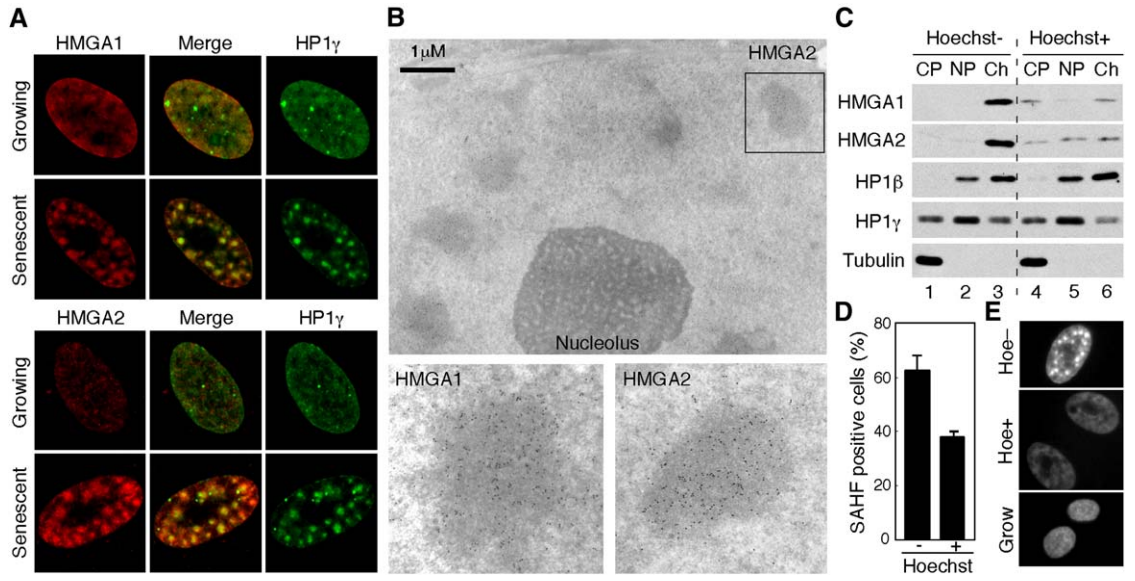

Figure 2. HMGA Proteins Are Enriched in SAHFs

(A) Representative confocal images of indirect immunofluorescence of HMGA1 or HMGA2, and HP1γ.

(B) Electron microscope images of senescent cells following immunogold labeling of HMGA1 or HMGA2. Lower panels; higher magnification of HMGA-containing SAHF.

(C–E) Hoechst competition assay. The amount of indicated proteins was determined by immunoblotting of indicated fractions in the presence (lanes 4–6) or absence (lanes 1–3) of Hoechst 33258 (Hoe) (C). The cells were assessed for SAHF formation (D). Representative images of the nuclei stained with DAPI are shown with the control nuclei of normal growing cells (E). Shown are the following: CP, cytoplasmic; NP, nucleoplasmic; Ch, chromatin fraction. Values represent the mean ± standard error of three experiments.

components (see below). Identical results were obtained using distamycin A, which also binds the minor groove of AT-rich DNA (data not shown) (Radic et al., 1992). Thus, HMGA proteins apparently associate with these heterochromatic structures via their canonical biochemical activity.

HMGA Proteins Promote SAHF Formation and Senescence

To determine whether HMGA proteins are sufficient to induce SAHFs or other senescence phenotypes, we ectopically expressed each *HMGA* gene in IMR90 cell populations. We used retroviruses that expressed HMGA tagged with hemagglutinin (HA) or fused to green fluorescent protein (GFP) from either the strong CMV promoter or the weaker LTR promoter, leading to different levels of transgene expression (Figure 3A). Cells expressing high levels of either HMGA protein underwent an acute cell cycle arrest and displayed features of senescence, including increases in $p16^{INK4a}$ and p53, a senescent morphology, and elevated SA-β-galactosidase activity (Figures 3A and 3B). These cells also acquired SAHF-like foci that contained HMGA and me-K9H3 (Figure 3C; data not shown).

Cells expressing HMGA1 from the weaker LTR promoter, contained near endogenous levels (Figure 3A), did not acutely arrest but grew similarly to controls—at least initially (Figure 3D). However, these cells underwent replicative exhaustion earlier (Figure 3D), showing a more rapid onset of senescent morphology and $p16^{INK4a}$ and p53 induction and SAHF formation (data not shown). Similar results were obtained for cells expressing physiologic levels of HMGA2 (Figure S4). Therefore, HMGA proteins are able to induce SAHFs and other senescence phenotypes when overexpressed and cooperate with other factors to accelerate senescence at physiologically relevant levels.

$p16^{INK4a}$ and HMGA Induce SAHFs in a Mutually Reinforcing Manner

Although HMGA proteins can induce $p16^{INK4a}$ when overexpressed, enforced expression of $p16^{INK4a}$ is sufficient to induce SAHFs (Narita et al., 2003), which contain HMGA (see above). We therefore performed epistasis tests to study the relationship between HMGA and $p16^{INK4a}$ in the senescence program. We first coexpressed HMGA1 (using the strong CMV promoter) with an *INK4a*-specific shRNA (sh-p16) or a control vector in IMR90 cells and measured DNA synthesis and SAHF formation using BrdU incorporation and DAPI staining, respectively. HMGA1-induced arrest and SAHF formation were clearly impaired in cells expressing sh-p16, despite having similar levels of transgene expression (Figure 3E). We also tested the effect of p53 inactivation using a *p53* shRNA (sh-p53) or dominant-negative mutant $p53^{175H}$ (DN) and found that p53 had less impact on HMGA-induced senescence (Figure 3E). Of note, cells expressing either shRNA alone or together reached replicative senescence earlier than

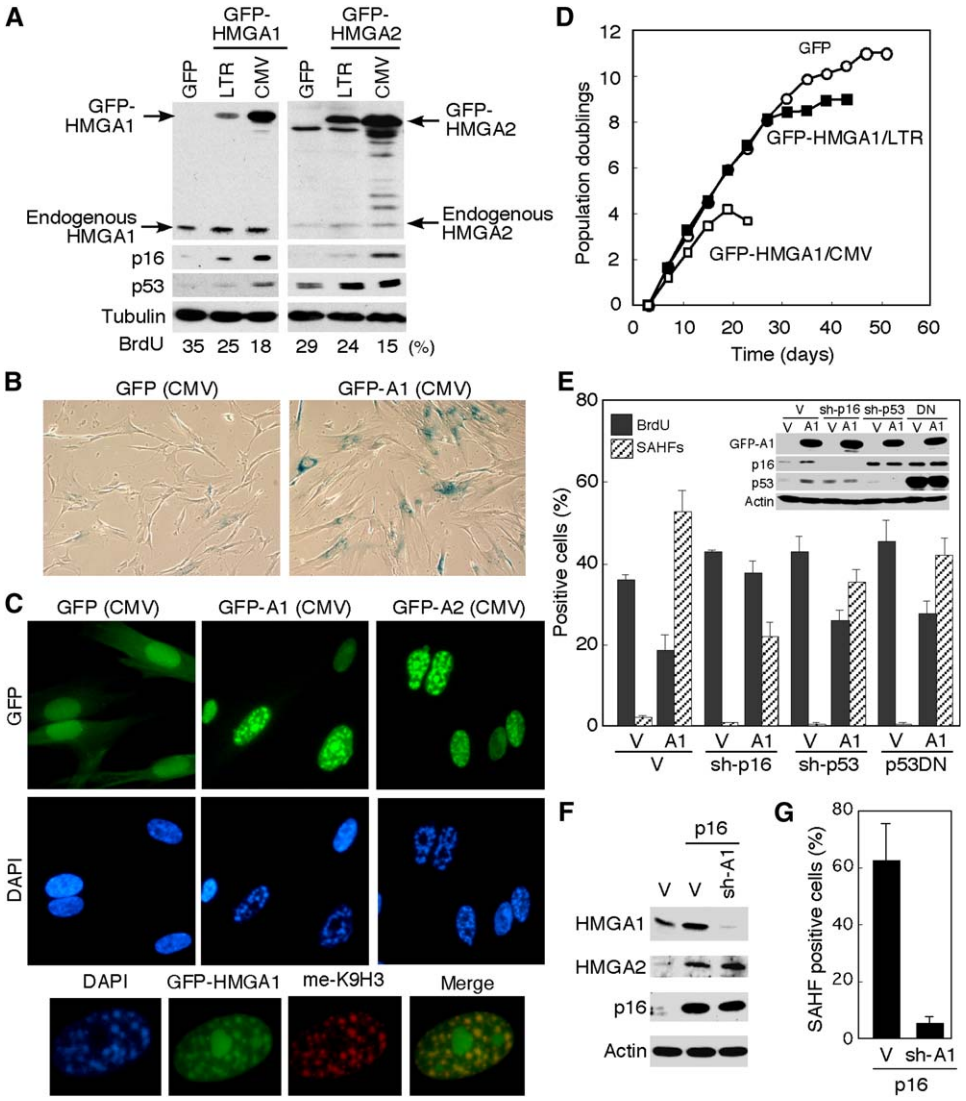

Figure 3. HMGA Proteins Promote SAHF Formation and Senescence

(A) IMR90 cells expressing the GFP-fusion HMGA proteins driven by LTR (pWZL) or CMV (pLPC) promoter were lysed and subjected to immunoblotting using antibodies against HMGA1, HMGA2, $p16^{INK4a}$, or p53. The percentage of the BrdU-positive cells is shown below each lane.

(B) IMR90 cells expressing GFP or GFP-HMGA1 from CMV promoter were assessed for SA-β-galactosidase activity.

(C) Fluorescent images of IMR90 cells expressing GFP or GFP-HMGA fusion proteins (GFP-A1 or GFP-A2) from the CMV promoter. IMR90 cells expressing GFP-HMGA1 were also immunolabeled for me-K9H3. Nuclei were counterstained with DAPI.

(D) Growth curves of IMR90 cells expressing a control GFP, GFP-HMGA1 from the retroviral LTR, or GFP-HMGA1 from CMV promoter.

(E) IMR90 cells expressing a control vector (V) or GFP-tagged HMGA1 (GFP-A1) from the CMV promoter together with a vector (V), an shRNA against *INK4a* (sh-p16) or *p53* (sh-p53), or dominant-negative mutant $p53^{175H}$ (p53DN) were assessed for BrdU incorporation and SAHF formation. The inset shows the expression levels of GFP-HMGA1 (GFP-A1), $p16^{INK4a}$, and p53 with actin as a loading control. Values are the mean ± standard error of three experiments.

(F and G) IMR90 cells expressing vector alone (V), or V, shRNA against *HMGA1* (sh-A1) together with $p16^{INK4a}$ were analyzed for expression of the indicated proteins by immunoblotting (F) and SAHF formation with DAPI staining (G) 7 days after retroviral transduction. Values are the mean ± standard error of three experiments.

controls, indicating that disruption of $p16^{INK4a}$ and/or p53 is not sufficient to completely bypass HMGA-induced arrest (data not shown).

We next coexpressed $p16^{INK4a}$ in the presence or absence of an *HMGA1* shRNA. $p16^{INK4a}$ also efficiently induced both HMGA proteins, which localized to SAHFs (Figure 3F; data not shown). Furthermore, the *HMGA1* shRNA completely prevented SAHF accumulation in response to $p16^{INK4a}$ (Figures 3F and 3G), although these cells eventually arrested. Thus, HMGA and $p16^{INK4a}$ act in a mutually reinforcing manner to promote SAHF formation.

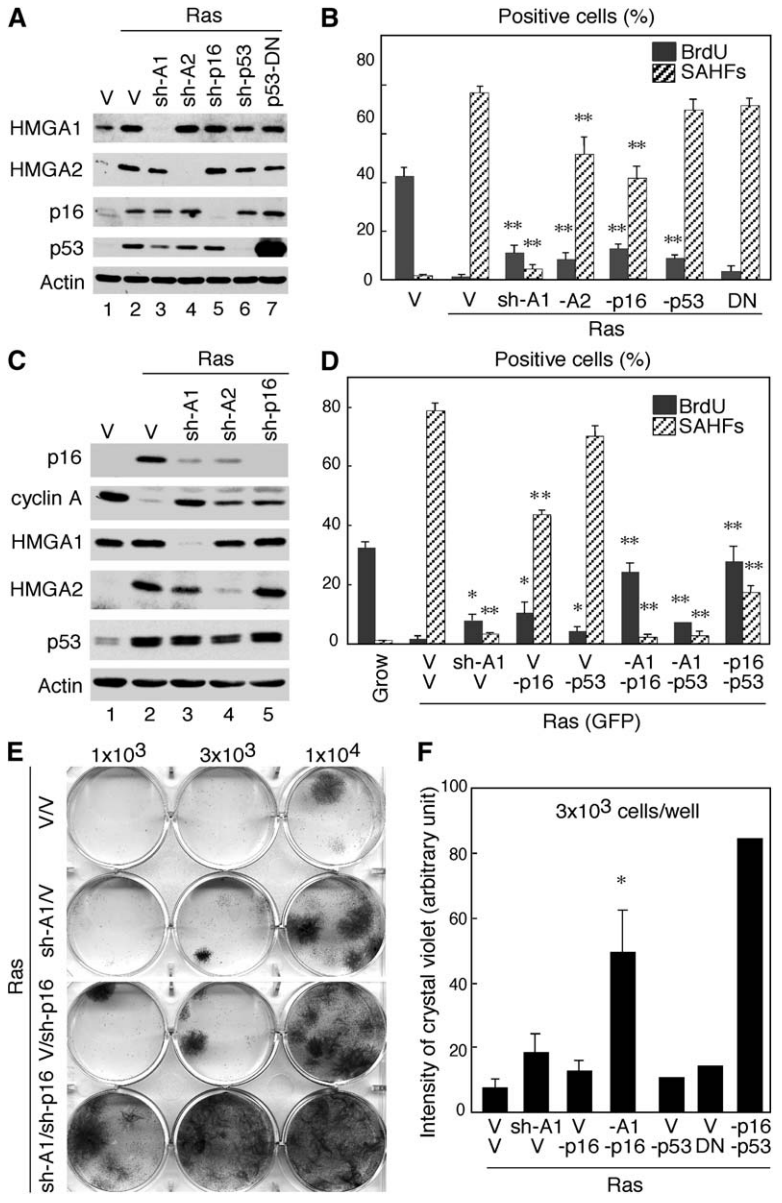

Figure 4. HMGA Proteins Are Required for SAHF Formation and Cooperate with p16 to Promote Senescence

(A) IMR90 cells expressing vector alone (V), or a combination of H-*ras* V12 (Ras) and vector (V), shRNA against *HMGA1* (sh-A1), *HMGA2* (sh-A2), *INK4a* (sh-p16), or p53 (sh-p53), or dominant-negative mutant $p53^{175H}$ (p53-DN) were analyzed for expression of the indicated proteins 7 days after retroviral transduction. Note that cells with sh-p16, sh-p53, or p53-DN still induce HMGA proteins that are chromatin bound (Figures 5A and S8), indicating that HMGA is not directly responding to $p16^{INK4a}$ or p53.

(B) The cell populations described in (A) were assessed for BrdU incorporation and SAHF formation, with values representing the mean ± standard error of at least three experiments. (**) denotes significance values of $p < 0.01$ relative to V/Ras cells.

(C) IMR90 cells expressing vector alone (V), or a combination of H-rasV12 (Ras) and vector (V), shRNA against *HMGA1* (sh-A1), *HMGA2* (sh-A2), or *INK4a* (sh-p16) were plated 1×10^4/3.5 cm at 9 days after retrovirus infection and assessed for the expression of the indicated proteins 32 days after infection. Cells were maintained in selective media to ensure retention of the *ras*-expressing retrovirus.

(D) IMR90 cells coexpressing *ras* and *GFP* [Ras (GFP)] with indicated combination of shRNAs (sh) against *HMGA1* (−A1), *INK4a* (−p16), and *p53* (−p53) were assessed for BrdU incorporation and SAHF formation in the GFP-positive population 7 days after lentiviral infection, as in Figure 4B. Values are the mean ± standard error of three experiments. (*) or (**) denotes significance values of $p < 0.05$ or $p < 0.01$, respectively, relative to V/Ras cells.

(E and F) The indicated cell populations were plated in three different densities 10 days after retroviral gene transduction and assessed for the colony formation with Crystal violet staining ~20 days later. (E). Cells were maintained in selective media (Blasticidin) to ensure retention of the *ras*-expressing retrovirus. The intensity of Crystal violet of 3×10^3/3.5 cm series was measured with ImageJ software, with values representing the mean ± standard error (n = 5) (F). (*) represents significance values of $p = 0.01$ relative to V/V/Ras cells. The mean value of two independent experiments for each sh-p53/Ras or p53 dominant-negative mutant $p53^{175H}$ (DN)/Ras population is also shown with a representative value of sh-p16/sh-p53/Ras population as a positive control.

HMGA Proteins Are Required for SAHF Formation and Complete Proliferative Arrest during Senescence

We also examined the contribution of each HMGA protein to various senescence phenotypes. Acute knockdown of each HMGA protein alone had no obvious effect on proliferation (data not shown). However, 7 days after transduction with oncogenic *ras*, cells expressing sh-HMGA1 (sh-A1) or sh-HMGA2 (sh-A2) (Figure 4A) showed reduced SA-β-galactosidase activity (Figure S5), increased BrdU incorporation, and decreased SAHF formation compared to controls (Figure 4B; see also Figure S6 for results using additional *HMGA* shRNAs). In fact, cells expressing sh-A1 were almost completely devoid of SAHFs (Figure 4B), even though they expressed high levels of endogenous $p16^{INK4a}$ (Figure 4A, compare lanes 2–4).

Both *HMGA* shRNAs enabled some cells to bypass senescence, leading to a modest increase in colony formation that was similar to that produced by an *INK4a* shRNA (Figure 4E; data not shown). HMGA1 and HMGA2 have similar structures and share some transcriptional targets (Baldassarre et al., 2001), suggesting that these proteins

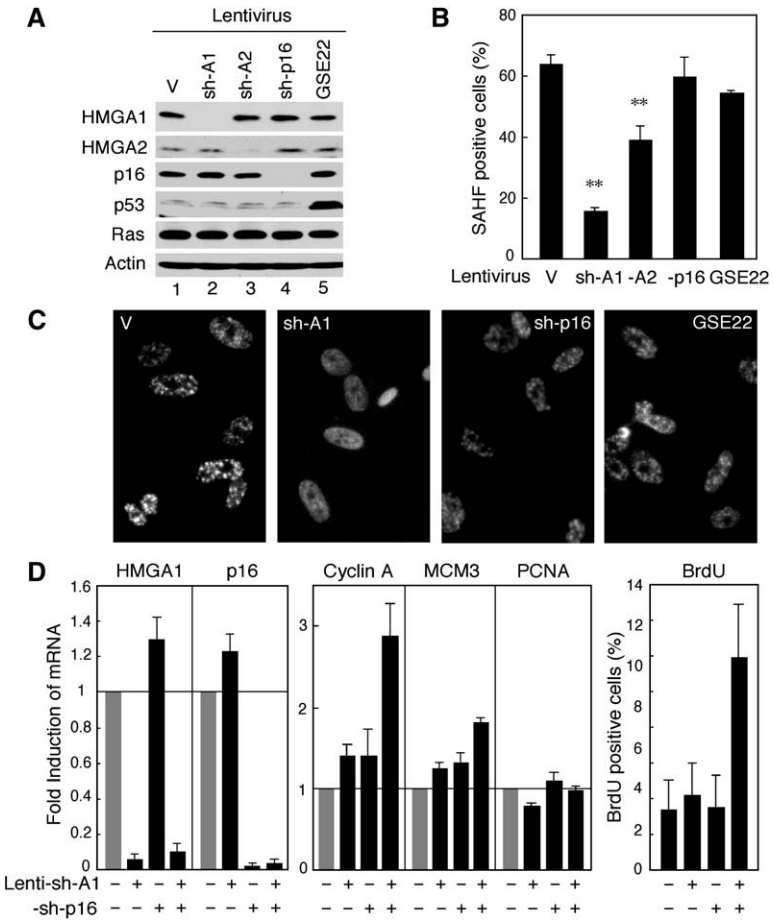

Figure 5. HMGA Proteins Are Essential Components of SAHF and Required for the Stable Suppression of Proliferation-Associated Genes

(A–C) Lentivirus-expressing miR30 control (V), shRNA (sh) against *HMGA1* (−A1), *HMGA2* (−A2), or *INK4a* (−p16), or dominant-negative p53 peptide (GSE22) was introduced into *ras*-senescent IMR90 cells. Five days later, cells were analyzed for expression of the indicated proteins by immunoblotting (A) and for SAHF formation with DAPI staining (B and C). Values represent the mean ± standard error of at least three experiments. (**) denotes significance values of $p < 0.01$ relative to vector (V)-containing cells.

(D) mRNA levels of indicated genes and DNA synthesis were determined by quantitative RT-PCR and BrdU incorporation 5 days after retroviral transduction of the indicated gene. Values represent the mean ± standard error of three independent experiments.

might have overlapping functions in senescence (see also Tables S1–S4). Accordingly, cosuppression of HMGA1 and HMGA2 did not enhance the phenotype produced by sh-A1 alone (data not shown), although we cannot rule out the possibility that more effective shRNAs would cooperate. Thus, HMGA proteins contribute to senescence but necessarily must act in concert with additional factors to do so.

HMGA shRNA-expressing cells that formed colonies, at 32 days after infection, retained *ras* expression but had much lower $p16^{INK4a}$ levels than senescent cells (Figure 4C, compare lane 2 to lanes 3 and 4), suggesting that reduced $p16^{INK4a}$ activity was also required for senescence bypass. We therefore examined the impact of cosuppressing HMGA1 and $p16^{INK4a}$ on proliferation following a senescence trigger (i.e., oncogenic *ras*). In acute proliferation assays, cells coexpressing both *HMGA1* and *INK4a* shRNAs showed a DNA synthesis rate that was substantially greater than cells expressing either shRNA alone and comparable to growing cells or cells coexpressing *p53* and *INK4a* shRNAs (Figure 4D). In contrast, cosuppression of *HMGA1* and *p53* did not show a synergistic effect on proliferation (Figure 4D). In long-term assays, cells expressing both *HMGA1* and *INK4a* shRNAs with *ras* formed colonies efficiently at numbers that approached those produced by cells coexpressing *p53* and *INK4a* shRNAs (Figures 4E and 4F). Therefore, consistent with their action in a nonlinear pathway, HMGA and $p16^{INK4a}$ cooperate to promote SAHF formation and senescence.

HMGA Proteins Are Essential Components of SAHFs and Are Required for the Stable Suppression of Proliferation-Associated Genes

The Hoechst competition experiments described above suggest that HMGA proteins are essential for maintaining SAHF integrity. To examine this more directly, senescent IMR90 cell populations were transduced with lentiviral vectors expressing the shRNAs targeting *HMGA1*, *HMGA2*, or *INK4a*, as well as dominant-negative p53 peptide (GSE22) (Beausejour et al., 2003). These vectors efficiently transduced senescent cells and suppressed their corresponding targets (Figures 5A and 5D; data not shown). SAHF morphology was unaffected by $p16^{INK4a}$ depletion, indicating that $p16^{INK4a}$ is required for SAHF establishment but not maintenance. SAHF morphology was also unaffected by p53 inactivation (Figures 5B and 5C). In contrast, SAHFs could be dissolved by suppression of either HMGA protein, with loss of HMGA1 producing an almost complete effect (Figures 5B and 5C). Consistent with

the Hoechst experiments (Figure 2C), HP1γ remained chromatin bound (Figure S7). Thus, HMGA proteins are essential components of these heterochromatic structures and promote senescence in parallel to HP1.

$p16^{INK4a}$ contributes to senescence, in part by triggering stable repression of E2F target genes (Narita et al., 2003). Since SAHFs have been associated with this activity (Narita et al., 2003), we assessed the impact of suppressing $p16^{INK4a}$—alone or in combination with suppressing HMGA1—on the expression of selected E2F target genes (Figure 5D). Senescent (SAHF-positive) IMR90 cells were transduced with lentiviral vectors expressing shRNAs targeting *HMGA1* and/or *INK4a*, and the resulting cell populations were examined for their ability to maintain E2F target gene suppression and to synthesize DNA 5 days later. Cosuppression of both *HMGA* and *INK4a* increased the expression of *cyclin A* and *Mcm3* more efficiently than either shRNA alone (Figure 5D). In contrast, none of the shRNAs tested increased *PCNA* expression at this time point, implying that proteins in addition to HMGA1 and $p16^{INK4a}$ can contribute to gene silencing or that *PCNA* is more responsive to residual HMGA and/or $p16^{INK4a}$ protein levels. A substantial percentage of cells cosuppressing $p16^{INK4a}$ and HMGA1 re-entered S phase, an effect that did not occur in cells suppressing either $p16^{INK4a}$ or HMGA1 alone (Figure 5D). Therefore, HMGA proteins cooperate with $p16^{INK4a}$ to stably repress E2F target genes in senescent cells.

HMGA Proteins Regulate Gene Expression Depending on Cellular Contexts

We also conducted a series of microarray experiments to identify genes affected by HMGA1 and HMGA2 in normal and senescent cells. IMR90 cells were transduced with shRNAs targeting either HMGA1 or HMGA2, simultaneously transduced with a control vector, or triggered to undergo senescence by oncogenic *ras*, and differentially expressed genes were identified using Affymetrix U133 Plus 2.0 expression arrays. Interestingly, the vast majority of genes that were differentially expressed (≥2-fold) in senescent cells expressing *HMGA* shRNAs were upregulated, indicating a prominent role for HMGA in gene repression (Figure 6A). This is in contrast to normal IMR90 cells, where there were similar numbers of up- and downregulated genes (Figure 6A), or ES cells, where HMGA1 mainly increases gene expression (Martinez Hoyos et al., 2004).

Although there was some overlap, most of the genes controlled by HMGA proteins in senescent cells were distinct from those affected by HMGA in normal cells (Figure 6B). Thus, whereas pathway annotation analysis of the HMGA-controlled genes identified genes linked to "cytokine-cytokine receptor interaction," as overrepresented in datasets from both normal (p = 0.00032 for HMGA1; p = 0.00033 for HMGA2) and senescent cells (Tables S1–S4, p = 0.00031 for HMGA1; p = 0.0010 for HMGA2), genes linked to the "cell cycle" were overrepresented only in datasets derived from cells triggered to senesce (p = 0.026 for HMGA1; p = 1.9E-10 for HMGA2).

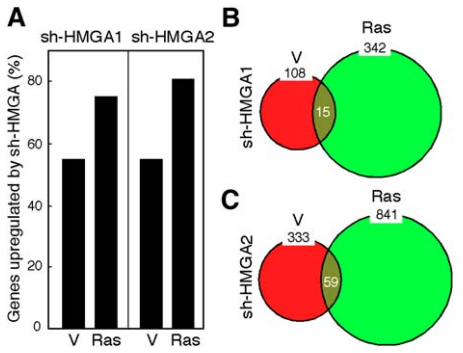

Figure 6. Regulation of Gene Expression by HMGA Proteins Is Context Dependent

Gene expression profiles of vector (V) or *ras*-expressing IMR90 cells transduced with an sh-RNA against *HMGA1* or *HMGA2* were compared to cells transduced with a control vector.

(A) Shown are the percentages of upregulated genes out of all the genes differentially expressed (≥ 2-fold) in response to sh-HMGA1 (left) or sh-HMGA2 (right). Similar results were obtained using more stringent cutoffs (3-fold or 4-fold difference) or various normalization methods (data not shown).

(B and C) Venn diagram showing the numbers of genes upregulated by sh-HMGA1 (B) or sh-HMGA2 (C) in growing (V) and *ras*-senescent (Ras) cells. The overlaps illustrate genes commonly upregulated in both cellular contexts.

Among the genes repressed by both HMGA proteins only in senescent cells were E2F targets, including *CDC2*, *MCMs*, and *cyclin A*. However, neither *INK4a* nor canonical p53-responsive genes were affected by HMGA in this analysis. Our data therefore associate HMGA with the repression of cell-cycle genes in senescent cells.

Oncogenic Lesions Bypass HMGA-Induced Senescence

Although both *HMGA1* and *HMGA2* can act as oncogenes, our results imply that each also contributes to cellular senescence, an antioncogenic program. Accordingly, we see that loss of $p16^{INK4a}$ and p53 attenuates the acute induction of senescence by HMGA proteins (Figure 3E; data not shown), suggesting that high HMGA levels may only be oncogenic if the senescence program is disabled. Interestingly, *HMGA2* is located near the *HDM2* and *CDK4* loci on chromosome 12q13-15 and is often coamplified with both *HDM2* and *CDK4* (Berner et al., 1997), which target the p53 and p16-Rb pathways, respectively. To directly determine whether these lesions bypass HMGA-induced senescence, we overexpressed HMGA2 with HDM2 and/or CDK4 in IMR90 cells and assessed their long-term proliferative potential. Here, cells were selected with three consecutive drug treatments to obtain the pure populations coexpressing three genes, a procedure which ultimately shortened their general replicative life span (Figure 7B). Still, cells coexpressing HDM2 and CDK4 bypassed HMGA2-induced arrest and SAHF formation (data not shown), displaying a dramatically extended life span even despite high levels of HMGA2 (Figures 7A and 7B).

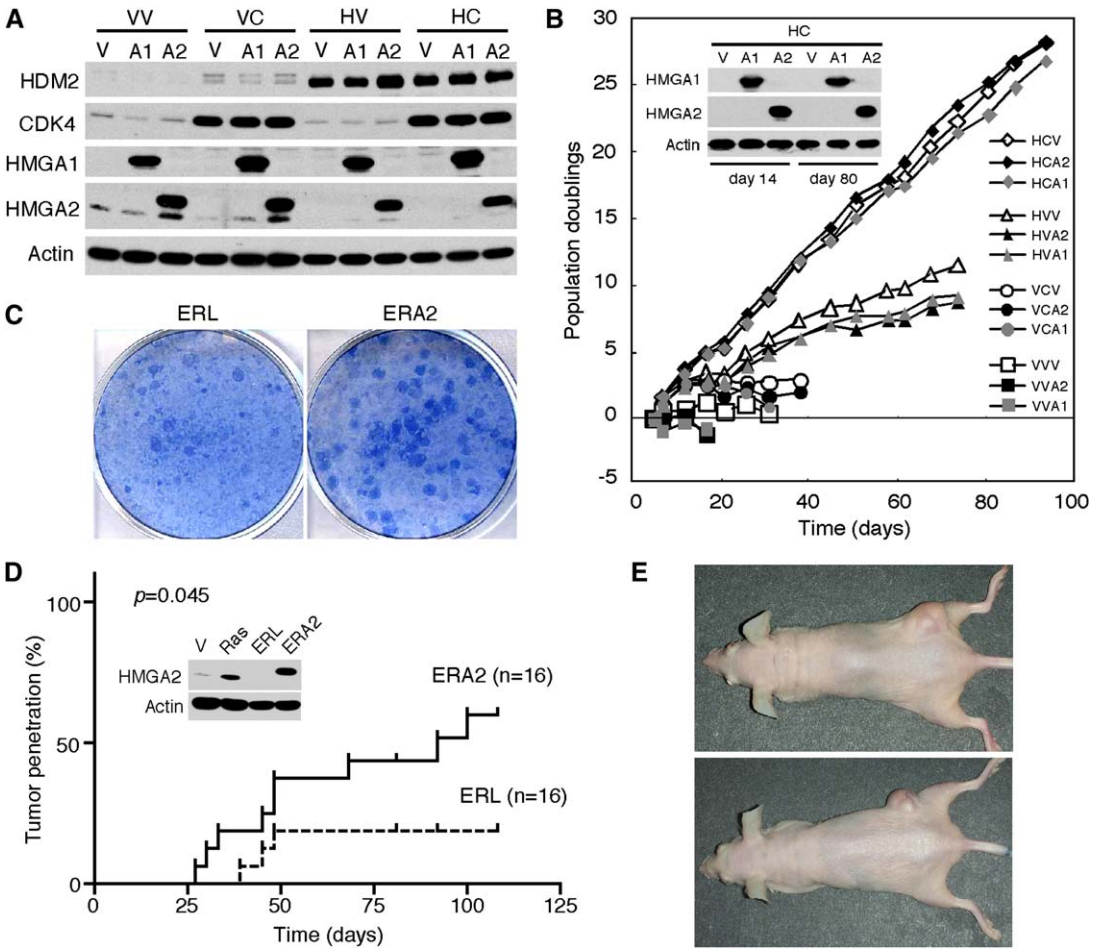

Figure 7. The Action of HMGA Proteins in Senescence Depends on Intact Senescence Machinery

(A) IMR90 cells expressing GFP-HMGA1 (A1), HA-HMGA2 (A2), HDM2 (H), CDK4 (C), or corresponding control vectors (V) in the indicated combinations were assessed for the indicated ectopic proteins with actin as a loading control.

(B) Population-doubling analysis of the cells described in (A). Cell number was plotted after completion of three consecutive selections for the transduced marker genes. The inset shows the expression of ectopic HMGA proteins over time.

(C) A representative focus formation assay using IMR90 cells expressing E1A (E), ras (R), and pLPC vector (L) or HA-tagged HMGA2/pLPC (A2).

(D) Tumorigenicity assay using two independent preparations of the cells described in (C) injected subcutaneously into immunocompromised mice.

(E) Two representative images of mice in (D) injected with E1A/ras cells (left flank) or E1A/ras/HMGA2 (right flank) containing cells.

Cells expressing either HDM2 or CDK4 alone had an intermediate phenotype (Figure 7B). Similar results were obtained with HMGA1 (Figure 7B). Therefore, the same lesions linked to *HMGA* amplification in human cancers circumvent its antiproliferative activity.

To determine whether disruption of senescence might expose the oncogenic potential of HMGA proteins, we introduced HA-tagged *HMGA2* into IMR90 cells coexpressing E1A (which inactivates Rb and prevents p53-mediated arrest) and oncogenic *ras*. These cells otherwise express extremely low levels of HMGA2 (Figure 1G, lane 5), fail to senesce, and are weakly transformed (Seger et al., 2002). As expected, control cells expressing E1A and ras formed some multilayered foci and were weakly tumorigenic following transplantation into immunocompromised mice (Figures 7C–7E). However, HMGA2 expression clearly enhanced these effects, leading to more transformed foci, increased tumor penetrance, and decreased tumor latency relative to controls. Of note, the level of HMGA2 protein that enhanced transformation of cells expressing E1A/ras was more than senescent cells expressing ras alone (Figure 7D, inset). Thus, HMGA2 can have antioncogenic or oncogenic properties in IMR90 cells depending on the integrity of the senescence machinery.

DISCUSSION

Our observations expand our understanding of HMGA biology, as well as their role in chromatin organization and cancer. We see that HMGA proteins accumulate on chromatin in senescent cells, where they promote and

maintain proliferative arrest. Although HMGA proteins may contribute to the transactivation of senescence-associated genes (see below), much of their antiproliferative activity appears to reflect their ability to produce a higher-order chromatin environment that serves as a buffer against the action of mitogenic transcription factors such as E2F. Thus, HMGA proteins act in a mutually reinforcing manner with $p16^{INK4a}$ to promote SAHF formation and contribute independently to the stable repression of E2F target genes. Accordingly, suppression of both HMGA and $p16^{INK4a}$ in growing cells acts synergistically to enable cells to bypass senescence and leads to the reactivation of some E2F target genes and S phase re-entry in senescent cells. Thus, HMGA proteins act as part of the program that initiates and maintains the senescent state.

HMGA proteins are involved in many cellular processes, including proliferation, differentiation, and apoptosis (Reeves, 2001). These HMGA activities are associated with its ability to facilitate assembly of the "enhanceosome" and create a chromatin environment conducive to transcription. Our study demonstrates that HMGA proteins can function in another process—cellular senescence. In principle, HMGA proteins could contribute to senescence through their enhanceosome activities, perhaps leading to the upregulation of senescence-associated genes such as $p16^{INK4a}$. However, our gene-profiling experiments indicate that their primary action in senescent cells is to promote gene repression, particularly of proliferation-associated genes. Although we cannot prove these effects are direct, the fact that HMGA proteins contribute to heterochromatin formation suggests that the primary role of these proteins in senescence is to contribute to a repressive chromatin environment that limits transcription.

Heterochromatin is characterized by the chromatin accumulation of certain modified histones such as me-K9H3, which produces a docking site for HP1. These biochemical alterations lead to chromatin compaction and gene silencing, although how this occurs is not fully understood. Our studies suggest that the HMGA proteins can be structural components of heterochromatin, at least in the context of SAHFs. Thus, in senescent cells HMGA proteins localize to condensed regions of DNA that also contain me-K9H3 and HP1. Moreover, enforced expression of either HMGA protein can trigger the formation of heterochromatic foci, and suppression of HMGA proteins, particularly HMGA1, can dissolve SAHFs in senescent cells.

Precisely how HMGA proteins contribute to heterochromatin formation remains to be determined. However, our studies strongly suggest that this activity involves its canonical ability to bind the minor groove of AT-rich DNA sequences since chemicals that compete with HMGA for binding the minor groove of DNA can produce a morphological decondensation of chromatin in senescent cells. Notably, these agents do not displace the heterochromatin protein, HP1, implying that HMGA and HP1 act in parallel to produce a highly condensed DNA state. This cooperative relationship may also underlie the mutual dependence of $p16^{INK4a}$ and HMGA in senescence—with $p16^{INK4a}$ acting in part to trigger histone methylation and recruitment of HP1 through its action on Rb (Nielsen et al., 2001; Vandel et al., 2001), and HMGA proteins contributing to a higher-order chromatin state. Importantly, the role of HMGA proteins in heterochromatin structure may not be limited to cellular senescence, since HMGA proteins have been noted on the inactive X chromosome and in pericentric heterochromatin (Chadwick and Willard, 2003; Harrer et al., 2004), which also contains DNA sequences that are AT rich (Dillon, 2004).

That HMGA proteins contribute to heterochromatic structures and gene repression is surprising in light of work linking HMGA proteins to more open chromatin configurations and transcriptional activation. These opposing observations suggest that the activity of HMGA proteins can be modulated, perhaps producing distinct effects on the local chromatin structure and transcription of target genes. Interestingly, HMGA proteins can be phosphorylated, acetylated, and methylated at specific amino acids and, perhaps like histones, the type, location, and extent of such modifications control their ability to promote open or closed chromatin states (Reeves, 2001; Sgarra et al., 2004). Indeed, the location of HMGA1 acetylation can influence transcription from the *IFN-β* locus (Munshi et al., 2001), and HMGA controls distinct subsets of genes in normal and senescent cells. In any case, by linking HMGA proteins to SAHFs and senescence, our study reenforces the importance of global chromatin remodeling for the senescence program.

The action of HMGA proteins in senescence provides insights into their role in cancer development. Previous studies link the *HMGA* genes to oncogenesis and show that high levels of HMGA proteins can promote proliferation, transformation, and tumorigenesis (Reeves et al., 2001; Takaha et al., 2004). Here we see that HMGA proteins can have antiproliferative activities as well. Thus, as has been described for TGF-β (Siegel and Massague, 2003), HMGA proteins may be both pro- and antioncogenic, depending on the cellular context. Consistent with this view, haploinsufficiency of *Hmga1* causes myelo-lymphoproliferative disorders in mice (Fedele et al., 2006). Overcoming the antiproliferative activities of HMGA proteins may be an important step during carcinogenesis.

EXPERIMENTAL PROCEDURES

Vectors and Viral Infections

The following retroviral and lentiviral vectors were used in this study: pBabe-Puro (Oncogenic *ras* [H-*ras*V12]), pBabe-EGFP (H-*ras*V12), pWZL-Hygro (H-*ras*V12, EGFP-tagged human *HMGA1* and *HMGA2*, *INK4a*, *E1A*, $p53^{175H}$, and *CDK4*) (Serrano et al., 1997), pWZL-Blasticidin (H-*ras*V12), pWZL-Neo (*HDM2*), pLPC-Puro (*E1A*, EGFP-tagged human *HMGA1* and *HMGA2*, and HA-tagged human *HMGA2*) (Narita et al., 2003), pSin-Puro ($p16^{INK4a}$ shRNA), pSin-Hygro ($p16^{INK4a}$ shRNA), pSin-Puro-miR30 (*HMGA1* and *HMGA2* shRNAs), pSin-Hygro-miR30 (*p53* shRNA), and pRRL.Sin-18 lentiviral vector (*INK4a*, *HMGA1* and *HMGA2* shRNAs, miR30 control, and GSE22) (Beausejour et al., 2003). Human *HMGA1* and *HMGA2* were cloned by RT-PCR from total RNA of IMR90 cells. Information on the RNAi vectors and target sequences are available in Supplemental Data. Human diploid

IMR90, WI38, and BJ fibroblasts (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics. Retroviral and lentiviral gene transfer was performed as described (Beausejour et al., 2003; Narita et al., 2003). Lentivirus supernatant was diluted with culture medium and applied to IMR90 cells 9 days after introduction of *ras*.

Cell Proliferation, Transformation, and SA-β-Gal Assays

BrdU incorporation was measured as described (Narita et al., 2003). For population-doubling analysis, cells were counted and 4×10^5 cells were plated on 10 cm plates every 3–4 days. For colony formation assays, 1×10^3, 3×10^3, or 1×10^4cells were plated on 3.5 cm plates. Cells were fixed with 4% formaldehyde and stained with Crystal violet (Sigma). Dye intensity was quantified using "ImageJ" software (http://rsb.info.nih.gov/ij/). For focus formation assays, 3×10^5 cells were plated on 3.5 cm plates. Cells were fixed and stained with Giemsa (Sigma). Cells were assessed for tumorigenicity in immunocompromised mice as described (Seger et al., 2002). SA-β-gal activity was detected as described (Narita et al., 2003). Data are presented as mean ± standard error. Differences were analyzed by Student's t test, and p value < 0.05 was considered to be statistically significant.

Gene Expression

Indirect immunolabeling was performed as described (Narita et al., 2003). Cells were pre-extracted in the CSK buffer with 0.3 mg/ml digitonin (Sigma) before fixation for HP1γ staining. The following primary antibodies were used: anti-HMGA1 (1:1000, provided by V. Giancotti), anti-HMGA2 (1:200, provided by V. Giancotti), anti-HP1γ (Chemicon, 1:1000), anti-BrdU (Pharmingen, 1:500), anti-GFP (Clontech, 1:50), anti-me-K9H3 (Upstate, 1:500), and anti-Ac-K9/14H3 (Upstate, 1:1000) antibodies. Alexa Fluor Conjugates (Molecular Probes) were used as the secondary antibodies, and DNA was visualized by using 4',6-diamidino-2-phenylindole (DAPI) (1 μg/ml). Confocal images were obtained using a Zeiss LSM 510 confocal laser-scanning microscope. Electron microscopy was performed as described (Narita et al., 2003). The primary antibodies against HMGA1 and HMGA2 and the secondary antibodies conjugated to 10 nm colloidal gold were used.

Western blotting analysis was carried out as described (Narita et al., 2003). Blots were probed with the following antibodies: anti-HMGA1 (1:4000, provided by V. Giancotti), anti-HMGA2 (1:1000, provided by V. Giancotti), anti-$p16^{INK4a}$ (H-156, Santa Cruz, 1:1000), anti-p53 (Do-1, Oncogene, 1:1000), anti-HP1β (Chemicon, 1:1000), anti-HP1γ (Chemicon, 1:5000), anti-Ac-K9/14H3 (Upstate, 1:4000), anti-cyclin A (Sigma, 1:1000), anti-HA (12CA5 hybridoma supernatant, 1:100), anti-CDK4 (provided by G.J. Hannon, 1:2000), anti-Hdm2 (2A10 and 4B11, 1:250, provided by A. Levine), anti-α-tubulin (B-5-1-2, Sigma, 1:5000), and anti-actin (ac-15, Sigma, 1:5000) antibodies. Quantitative RT-PCR was performed as described (Narita et al., 2003).

For microarray analysis, total RNA was purified by RNeasy columns (Qiagen) and utilized for cRNA preparation by Message AmpII (Ambion). cRNA was hybridized to U133 Plus 2.0 microarrays (Affymetrix) according to the manufacturer's instructions. Two biological replicates were performed for each experimental condition. Data were analyzed with GeneTraffic UNO (Stratagene) and the with KEGG pathway database (http://apps1.niaid.nih.gov/david/).

Cell Fractionation, Chromatin Isolation, and Protein Sequencing

Cell fractionation was performed as described with minor modifications (Mendez and Stillman, 2000). Cells were resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, protease inhibitor cocktail [Complete, Roche], 0.1 mM phenylmethylsulphonyl fluoride). Digitonin (Sigma, 0.3 mg/ml) was added, and the cells were incubated for 10 min on ice. Nuclei were collected by low-speed centrifugation (4 min, 1300 × *g*, 4°C) and supernatants were isolated as cytoplasmic factions. Nuclei were washed once in buffer A and lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors as described above). After being incubated on ice for 30 min with occasional vortexing, the insoluble chromatin factions were isolated by low-speed centrifugation and supernatants served as nucleoplasmic fractions. After vigorous washing with buffer B, the chromatin pellets were resuspended in DNase I (Roche) solution (2×10^3 units/ml DNase I, 20 mM Tris [pH7.5], 10 mM $MgCl_2$) and incubated on ice for 1 hr. Samples were subjected to SDS-PAGE. The excised gel bands were trypsinised following standard procedures and analyzed by LC-MS/MS. The resulting spectra were analyzed using the SONARS search engine.

Hoechst Competition Assay

ras-induced senescent IMR90 cells (postselection day 7), plated on coverslips, were incubated with 10 μg/ml Hoechst 33258 (Molecular Probes) for 2 days. The cells were fractionated as described above, followed by immunoblotting, or fixed with 4% formaldehyde, followed by DNA staining in 0.2% Triton X-100 and DAPI/PBS.

Supplemental Data

Supplemental Data include seven figures experimental procedures, references, and four tables and can be found with this article online at http://www.cell.com/cgi/content/full/126/3/503/DC1/.

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Note Added in Proof

In marked contrast to our findings in normal diploid fibroblasts, a recent report indicates that HMGA2 can bind Rb and counteract its ability to inhibit E2F activity, leading to enhanced E2F target gene activation in tumor cells. These observations further underscore the context dependency of HMGA2 action in cellular proliferation and transformation, even at the level of E2F target genes.

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