Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence

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Epigenetic changes to chromatin are thought to be essential to cell senescence, which is key to tumorigenesis and aging. Although many studies focus on heterochromatin gain, this work demonstrates large-scale unraveling of peri/centromeric satellites, which occurs in all models of human and mouse senescence examined. This was not seen in cancer cells, except in a benign senescent tumor in vivo. Senescence-associated distension of satellites (SADS) occurs earlier and more consistently than heterochromatin foci formation, and SADS is not exclusive to either the p16 or p21 pathways. Because Hutchinson-Guilford progeria syndrome patient cells do not form excess heterochromatin, the question remained whether or not proliferative arrest in this aging syndrome involved distinct epigenetic mechanisms. Here, we show that SADS provides a unifying event in both progeria and normal senescence. Additionally, SADS represents a novel, cytological-scale unfolding of chromatin, which is not concomitant with change to several canonical histone marks nor a result of DNA hypomethylation. Rather, SADS is likely mediated by changes to higher-order nuclear structural proteins, such as LaminB1.

Introduction

Cultured human primary cells have a limited life span and ultimately become incapable of further division despite remaining metabolically active. This irreversible exit from the cell cycle, widely referred to as cellular senescence, has important implications not only for aging and stem cell biology but also as a key anti-tumorigenesis mechanism. Senescence can be induced by a variety of mechanisms, including shortened telomeres (replicative senescence), oncogene expression, oxidative stress, and replicative exhaustion, or via expression of regulatory factors, such as the ubiquitin ligase SMURF2 (Zhang and Cohen, 2004; Zhang, 2007).

Cellular senescence is accompanied by changes in gene expression and chromatin packaging; however, the absence of cell cycling is the only hallmark that consistently distinguishes senescent cells (Herbig et al., 2004; Cristofalo, 2005; Di Micco et al., 2011; Kosar et al., 2011). Formation of senescence-associated heterochromatic foci (SAHF) has received much attention, not only as a senescence marker but also as a proposed mechanism to promote and stabilize the senescent state (Narita et al., 2003; Zhang et al., 2007). Although SAHF exhibit repressive chromatin modifications, including H3K9Me3, H4 hypoacetylation, macroH2A, and HP1 (Adams, 2007), some marks commonly associated with heterochromatin are lost during senescence and aging, such as linker histone H1 and DNA methylation (Funayama et al., 2006; Misteli, 2010).

Although SAHF are common in human senescence, they are not found in all senescent human cells or any senescent mouse cells (Narita et al., 2003; Kennedy et al., 2010). In particular, cells from patients with Hutchinson-Guilford progeria syndrome (HGPS) and from aged individuals tend to show loss of visible heterochromatin blocks and associated marks (Shumaker et al., 2006; Misteli, 2010). These inconsistencies leave open the question of whether or not cells in premature aging syndromes such as HGPS undergo the same process for loss of proliferative capacity as normal primary fibroblasts senescing in culture. Furthermore, even within normal cultured senescing fibroblasts it is not known whether SAHF are a key part of the senescence pathway or arise as a consequence of a senescent end state (Narita et al., 2003; Zhang et al., 2007; Kennedy et al., 2010; Di Micco et al., 2011). It would thus be important to identify a broad epigenomic...
change to chromatin that consistently occurs during senescence in a variety of human and mouse systems and during premature arrest in cells from patients afflicted by HGPS.

In contrast to the formation of excess facultative heterochromatin, constitutive heterochromatin in senescent cells has received little attention. Here, we demonstrate that the peri/centromeric satellite heterochromatin undergoes a striking decondensation in senescent cells. This dramatic change to structures key to cell division occurs consistently in a variety of senescence models, is not exclusive to either known senescent pathway, and happens independently of and before SAHF formation. It has also been observed in senescent human and mouse cells and appears to be prevalent in vivo in benign prostate intraepithelial neoplasia (PIN) tumors. Importantly, this change to satellite heterochromatin is particularly prevalent in cultured fibroblasts from two HGPS patients. Thus, what we term senescence-associated distension of satellites (SADS) is a new marker that constitutes an early and potentially key event in the process of cell senescence. We further show that SADS retain several canonical histone hallmarks of heterochromatin, suggesting that this visible unraveling hallmarks change to higher-order chromatin folding potentially at an unprecedented level.

Results
Marked distension of satellite DNA in a subset of primary human fibroblasts

In a culture of proliferative normal diploid fibroblasts (Tig1) hybridized with a probe to α-satellite (α-sat) DNA, we first noted that a small subset of cells appear to have dramatically distended satellites, such that the normally compact round centric-satellite signals (Fig. 1 A, left) form linear extensions that appear “strung out” (Fig. 1 A, right). These thin threads of distended DNA can be difficult to reproduce in print, but were unambiguous through
the microscope (see online supplemental material). This highly distended pattern was not a sporadic variation peculiar to a few centromeres, but evident in most if not all α-sat signals in a given cell. For quantitative scoring (Fig. 1 E), a cell nucleus had distended satellites when the majority of signals lost their discrete round shape, had threadlike extensions, and elongated along a longitudinal axis. Furthermore, satellite II DNA, found at the pericentromeres of several chromosomes, showed a similarly dramatic loss of its normally tight packaging (Fig. 1 A). Similar patterns were seen in a variety of diploid fibroblast lines including IMR90, WI38, BJ, and LF1, but not in several tumor-derived immortal cell lines. Although some of the fibroblasts with distended satellite DNA had enlarged nuclear morphology (Fig. 1 A, right) others did not (Fig. 1 B); this and other evidence rules out that satellite distention reflects global DNA decondensation throughout the genome.

**Satellite distension is a hallmark of cells undergoing replicative senescence in culture**

The “fried egg” morphology of some cells with markedly distended satellites in early passage cultures suggested that they may represent senescent primary fibroblasts. Thus we compared early passage, proliferative cultures to those that had undergone replicative senescence. Remarkably, 84% of cells in senescent early passage, proliferative cultures to those that had undergone senescence (Fig. 1, B and E; n = 3) had distended α-sat, compared with only 14% of early passage fibroblasts (Fig. 1 E). These cells had several other markers of senescence, including positive senescence-associated β-galactosidase (β-gal) staining, up-regulation of p16 and p21, and often SAHF (Fig. 1, C, D, and F–J). We also examined several transformed cell lines (HeLa, PC3, and U2OS) and despite numerous genetic and epigenetic aberrations in these neoplastic lines we found essentially no cells in these cultures with distended satellite (Fig. 1 K).

Next, to ensure satellite decondensation is not a property of quiescent cells, we examined Tig1 cells serum starved for 48 h or allowed to recover in normal serum for 24 h. Compared with controls, the percentage of cells exhibiting satellite unraveling did not change as a result of serum starvation (Fig. 2 A). Thus, satellite distension is not a property of stressed cells undergoing quiescence, nor a function of general epigenetic aberration in tumor cells, suggesting it is a regulated chromatin change specific to the senescence program.

We also used BrdU incorporation in replicating DNA to discriminate proliferative versus nonproliferative cells, to address whether the ~15% of cells exhibiting satellite distension in low/mid passage cultures are still cycling or are indeed senescent. A 20-min BrdU pulse in low passage Tig1 fibroblasts demonstrated that the subset of cells exhibiting satellite distension rarely incorporate BrdU (Fig. 2, B–D). As shown in Fig. 2 D, 35.3% of the cells with compact centromeric DNA also labeled with BrdU, whereas <1% (0.64%) had both BrdU and decondensed satellite, demonstrating that cells with satellite distension do not simultaneously replicate DNA. Similar results were seen in late passage fibroblasts (five passages from full senescence; Fig. 2 E). We then used a 48-h BrdU pulse to ask what percentage of senescent cells (defined by lack of DNA replication over 2 d) exhibit satellite distension. Importantly, in both early and late passage cultures, almost all BrdU-negative cells had decondensed satellite DNA, indicating this is a consistent change characteristic of senescent cells (Fig. 2 F).

Exposing cells to BrdU for a long period (48 h) is helpful to mark cells that are no longer cycling, but is long enough that some cells could label during their last S phase and then withdraw from the cell cycle. This would occur more frequently in late passage cultures in which many cells are entering senescence. Consistent with this, of the 15% of early passage cells that contained distended satellite DNA (Fig. 2 D), only 1.8% of these also incorporated BrdU during the 48-h label (Fig. 2 G). In contrast, in late passage, rapidly senescing cultures, ~25% of cells had distended satellites (Fig. 2 E) and up to 38% of these also labeled with BrdU (Fig. 2 G). This difference was observed in multiple experiments and suggests that within 48 h of the last DNA replication, cells entering senescence have already undergone major structural changes to satellite DNA. To test this further, the 48-h label was done on a fully senescent culture; in this case ~85% of cells had distended satellites, but only 6% of these stained positive for BrdU (Fig. 2 G).

**SADS is a hallmark of senescence induced by SMURF2 expression, oxidative stress, or oncogenic Ras**

We next addressed whether SADS occur in human cells induced to senesce via mechanisms that do not include telomere shortening (replicative senescence), thus we tested this in cells induced to senesce by three other methods: overexpression of SMURF2, oxidative stress, and oncogenic Ras. Previous work has shown that up-regulation of the ubiquitin ligase SMURF2 induces early onset senescence in cycling fibroblasts (Zhang and Cohen, 2004). Subsequently, several days after infection with SMURF2, cultures were largely senescent as judged by the presence of SADS (Fig. 3, A, B, E, and F), SAHF (Fig. 3, E and F; and Fig. S1 C), and β-gal staining (Fig. S1, A and B). Importantly, in multiple experiments the majority of infected cells (up to 90%) exhibited satellite distension within 10 d, whereas cells infected with a control vector did not (Fig. 3, E and F). Additionally, we induced early passage Tig1 fibroblasts to senesce by either oxidative stress or overexpression of oncogenic Ras, and subsequently observed that neither culture became confluent, many cells contained SAHF; and 73% and 71% of the cells had distended satellites, respectively (Fig. 3, C and D). These findings show that satellite distension is not exclusive to replicative senescence, but can be induced fairly rapidly by expression of the senescence regulator SMURF2, through oxidative stress or oncogenic Ras.

**Satellite distension is not exclusive to either p21 or p16 senescence pathways**

Previous work has revealed two pathways that fibroblasts use to undergo senescence, either the p53/p21 or p16/Rb (Zhang, 2007). Interestingly, cells do not up-regulate both p21 and p16 until late in senescence (Herbig et al., 2004). Although SAHF formation occurs primarily via the p16 pathway, cells such as BJ fibroblasts, which lack significant p16 expression, do not form SAHF (Narita et al., 2003; Fig. 3, A and B; and Fig. S1 C). Thus it was important...
Satellite distension was only slightly reduced compared with SMURF2-induced LF1 senescent fibroblasts (Fig. 3, E and F). Although there is a near complete loss of SAHF in BJ cells that lack substantial p16 expression, SADS are still highly prevalent. As further evidence that SADS do not require p16 we examined p16 null mammary epithelial cells, which also formed distended satellites (Fig. S1, F–I). Hence, SADS does not require p16 and is not restricted to either of the two established senescence pathways.

Satellite distension is an early event in senescence and independent of SAHF formation

Several observations support that SADS occurs early in cell senescence, well before and independent of SAHF formation. As shown in Fig. 3 (A and B), we observed cells with decondensed satellite distension was only slightly reduced compared with SMURF2-induced LF1 senescent fibroblasts (Fig. 3, E and F). Although there is a near complete loss of SAHF in BJ cells that lack substantial p16 expression, SADS are still highly prevalent. As further evidence that SADS do not require p16 we examined p16 null mammary epithelial cells, which also formed distended satellites (Fig. S1, F–I). Hence, SADS does not require p16 and is not restricted to either of the two established senescence pathways.
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cultures of normal mouse embryonic fibroblasts (MEFs). In many of these cells, the normally round chromocenters, organized clusters of the pericentric major satellite visible by DAPI staining, did indeed show structural aberrations (Fig. 4, B and D) exemplified by irregular shapes that coalesced, frequently with fibrous interconnections. In situ hybridization to the smaller blocks of minor satellite DNA (the centromere proper) demonstrated that 86% of cells in a culture of senescent MEFs (Fig. 4 E, β-gal staining) no longer formed small, round, and compact signals (Fig. 4 A, top), but clearly was extended, irregular, and often threadlike (Fig. 4, A–C). Therefore, SADS is a prominent chromatin change common to both mouse and human senescence.

Because some markers of human senescence, including SAHF, have not been verified in vivo (Kreiling et al., 2010), we obtained sections of premalignant PIN tissue (Fig. 4, F and G) known to have high levels of senescence (Chen et al., 2005). We then used β-gal staining to verify the presence of senescence in this tissue (Fig. 4 H) and DNA FISH to α-sat and sat II sequences in serial sections to reveal that distended satellite signals are present in cells within the positive β-gal–stained regions of PIN tissue (Fig. 4, I and J). In contrast, examination of tissue sections from several malignant cancers that lack senescent cells, including...
This apparent contradiction leaves open the question of whether HGPS cells undergo proliferative arrest by mechanisms distinct from senescence in normal cells. Therefore, we obtained primary HGPS skin fibroblasts to examine if satellite distension is evident in these cells as they arrest. We examined three different early passage cultured fibroblast lines: a 1-yr-old male (HGADFN155 passage 10, PD 21), an 8-yr-old female (HGADFN167 passage 11, PD 20), and that female’s 37-yr-old healthy biological mother (HGMDFM090 passage 13, PD 23). This demonstrated that although a fraction of cells had compact satellites (Fig. 5, A and E), satellite distension is indeed a hallmark of many cultured HGPS cells (Fig. 5, A, D, and F). For example, almost 50% of the 1-yr-old boy’s cells, which displayed a marked HGPS phenotype (blebbed and contorted nuclei), had distended satellites. Additionally, cells with SADS were confirmed to be non-cycling by a 20-min BrdU pulse in the 8-yr-old girl’s cell line, with just 1.5% of total cells having both SADS and BrdU label versus 29% with SADS and no BrdU (Fig. 5 B). We then asked

breast, thyroid, pancreatic, and prostate, showed that the satellites were condensed (Fig. 4, K and L; and not depicted), demonstrating that only in the senescent PIN tumor were there large sections of cells with distended satellites, which indicates that SADS are an in vivo marker characteristic of senescent cells.

SADS occurs in HGPS patient cells undergoing premature proliferative arrest

We next addressed whether SADS would also be seen in cells of patients with a devastating human disease that is an important model of in vivo aging—HGPS. HGPS patients express a mutated form of lamin A/C that causes global defects in the nuclear heterochromatic compartment. Consistent with the rapid aging phenotype, HGPS cells undergo premature senescence in culture, providing an in vivo model for human aging. However, neither in vivo nor in vitro do HGPS cells exhibit gain of visible heterochromatin blocks (SAHF) or heterochromatic marks. Instead, a general decrease in H3K9me3, HP1, and NURD-associated factors has been reported (Scaffidi and Misteli, 2006; Shumaker et al., 2006; Pegoraro et al., 2009). This apparent contradiction leaves open the question of whether HGPS cells undergo proliferative arrest by mechanisms distinct from senescence in normal cells. Therefore, we obtained primary HGPS skin fibroblasts to examine if satellite distension is evident in these cells as they arrest. We examined three different early passage cultured fibroblast lines: a 1-yr-old male (HGADFN155 passage 10, PD 21), an 8-yr-old female (HGADFN167 passage 11, PD 20), and that female’s 37-yr-old healthy biological mother (HGMDFM090 passage 13, PD 23). This demonstrated that although a fraction of cells had compact satellites (Fig. 5, A and E), satellite distension is indeed a hallmark of many cultured HGPS cells (Fig. 5, A, D, and F). For example, almost 50% of the 1-yr-old boy’s cells, which displayed a marked HGPS phenotype (blebbed and contorted nuclei), had distended satellites. Additionally, cells with SADS were confirmed to be non-cycling by a 20-min BrdU pulse in the 8-yr-old girl’s cell line, with just 1.5% of total cells having both SADS and BrdU label versus 29% with SADS and no BrdU (Fig. 5 B). We then asked
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DNA signals (Fig. 1 K and see Fig. 7 F). Thus, visible satellite distension was not related to satellite RNA expression. To quantify the extent of this striking satellite distension, we measured the signal using a probe specific to the \( \alpha \)-sat on human chromosome 17 (Chr. 17), in proliferative versus senescent Tig1 fibroblasts. The Chr. 17 \( \alpha \)-sat in senescent, SAHF-positive Tig1 fibroblasts had a mean length of 5.44 µm, in contrast to 0.88 µm in cycling cells (Fig. 6, A–E). As shown in Fig. 6 D, the shortest Chr. 17 satellite signal in SAHF-positive cells was more extended than the largest found in cycling cells. This increased satellite length does not merely reflect changes in nuclear size because large cycling nuclei with condensed satellites were observed as well as small nuclei with decondensed satellites (Fig. 1 B) and mean satellite length increased over sixfold, whereas nuclear diameter increased slightly less than twofold (Fig. 6, C and E).

In addition, the signal size of a randomly selected 156-kb BAC (containing the Chr. 21 Dyrk1 locus) showed no decondensation in senescent cells (Fig. 6, F–H), and there was no visible difference in the condensation of traditionally heterochromatic telomere signals in senescent (SMURF2 induced) and nonsenescent control cells (Fig. 6, I–L). Hence, satellite distension is not merely a reflection of nuclear size nor indiscriminate chromatin decondensation.

Satellite unfolding occurs on chromatin that retains canonical histone marks of heterochromatin

Several aspects of our findings suggest that SADS represents a unique change to higher-order chromatin folding, of a much different nature than in other studies of DNA decondensation. Changes in DNA condensation are known to be linked to active chromatin and certain histone modifications, but such molecular-scale changes are below the resolution of light microscopy and can only be determined by techniques such as DNase sensitivity. In contrast, SADS can be readily seen despite the limited resolution of light microscopy. To address whether SADS is linked to expression of the corresponding satellite sequences, we examined satellite RNA expression in senescent cells using well established techniques (Lawrence et al., 1989). In primary normal fibroblasts with visible SADS, sat II RNA is not substantially expressed (see Fig. 7, A and B) despite the dramatic decondensation of the DNA. In contrast, we and others have shown that sat II RNA is highly expressed in many rapidly cycling cancer cells (Ting et al., 2011; unpublished data), including cancer cell lines that we show have tightly packaged satellite DNA signals (Fig. 1 K and see Fig. 7 F). Thus, visible satellite distension was not related to satellite RNA expression.

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SADS is a unique higher-order unfolding of chromatin distinct from DNA decondensation linked to expression

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Satellite unfolding occurs on chromatin that retains canonical histone marks of heterochromatin

Because SADS represents such a marked change in chromatin packaging, we anticipated that heterochromatin-associated modifications and possibly centromere-specific CENP proteins would change dramatically. We found that both CENP-A and -B

Figure 5. SADS is characteristic of HGPS cells that undergo premature arrest. (A) Frequency of distended satellites in early passage cells from two HGPS patients (1-yr-old HGADF1N155 and 8-yr-old HGADF1N167) and the older healthy mother of HGADF1N167, HGMDFM090 (\( n = 100 \) in one of three representative experiments). (B) A 20-min BrdU pulse demonstrates that very few early passage HGADF1N167 cells with decondensed satellites label, confirming that cells with SADS are rarely in S phase (\( n = 202 \)). (C) Almost all cells that did not incorporate BrdU during a 48 h label had distended satellites (\( n = 100 \)). (D–F) DNA FISH to \( \alpha \)-sat (green) and sat II (red) shows that some HGPS cells have normal appearing compact satellites (E), whereas senescent cells in both the HGADF1N167 (D) and HGADF1N155 (F) cell lines contain SADS. Also notice the blebbed nuclear membrane (DAPI; F) characteristic of laminopathies. Arrows and arrowheads indicate a particularly distended \( \alpha \)-sat signal that is enlarged in the corresponding insets.
H1 depletion occurs after SADS form, consistent with other evidence that this H1 loss is concomitant with SAHF formation (Fig. S3, D–F). We and others also noted that SAHF stain strongly for repressive heterochromatin marks H3K9me3 and HP1γ (Narita et al., 2003; Zhang et al., 2005), and the accumulation of these marks on SAHF is concomitant with sharply lower levels of H3K9me3 and HP1γ in nucleoplasmic regions between SAHF, where the distended satellites reside (Fig. 1 B; Fig. S3, A–C; and Fig. S4 C; Funayama et al., 2006). Although SADS had formed in many cells that had clearly not redistributed H3K9Me3 (Fig. S3, B and C), the threadlike nature of SADS makes it difficult to determine by molecular cytology if the canonical heterochromatin marks (H3K9Me3 and H3K27Me3) are still on the DNA.

Therefore, to address H3K9Me3 and H3K27Me3 distribution at higher resolution, we analyzed the α-sat sequences for still associate with distended satellites in senescent cells. Consistent with CENP-B remaining bound across the broader segment of α-sat DNA, the normally compact CENP-B signal becomes visibly decondensed in senescent cells (Fig. 7 C and Fig. S2, A and B). In contrast, CENP-A, which binds to the much smaller α-sat core, was not visibly distended (Fig. S2, A and B, see line-scans comparing CENP-A and -B). Although CENP-A is still present, it did appear dimmer in many (but not all) senescent cells, consistent with a recent report of overall lower CENP-A levels in senescent cells (Maehara et al., 2010).

We next examined several heterochromatin modifications typically associated with DNA condensation, which we anticipated to be depleted from highly distended satellite DNA, such as linker histone H1, which has been shown to decrease during senescence (Funayama et al., 2006). We noted that in cells with SADS 64% still had high levels of H1, suggesting that nuclear H1 depletion occurs after SADS form, consistent with other evidence that this H1 loss is concomitant with SAHF formation (Fig. S3, D–F). We and others also noted that SAHF stain strongly for repressive heterochromatin marks H3K9me3 and HP1γ (Narita et al., 2003; Zhang et al., 2005), and the accumulation of these marks on SAHF is concomitant with sharply lower levels of H3K9me3 and HP1γ in nucleoplasmic regions between SAHF, where the distended satellites reside (Fig. 1 B; Fig. S3, A–C; and Fig. S4 C; Funayama et al., 2006). Although SADS had formed in many cells that had clearly not redistributed H3K9Me3 (Fig. S3, B and C), the threadlike nature of SADS makes it difficult to determine by molecular cytology if the canonical heterochromatin marks (H3K9Me3 and H3K27Me3) are still on the DNA.

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these histone modifications, using publically available ChIP-seq data (Chandra et al., 2012). Surprisingly, the number of reads in $\alpha$-sat sequences for heterochromatin marks H3K9Me3 and H3K27Me3 was tightly correlated in cycling and senescent cells, indicating that there was not a change in the level of these marks despite satellite distension (Fig. 7 D). These observations are in stark contrast to the differences in these histone modifications on satellite DNA between undifferentiated ES cells and cycling IMR-90 fibroblasts (Fig. 7 D) even though the packaging of these satellites remains visibly unchanged (not depicted).
We then examined the histone modification H3K4Me3, which is associated with gene expression, and noticed that levels of this mark changed slightly between both cycling and senescent cells and less so between ES and cycling fibroblasts (Fig. 7 D). Collectively, these results indicate that SADS formation involves higher-order unfolding of satellite chromatin that does not require changes in canonical histone marks associated with condensed heterochromatin (H3K9Me3 and H3K27Me3) or decondensed euchromatin (H3K4Me3).

We also considered whether loss of DNA methylation could trigger SADS formation. Interestingly, hypomethylation of satellite DNA is prevalent in most cancer samples (Ehrlich, 2009), yet the 13 different cancer samples (cell lines and tumors) examined here did not exhibit SADS (with the exception of the senescent benign PIN tissue). Surprisingly, we noted that 5-azacytidine (5-AzaC) induced SADS in U2OS cancer cells after several days of treatment (Fig. 7, E–G). However, these cells no longer cycled, presumably because 5-AzaC is known to induce senescence in normal and cancer cells through activation of the p16/INK4a promoter (which is often hypermethylated in cancer; Fang et al., 2004; Schnekenburger et al., 2011). To validate the interpretation that SADS formation after 5-AzaC treatment was a result of senescence, rather than a direct result of DNA hypomethylation, we examined untreated U2OS cells by a DNA methylation-sensitive Southern blot that confirmed that untreated U2OS sat II at 1q12 was hypomethylated (Fig. 7 H). In contrast, cycling Tig1 fibroblasts had methylated satellites as expected (and results suggest little or no change to satellite methylation upon senescence; Fig. 7 H and Fig. S5 D). Further evidence that DNA demethylation does not cause SADS came from examination of α-sat and sat II 1q12 in fibroblasts from a patient with immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome (Fig. S5, A and B), a disease characterized by a lack of DNA methylation on 1q12 caused by a mutation in DNA methyltransferase3b (Ehrlich et al., 2008). Measurement of sat II DNA 1q12 signals in interphase nuclei showed at most a very slight increase and was similar to normal fibroblasts or untreated U2OS cells (Fig. 7, E–G; and Fig. S5, B and C). Similarly, the α-sat signals did not show significant distension (Fig. S5 A). Collectively, these results indicate that the striking unfolding of satellite heterochromatin within nuclei of noncycling cells is tightly linked to cell senescence and is not triggered by DNA hypomethylation.

The magnitude of satellite DNA distension described here suggests that senescence likely involves changes to nuclear structural proteins that play a role in higher-order folding of the chromatin fiber. In this context, it is significant that we show a prevalence of SADS in HGPS cells (Fig. 5), considering that defects in lamina have been shown to have an impact in the nuclear organization of centromeric satellites (Taimen et al., 2009), that lamina-associated domains are enriched in pericentric satellite DNA (Guelen et al., 2008), and that SADS frequently extend along the nuclear periphery or stretch from central regions all the way to the lamina (e.g., Figs. 1 B, 6 B, and S5 C). Recent work also shows marked decreases in LaminB1 levels in both HGPS and normal cell senescence (Shimi et al., 2011; Freund et al., 2012). Consequently, we performed α-sat DNA FISH and antibody staining to LaminB1 in mid passage WI-38 fibroblasts and found that 95% of cells with normal LaminB1 levels had condensed satellites (Fig. 7, I and K). In contrast, in cells with obviously depleted LaminB1 staining, 73% had clearly distended satellites (Fig. 7, J and K; and Fig. S2, C and D). As further considered in the Discussion, this suggests that changes in LaminB1 and potentially other nuclear structural proteins may contribute to higher-order unraveling of satellite DNA during senescence.

**Discussion**

Epigenetic changes to chromatin are thought key to senescence and have received substantial attention; however, this study demonstrates a prominent, genome-wide chromatin change that has eluded discovery and runs counter to expectations that senescence is marked primarily by gain of large blocks of heterochromatin. We reveal that large-scale distension or “unraveling” of centric and pericentric satellite heterochromatin is a distinguishing mark of senescent cells that occurs in all senescence models studied here, with both high sensitivity and specificity. Furthermore, this distension occurs early in the senescence process and, unlike SAHF formation, is not exclusive to either the p16 or p21 senescence pathways.

Although SADS occurs earlier and more consistently than SAHF formation, that both occur in the same senescent nuclei resolves the seemingly incompatible or paradoxical findings regarding gain versus loss of heterochromatin marks in senescent and aged cells (Funayama et al., 2006; Narita et al., 2006; Scaffidi and Misteli, 2006; Kreiling et al., 2010; Kosar et al., 2011). Our findings clearly show that loss of constitutive heterochromatin and gain of facultative heterochromatin occurs in the same nucleus. Although bright accumulations of many heterochromatin marks, such as H3K9me3 or HP1γ, become prominent in senescent cells, the whole nucleus does not gain these heterochromatin marks; rather, senescence is characterized by redistribution of condensed and decondensed genomic regions.

**A unifying epigenetic feature of all senescence models examined including mouse and HGPS**

An important point demonstrated here is that SADS occurs during permanent cell cycle withdrawal for all of many different cell types and methods examined to induce cell senescence. Human cells that lack SAHF (BJ fibroblasts) still form SADS but, more importantly, SADS bridges what has been a major gap between mouse and human cell senescence: both the major and minor satellites distend with threadlike extensions in senescent MEFs. This clarifies our previous observation that the chromocenters of Dicer-depleted MEFs, which senesce prematurely, lose their round compact shape and appeared to connect and coalesce (Mudhasani et al., 2008). Additionally, we observed distended satellite heterochromatin in senescent cells within PIN cancer tissues, which is significant because other heterochromatin changes (SAHF) have not been observed in vivo (Kreiling et al., 2010).
were not yet depleted for histone H1, which is consistent with
may trigger senescence. SADS could also be seen in cells that
but not both, but we see SADS irrespective of which pathway
here, early in senescence cells often express either p21 or p16,
viously reported (Herbig et al., 2004) and indicated by results
SADS can be observed before nuclear enlargement. As pre-
dent before SAHF formation, but never vice versa, and similarly,
early in senescence cells often express either p21 or p16,
noncycling cells and occurs in cells that first
up-regulate either the p21 or p16 pathway. In
late stage senescence, both pathways are up-
regulated, and the p16 pathway promotes for-
mation of SAHF. During this process there is a
reorganization of several heterochromatin fac-
tors as seen by immunofluorescence including
H3K9Me3 and HP1γ, whereas H1 levels uni-
formly decrease with the formation of SAHF.
Whereas nuclear diameter increases progres-
sively and is most pronounced in cells with
SAHF, SADS are seen in some cells before nu-
clear enlargement. The nuclear lamina protein (LaminB1)
may play a role in the higher-order
folding of the DNA as it is diminished in most
cells before SADS form. (B) Sat DNA in cycling
cells is tightly packaged and this packaging is
lost during senescence as the DNA distends.
Because three canonical histone modifications
remain unchanged during senescence the dif-
erence in DNA packaging may be attributed to
changes in higher-order folding.

The importance of SADS providing a unifying theme of
senescent cells is especially evident in relation to the debate
regarding HGPS. Interestingly, HGPS cells do not exhibit in-
creases in repressive marks typical of normal senescent fibro-
blasts; rather they show depletion of repressive factors (Scaffidi
and Mistel, 2005; Shumaker et al., 2006). Thus there has been
a lingering question as to whether chromatin changes in HGPS
cells reflect mechanisms of cell cycle arrest that are fundamen-
tally distinct from that of normal cell senescence. Importantly,
early passage fibroblasts from two young HGPS patients under-
going proliferative arrest showed a high rate of SADS. There-
fore, this marked distension of satellites constitutes a more
universal epigenetic program that underlies the biology of both
HGPS and normal cell senescence.

Integration of SADS into the senescence
process: A model
Fig. 8 A provides an overview of SADS formation relative to
several changes characteristic of senescent cells. Multiple points
of evidence demonstrate that unraveling of compact satellite
dNA is a relatively early step, and the rapid nature with which
SADS occurs contrasts sharply with the broader changes to
chromatin seen by FAIRE analysis 3–4 mo after entry into se-
nescence (De Cecco et al., 2013). For example, BrdU labeling
of cultures nearing senescence showed satellite distension pres-
cent in cells within 48 h of the last S phase. SADS are often evi-
dent before SAHF formation, but never vice versa, and similarly,
SADS can be observed before nuclear enlargement. As pre-
viously reported (Herbig et al., 2004) and indicated by results
here, early in senescence cells often express either p21 or p16,
but not both, but we see SADS irrespective of which pathway
may trigger senescence. SADS could also be seen in cells that
were not yet depleted for histone H1, which is consistent with
other evidence that loss of H1 is a late event (Funayama et al.,
2006). Additionally, many nuclei containing SADS had not re-
distributed heterochromatin marks (H3K9Me3 and HP1γ) onto
SAHF. Hence, our model conveys that satellite DNA distension
occurs before and irrespective of a more global redistribution of
facultative heterochromatin.

SADS is large-scale chromatin unfolding
facilitated by nuclear structural proteins
rather than histone modifications
We have referred to the changes in satellite packaging reported
here, and modeled in Fig. 8 B, as distension rather than decon-
densation to emphasize that the change in packaging is on a differ-
ent scale than the decondensation that has been widely studied in
the control of gene expression. To our knowledge, such a marked
extension of chromatin in an interphase nucleus is relatively
unique in biology and may be unprecedented. That a single cen-
tromeric α-sat was often as long as 5 µm (Fig. 6) suggests that
these α-sat blocks (ranging from ~0.5 to 3 Mb) exhibit significant
decompaction ~5× below the level of condensed chromatin and
lower than levels seen in a large genic locus (Lawrence et al.,
1990). This clearly cytological scale distension, coupled with
the surprising finding that the DNA is still packaged in nucleo-
somes that retain three canonical histone marks (Fig. 7 D), leads
us to propose that SADS involves changes of higher-order chroma-
tin loops, such as release of satellite DNA from scaffolding and
other chromosomal structural proteins, rather than modifications of
core histones.

Given that HGPS is caused by mutations in LaminA/C
and is characterized by higher levels of SADS, and that LaminB1
is depleted in HGPS and senescent normal fibroblasts, an im-
portant possibility is that changes to these scaffold proteins
could play a role in satellite distension (Shimi et al., 2011;
Freund et al., 2012). Although our evidence indicates that cells with SADS do not always have low LaminB1 staining, SADS did not appear to occur in cells that have high LaminB1 staining (Fig. 8 A, model). Therefore, LaminB1 may be one of several proteins that anchor satellite heterochromatin in nuclei and its loss may contribute to increased destabilization of satellite architecture and subsequent higher-order unfolding of DNA.

Potential implications of SADS for cell cycle withdrawal
This discovery raises the possibility that the early unraveling of satellite heterochromatin could contribute to permanent cell cycle withdrawal. In this context, it is interesting to note that the cell cycle regulator Rb has been reported to interact with the nuclear lamina and play a role in the stabilization of satellite heterochromatin (Gonzalo and Blasco, 2005; Dorner et al., 2007; Marji et al., 2010). In addition, various other components of the interphase centromere/kinetochores could be impacted; for example, CENP-F, which interacts with Rb and blocks cells at the G1–S transition (Du et al., 2010), or CENP-G, which associates with α-sat throughout the cell cycle and may interact with the nuclear matrix (He et al., 1998).

Hence, the discovery of SADS provides the field with a prominent, early, and consistent marker of senescence, and it is likely to spark new avenues of research into how unraveling of interphase centromeres arises. SADS also helps to resolve a paradox between the loss of heterochromatin reported within some aging and senescence models and the gain of heterochromatin reported in others by showing that all cell types studied here consistently lose higher-order folding in satellite DNA. Finally, the magnitude of centromeric heterochromatin distension revealed here opens new avenues of inquiry into higher-order chromatin packaging, as well as its relationship to cell senescence.

Materials and methods

Cell culture
Lung fibroblasts including Tig1, WI-38, and IMR-90 (Coriell) as well as ICF cells (GM08747; Coriell) were grown in MEM containing 15% FBS. LF1 and B fibroblasts, LF1.p21 knockouts, and HGPS cell lines (The Progeria Research Foundation) were grown in DMEM containing 15% FBS. MEFs (a gift from D. Carone, University of Massachusetts Medical School, Worcester, MA) and neoplastic cancer lines (PC3, HELA, and U2OS; American Type Culture Collection) were grown in DMEM with 10% FBS. MEFs (a gift from D. Carone, University of Massachusetts Medical School, Worcester, MA) and neoplastic cancer lines (PC3, HELA, and U2OS; American Type Culture Collection) were grown in DMEM with 10% FBS. 48R human mammary epithelial cells (from normal female breast tissues) were maintained in mammary epithelial cell growth medium. Cells were passaged 1:2 when confluent, and hybridized with an α-sat probe (as described in “In situ DNA and RNA hybridization”) and incubated with a BrdU antibody (Partec) at a 1:200 dilution and washed before detection as described earlier. Finally, senescence-specific β-gal staining was performed according to Dimri et al. (1995).

Image analysis
The threadlike satellite sequences may be hard to see in print but were unmistakable through the microscope. As such, sections of images were often magnified for reader convenience and we encourage the viewing of our images in a digital format. We performed the imaging using an Axiovert 200 or an Axio phot microscope (Carl Zeiss) equipped with a 100× PlanApo objective (NA 1.4) and Chroma 83000 multibandpass dichroic and emission filter sets, set up in a wheel to prevent optical shift. Images were captured with a camera (Orca-ER) or a 200 series CCD camera (Photometrics) using AxioVision 4.8 software (Carl Zeiss). Z stacks were a compilation of 15–30 images taken at an interval of 0.1 to 0.25 μm, and subsequent image deconvolution was done using AxioVision 3D software theoretical PSF (Carl Zeiss) and either the nearest neighbor or fast iterative algorithm. Distances were measured using either Axiovision 4.8 or MetaMorph 4.6 software. Most experiments were performed a minimum of three times and scored by at least two independent investigators. All findings were easily visible by eye through the microscope (unless otherwise noted).

Analysis of ChIP-seq data
Publicly available ChIP-seq data (Chandra et al., 2012) were collected and analyzed. ChIP-seq and input sequence reads were mapped to the GRC37.p11 (hg19) reference genome using bowtie (v. 0.2.8; Langmead et al., 2009) and read densities and regions of statistically significant enrichment (“peaks”) were determined using model-based analysis of ChIP-Seq (v. 1.4.1; Zhang et al., 2008) using default settings. Tandem repeat sequences in hg19 were annotated using RepeatMasker (Smart and Green, 1996–2010). For the purposes of visualization, read densities were normalized by scaling such that mean read densities for common ChIP-seq peaks were equal to 100% and similar to the mean read densities for the peak of MA norm (Shao et al., 2012). The RepeatMasker α-sat sequences were divided into 100-bp fragments over which the mean normalized read densities were compared.

Western blots
35 μg of each lysate was run over 15% SDS-PAGE and electroblotted onto 0.2 μm of immunoblot PVDF (Bio-Rad Laboratories) for 90 min at 350 mA, 4°C. Western analysis was performed in PBS and 0.1% Tween 20, with or without 5% nonfat dry milk powder as necessary. Antibody concentrations...
and incubations are as follows: 1:500 rabbit α-p16 overnight at 4°C, 1:1,000 mouse α-p21 overnight at 4°C, 1:8,000 mouse α-tubulin 1 h at room temp, 1:1,000 HRP goat α-mouse (Thermo Fisher Scientific) 1 h at room temp, and 1:1,000 HRP α-rabbit (GE Healthcare) 2 h at room temperature.

**Methylation-sensitive Southern blots**

Cells were pelleted and resuspended in 50 µl of lysis buffer (400 mM Tris-Cl, 60 mM EDTA, 150 mM NaCl), and 1% SDS), incubated with RNase for 30 min at 37°C, treated with ProK overnight at 50°C, and then subjected to a phenol chloroform extraction. 10 µg of DNA was then digested overnight with HpaII,MspI, or BstB1 (New England Biolabs, Inc.) and run on a 1% agarose gel. DNA was transferred overnight to a nylon membrane using capillary action and fixed with 0.4 M NaOH. DNA of interest was detected using the Bright Star Bioblot kit (Ambion) with a nick-translated biotin-labeled probe for sat II 1q12 (prepared as described in “In situ DNA and RNA hybridization”). The membrane was imaged with Chemidoc (Bio-Rad Laboratories).

**Online supplemental material**

Videos 1 and 2 show a moving 3D rendering and a movie of each slice in the Z stack of Fig. 1 A. Fig. S1 reinforces that SADS is a property common to multiple different senescence models and pathways. Fig. S2 shows the CENP-A and -B distribution on individual centromeres of senescent cells as well as lamin B1 levels in cycling and senescent cells. Figs. S3 and S4 use immunofluorescence to illustrate changes in H3K9Me3, H1, and HP1 as well as LaminB1 levels in cycling and senescent cells. Figs S3 and S4 use methylation on satellite distension. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201306073/D1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201306073.dv.

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Figure S1.  **SADS is a property common to many different senescence models and pathways.** (A and B) β-Gal staining shows that LF1 fibroblasts infected with SMURF2 (B) are senescent, whereas cells infected with a control (A) are not. (C) Percentage of B) and LF1 cells with SAHF plotted as an independent variable (n = 200, representative of one of three repeats). (D and E) p21 staining (red) is not present in LF1 p21 knockout senescent cells as judged by the presence of SAHF (DAPI; negative antibody control). (F–H) SADS form in human breast epithelial p16 knockout cells as judged by distended α-sat (green) and sat II (red; G) and blown up in H, while others have tight satellite signals indicative of cycling cells (F). (I) Western blot demonstrating that senescent LF1 p21^{−/−} and 48R mammary epithelial cells lack p21 and p16 expression, respectively.
Figure S2. **CENP-A and -B distribution on individual centromeres of senescent cells and LaminB1 levels in cycling and senescent cells.** (A) CENP-A and -B staining in a senescent cell (top), cycling cell (middle), and late senescent cell with prominent SAHF (bottom). CENP-B staining distends in senescent cells (red), whereas CENP-A (green) does not. CENP-A staining levels on individual centromeres showed some variability, but were generally brighter in cycling and early senescent cells (middle and top) than late senescent cells (bottom). This is quantified by a line scan (expanded regions in A) shown in B where signal intensities (CENP-A, green; CENP-B, red) are plotted along the length of the drawn line. (C and D) LaminB1 (red) levels are not always lower in cells with distended satellites (green).
Figure S3. **H3K9Me3 distribution changes and H1 levels decrease with senescence.** H3K9Me3 (red) has a general nucleoplasmic signal in cycling (A, top) and senescent cells (B, middle). H3K9Me3 overlaps SAHF (DAPI) in late senescent cells (C, right). (D–F) Although both cells have distended α-sat (green), the top cell lacks SAHF (DAPI) and has robust H1 staining (red; 64%) and the bottom cell has SAHF and severely diminished H1 staining.
Figure S4. **HP1-γ redistributes from satellites to SAHF as cells undergo senescence.** (A and B) HP1-γ (green) and CENP-B (red) colocalize in cycling cells with condensed centromeres (A) but not in cells with distended centromeres (B) in which HP1-γ gathers at PML bodies and no longer colocalizes with the centromeres. The arrowhead indicates a particularly decondensed centromere (magnified in the inset). The merged image in B combines two different planes. (C) In late stage senescent cells, HP1-γ colocalizes with SAHF (DAPI; gray inset) and centromeres are often found in areas that lack intense HP1-γ staining (magnified on right).
Unfolding of satellite DNA occurs with senescence

Figure S5. Effects of hypomethylation on satellite distension. (A and B) Cycling ICF cells do not display SADS or marked distension of nuclear α-sat (A) or sat II (B) on 1q12. (C) Appearance of sat II 1q12 (green) signals that distend in senescent Tig1 fibroblasts (bottom right), but are compact in cycling cells (top left). The inset shows a separate senescent cell with 1q12 signal that extends to the nuclear periphery. (D) A DNA methylation-sensitive Southern blot with ICF, senescent Tig1, and cycling Tig1 cells digested by HpaII (H) or MspI (M) and detected with a probe for sat II 1q12 sequences. The arrow points to a band size that is lacking in some of the methylation-sensitive HpaII lanes, indicating that the 1q12 region is methylated in the corresponding cell types. It is important to note that 1q12 remains methylated in both cycling and senescent cells.

Video 1. A 3D rendering of Fig. 1 A. The video depicts a 3D reconstruction of Fig. 1 A created using Axiovision software where a cycling (left) and senescent (right) cell are hybridized with α-sat (green) and sat II (red) in DAPI-stained nuclei.

Video 2. A Z stack from Fig. 1 A. A cycling (left) and senescent (right) cell hybridized with α-sat (green) and sat II (red) in DAPI-stained nuclei. Each frame correlates to a successive slice (~0.1–0.25 µm apart) in the Z stack obtained with Axiovision software.