Locus-specific induction of gene expression from heterochromatin loci during cellular senescence

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Abstract

Senescence is a fate-determined state, accompanied by reorganization of heterochromatin. While lineage-appropriate genes can be temporarily repressed through facultative heterochromatin, stable silencing of lineage-inappropriate genes often involves the constitutive heterochromatic mark, histone H3K9me3. The fate of these heterochromatic genes during senescence is unclear. Here we show a small number of lineage-inappropriate genes, exemplified by the skin genes *LCE2* in fibroblasts, are derepressed during senescence from H3K9me3 regions. DNA FISH experiments reveal that these gene loci, which are condensed at the nuclear periphery in proliferative cells, are decompacted during senescence. Decompaction of the locus is not sufficient for *LCE2* expression, which requires p53 and C/EBPβ signalling. *NLRP3*, which is predominantly expressed in macrophages from an open topologically associated domain (TAD), is also derepressed in senescent fibroblasts, potentially due to the local disruption of the H3K9me3-rich TAD that contains it. The role of NLRP3 has been implicated in the amplification of inflammatory cytokine signalling in senescence and aging, underscoring the functional relevance of gene induction from 'permissive' H3K9me3 regions in senescent cells.

Senescence is characterized by persistent proliferative arrest triggered by diverse stimuli, including excessive mitotic stress induced by persistent oncogene activation as in oncogene-induced senescence (OIS)[1]. Senescent cells can secrete various factors, including cytokines, growth factors, and extracellular matrix modifying enzymes, which have a profound impact on the tissue environment[2]. The senescence-associated secretory phenotype (SASP) can have adverse effects in vivo, particularly when it persists, and contributes to aging and age-associated disorders, including cancer[2–4].

Senescence is often accompanied by alterations to the chromatin state[5,6]. Active chromatin marks and chromatin accessibility are dynamically altered during senescence, and such alterations are well correlated with the senescence-associated gene expression profile, including the SASP[7–11]. In contrast, heterochromatic marks, particularly H3K9me3, are largely static during OIS in some contexts[12–14]. However, the higher-order structure of the heterochromatic regions can be drastically altered during senescence. For example, senescence-associated heterochromatin foci (SAHFs) are formed through the spatial reorganization of pre-existing H3K9me3- and H3K27me3-marked chromatin[12,14]. Senescence-associated distension of satellites (SADS), which involves the decompaction of (peri-)centric constitutive heterochromatin, is also observed without alteration of H3K9me3 and H3K27me3 levels at these regions[9,15]. These studies suggest that the 3D structural alterations of heterochromatic regions during senescence can occur without loss or gain of repressive marks.

Epigenetic gene regulation is critical for defining cell-types and lineages. Tissue specific gene silencing is typically attributed to facultative heterochromatin, marked by H3K27me3. In committed progenitor cells, it has been suggested that H3K27me3 is

involved in the silencing of lineage-specific genes that need to be induced upon terminal differentiation[16]. In contrast, consistent with its close association with constitutive heterochromatin, H3K9me3 is mainly enriched in the non-coding parts of the genome, such as repetitive regions, regardless of the cell lineage. In addition, however, it has become evident that H3K9me3-enriched heterochromatin also contributes to silencing of genes specifically expressed in other tissues (i.e. lineage-inappropriate genes), thereby ensuring the stability of cell identity[16,17]. However, how such cell type specific genes are regulated during senescence is not clear.

Here we show evidence that lineage-inappropriate genes can be expressed from H3K9me3-heterochromatic domains during senescence. This senescence-associated 'leakage' of lineage-inappropriate genes is accompanied by the physical decompaction of the heterochromatic regions, which gain active chromatin features. This is in marked contrast to the desilencing mechanism for lineage appropriate genes during terminal differentiation. We propose that there is another layer to the mechanism of gene expression regulation, which can transcend the epigenetic barrier in matured cells.

Results

Aberrant expression of lineage-inappropriate genes during senescence

While investigating transcriptomic datasets, which we previously published, from IMR90 human diploid fibroblasts (HDFs) induced into senescence by different stressors[18–20], we noticed that multiple *Late cornified envelope* (*LCE*) genes were up-regulated in oncogenic $HRAS^{G12V}$-induced senescence (RIS) or DNA damage-induced senescent (DDIS) IMR90 cells. This was surprising, since the *LCE* genes (consisting mainly of three sub-clusters *LCE1-3*) represent a tissue-specific gene cluster. The *LCE* gene cluster is located in the Epidermal Differentiation Complex (EDC) (~1.5 Mb) locus on chr. 1q21, where a number of cornification genes are clustered and are induced

during epidermal terminal differentiation in the stratum corneum[21,22]. These structural proteins are then enzymatically cross-linked to reinforce cornification at the very late stage of epidermal differentiation. Thus, the *LCE* genes are lineage-inappropriate in fibroblasts. Indeed, expression of genes within the EDC locus was barely detectable in IMR90 cells in any of the other conditions we tested, including not only normal proliferative, but also quiescence, transformation (via co-expression of $HRAS^{G12V}$ and the adenoviral oncoprotein E1A[18]), and acute DNA damage (Extended Data Fig. 1a). The p53 tumor suppressor plays a critical role in senescence[1], and the upregulation of most *LCE* genes during senescence was diminished upon introduction of a short-hairpin against p53 (sh-p53) in RIS cells (Extended Data Fig. 1a), reinforcing the correlation between upregulation of some *LCE* genes in fibroblasts and the senescence phenotype. A similar induction of *LCE* genes was also observed in multiple publicly available datasets for senescent HDFs with different senescence triggers, except for a dataset of BJ replicative senescence (RS) (Extended Data Fig. 1b)[23–25]. This led us to hypothesize that a potentially new mechanism for derepressing tissue-specific genes in non-orthotopic tissues underlies the *EDC gene* induction during senescence.

We first validated the expression of the LCE2 proteins (encoded by *LCE2A-D*) in IMR90 cells. While LCE2, probed by an anti-pan-LCE2 antibody[26], was undetectable in proliferating cells, RIS IMR90 cells exhibited a comparable amount of LCE2 to differentiated human keratinocytes (Fig. 1a). Involucrin, encoded by another EDC gene *IVL*, was induced during keratinocyte differentiation, as expected, but it was also modestly detected in RIS fibroblasts (Fig. 1a). Consistent with the transcriptomic data (Extended Data Fig. 1a), the up-regulation of the LCE2 proteins during RIS was reduced when p53 was knocked-down (Extended Data Fig. 1c). To gain the insight into the mechanism for the aberrant expression of these genes, we next characterized

146 the chromatin state of the EDC locus in IMR90 cells using ChIP-seq and RNA-seq
147 datasets, which we have previously published[12,14,20]. We found that, in both
148 proliferative and RIS IMR90 cells, the majority of the EDC locus was enriched for
149 H3K9me3 flanked by H3K27me3 (Fig. 1b). Consistently, our RNA-seq analysis
150 revealed that the transcriptional activity of the genes within the EDC of proliferative
151 IMR90 cells was very low. However, despite the apparent lack of reduction in the
152 H3K9me3 coverage (if anything there was a slight increase) on the EDC during RIS
153 (Fig. 1b, c), the central region of the EDC, corresponding to the region around *LCE2*
154 genes, became transcriptionally active (Fig. 1b). We next examined the permissive
155 nature of transcription at this region by ATAC-seq (Assay for Transposase-Accessible
156 Chromatin using sequencing[27]) and H3K27ac ChIP-seq[10]. ATAC-seq maps chromatin
157 accessibility, whereas H3K27ac is associated with active transcription and, together
158 with high chromatin accessibility, marks active regulatory regions
159 (promoters/enhancers)[28]. The EDC locus of RIS, but not proliferative, IMR90 cells
160 exhibited H3K27ac enrichment, which coincided with ATAC-seq peaks ~50 kb
161 downstream of the *LCE2* cluster, suggesting that active enhancers are formed within
162 the 'heterochromatic' EDC during RIS, albeit modest ones (Fig. 1d). The data suggest
163 that tissue-specific genes can be expressed from H3K9me3-rich regions in non-
164 orthotopic tissues during senescence. In contrast, while a cluster of olfactory receptor
165 (*OR*) genes, another tissue-specific genes on chr. 1q22[29], was also broadly enriched
166 for H3K9me3, this cluster did not produce any detectable transcripts, nor H3K27ac or
167 ATAC-seq peaks in both proliferating and RIS IMR90 cells (Fig. 1d). Thus, the
168 mechanism of gene expression from H3K9me3-heterochromatin appears to be locus
169 specific.
170
171 A potential tumor suppressor activity of LCE1 (homologous to LCE2 and also encoded
172 from the EDC locus) has previously been proposed[30]. To test whether ectopic

expression of an LCE2 protein promotes replicative exhaustion in normal fibroblasts, we overexpressed LCE2A in IMR90 cells and performed population doubling and colony formation assays. LCE2A-expressing cells showed a reduced capacity in proliferation and colony formation (Extended Data Fig. 1d, e). These results potentially suggest a functional relevance of desilencing of *LCE* genes in normal fibroblasts.

Distinct epigenetic landscapes of EDC genes in senescent cells relative to keratinocytes

To directly compare the chromatin status of the EDC between fibroblasts and keratinocytes, we performed ChIP-seq for key histone marks before and after terminal differentiation in primary human keratinocytes. It has been suggested that EDC genes in undifferentiated keratinocytes are facultatively silenced by the polycomb repressive complexes (PRCs) and the corresponding repressive histone mark H3K27me3[31,32]. We confirmed these earlier observations using ChIP-seq: In contrast to IMR90 cells, in keratinocytes H3K27me3, but not H3K9me3, was enriched across the EDC locus (Fig. 1b). We also confirmed that the H3K27me3 level was reduced during differentiation, whereas H3K27ac-peaks were increased (Fig. 1b, c). These data indicate that the dynamic of the epigenetic landscape at the EDC locus is different between keratinocyte differentiation (where EDC genes are lineage-appropriate) and senescence in fibroblasts (where EDC genes are lineage-inappropriate).

The EDC locus is structurally altered in senescent fibroblasts

Senescence is often accompanied by alterations of high-order chromatin structure[33], highlighted by both heterochromatin reformation (represented by SAHFs[34]) and decompaction (represented by SADS[15]) without substantial alterations in H3K9me3. To characterize the chromatin structure of the EDC, we performed DNA fluorescent *in situ* hybridization (FISH) experiments with multiple Bacterial Artificial Chromosome

(BAC) probes tiled across the EDC locus (Fig. 2a, bottom). In proliferating IMR90 cells, these FISH signals were highly condensed and tended to be located at the nuclear periphery, which provides a repressive environment (Fig. 2b, Extended Data Fig. 2a). In contrast, in RIS IMR90 cells, FISH experiments using the same probes exhibited a decompacted pattern with an extended fluorescent signal length (Fig. 2b, c). Although less prominent, EDC decompaction was also observed in DNA damage-induced senescence (DDIS) (d7) and replicative senescence (RS) IMR90 cells (Fig. 2c). It was not detected after acute DNA damage (acDD) (d1) or in E1A+RAS-expressing senescence-bypassed transformed cells, suggesting that the EDC decompaction is highly associated with senescence (Fig. 2c). Of note, the morphology of the OR locus, which was not derepressed during RIS (Fig. 1d), remained unchanged in RIS IMR90 cells (Extended Data Fig. 2b).

In primary keratinocytes isolated from human skin, the EDC FISH probes also exhibited a compacted pattern and we failed to detect any significant decompaction during differentiation (Fig. 2b, c). Interestingly, this locus tended to locate at the nuclear interior, which is often transcriptionally permissive, both before and after differentiation (Extended Data Fig. 2a), underscoring the distinct mechanisms that derepress the EDC genes in senescent fibroblasts relative to differentiated keratinocytes. Note, the inner nuclear localization of the EDC in the keratinocytes is consistent with a previous study, showing that relocation of the EDC locus from nuclear periphery to inner nuclear regions already occurs during embryonic development, at least in mice[35].

We next conducted a time series analysis of the EDC morphology using IMR90 cells expressing oncogenic $HRAS^{G12V}$ in a 4-hydroxytamoxifen (4OHT) inducible form ($ER:HRAS^{G12V}$), where senescence is progressively established over ~6 days[19]. Our FISH analysis showed that the EDC signal length became increasingly extended over

the time course (Fig. 2d). Similarly, LCE2 expression was also more evident at later time points (Fig. 2e). Consistently, in our published microarray data[19], *LCE2* upregulation was most prominent at the late stage of RIS (Extended Data Fig. 1a), indicating that local decompaction of the EDC and desilencing of the *LCE2* genes are temporally correlated during RIS. It has been shown that SADS is established during the early stages of senescence[15], whereas SAHFs, which involve the chromosome-wide spatial segregation of transcriptionally active (inter-SAHFs) and inactive (intra-SAHFs) regions, progressively accumulate during senescence[36]. Although decompaction of the EDC is reminiscent of SADS, the kinetics of the EDC decompaction appeared to be closer to the kinetics of SAHF formation (Fig. 2f). Within RIS cell populations, the EDC was significantly decompacted in SAHF-positive cells compared to SAHF-negative cells (Extended Data Fig. 2c). Immunolabelling analysis also revealed that the majority of SAHF-positive cells were LCE2-positive (Extended Data. Fig. 2d). These data reinforce a close correlation between EDC decompaction and SAHF formation, although any mechanistic relationship between SADS and EDC decompaction is not excluded. In agreement with this, in IMR90 cells, EDC decompaction was most prominent in RIS IMR90 cells compared to DDIS and RS IMR90 cells, where SAHF formation is modest (Fig. 2c, Extended Data Fig. 2e). Note, *LCE* gene induction in DDIS and RS was also weaker than in RIS (Extended Data Fig. 1b) and LCE2 protein levels were undetectable or variable in DDIS or RS IMR90 cells, respectively (Extended Data Fig. 2f). In addition, we have shown that different HDFs develop SAHFs to varying degrees: for example, SAHF-formation was weak in BJ cells, compared to IMR90 and WI38 cells during RIS (Extended Data Fig. 2g)[37]. Up-regulation of LCE2 proteins in RIS BJ cells was marginal with no significant decompaction of the EDC (Extended Data Fig. 2h, i).

253 We next examined a spatial correlation between SAHFs and the structure of the EDC
254 locus. In the multi-probe FISH analyses, the EDC appeared to be located at inter-
255 SAHFs 'open' regions (Fig. 2b, Extended Data Fig. 2a). To validate this, we conducted
256 FISH experiments co-staining both the EDC and OR loci (using a single probe for each
257 locus, as depicted in Fig. 2a, top) and found that the colocalization frequency of the
258 OR locus with SAHFs was significantly higher than that of the EDC locus with SAHFs
259 (Fig. 2g). Interestingly, both the EDC and OR loci were enriched for Lamin B1 (LMNB1)
260 and each formed a single lamina-associated domain (LAD) (Fig. 2a)[14]. Lamin B1 levels
261 are reduced during senescence[38] and we have previously shown that this reduction in
262 Lamin B1 occurs predominantly from H3K9me3 regions, and that this liberation of
263 H3K9me3 heterochromatic regions from the nuclear envelope is correlated with SAHF
264 formation[12]. Indeed, the reduction in Lamin B1 was particularly prominent at the OR
265 locus, compared to the EDC locus (Fig. 2a), further supporting the model that SAHF
266 formation involves a spatial reorganization of pre-existing heterochromatin[12]. These
267 data suggest that at these two lineage-specific gene clusters, both of which are
268 enriched for H3K9me3 in HDFs, distinct gene regulatory mechanisms operate: the
269 EDC locus is derepressed outside of SAHFs by physical decompaction, while the OR
270 locus is incorporated into SAHFs and remains condensed and silenced.
271
272 To test the direct correlation between EDC decompaction and SAHF formation, we
273 knocked down HMGA1, which is an essential structural component of SAHFs, during
274 RIS induction (RAS+shHMGA1) (Fig. 3a)[37]. The EDC decompaction was partially, but
275 significantly inhibited in RAS+shHMGA1 cells although EDC decompaction in these
276 cells was still significant compared to the basal level (Fig. 3b). RNA-seq analysis
277 revealed a trend of reversal of the desilencing of most EDC genes in RAS+shHMGA1
278 cells (Fig. 3c). Consistently, the induction of LCE2 proteins was also weaker in the
279 presence of shHMGA1 (Fig. 3d). Although HMGA1-dependent heterochromatin

compartmentalization does not fully explain the EDC decompaction/desilencing, these data collectively suggest that decompaction of the EDC and potential derepressing of EDC genes are correlated with the senescence-associated 3D reorganization of chromosome bodies, which might also promote SAHF formation.

It was shown that ectopic p16 (a CDK-inhibitor) is sufficient for inducing SAHF-positive senescence[34], but those cells show a distinct gene expression profile, lacking typical persistent DNA damage response (pDDR) and inflammatory senescence-associated secretory phenotype (SASP) (Fig. 3e)[39]. To further gain insight into the relation between EDC decompaction and these senescence effector phenotypes, we measured EDC decompaction and gene expression from this locus in p16-induced senescent IMR90 cells. While less pronounced than RIS cells, p16-senescent cells exhibited both SAHFs and EDC decompaction (Fig. 3f, g). Our RNA-seq data, however, showed little change in the EDC genes in p16-senescent cells, compared to RIS or even DDIS, the latter in which SAHF formation and desilencing of the EDC genes were milder than in the RIS cells (Fig. 3h). The data suggest that SAHFs and EDC decompaction are not sufficient for the EDC gene induction, which probably requires additional signalling or specific transcription factor activation.

One candidate for such signalling is the p53 pathway: consistent with the literature[39], the p53 downstream target, p21, was not upregulated in p16-senescent IMR90 cells (Fig. 3e). Additionally, consistent with the immunoblotting results (Extended Data Fig. 1c), induction of *LCE2* genes (except for *LCE2A*) during RIS was partially p53-dependent (Fig. 3i, j). Thus, we tested whether the *LCE2* genes are direct targets of p53. We renalysed our p53 ChIP-seq data[18] and found that p53 mainly binds the *LCE2A* gene body, concomitant with increased deposition of H3K27ac (an active enhancer mark), in RIS cells (Fig. 3k). These results suggest that p53 may control at least some EDC genes through directly binding to the enhancer during RIS, although

p53-dependent downstream signalling may also contribute. Note, p53-depletion had no significant impact on the EDC decompaction (Fig. 3l).

The level of a key transcription factor that drives the inflammatory SASP, C/EBPβ, is upregulated during RIS[40]. Consistent with the literature[39], the SASP was largely absent in p16-induced senescent cells, where we found little upregulation of *CEBPB* (Fig. 3e). Interestingly, induction of *LCE2* genes during RIS was inhibited by depletion of C/EBPβ (Fig. 3i, j). This prompted us to map C/EBPβ binding sites in RIS cells and found C/EBPβ peaks around promoters of *LCE2* genes, suggesting that the *LCE2* genes may be C/EBPβ-targets. Furthermore, co-depletion of p53 and C/EBPβ showed stronger effects than singular knockdown (Fig. 3j), suggesting a co-regulation of *LCE2* genes by these two transcription factors, at least in part through direct binding. Together, it is possible that deregulation of the EDC locus consists of two steps: first, decompaction of the locus and second, activation of specific signalling and/or transcription factors.

Aberrant expression of lineage-specific genes and chromatin decompaction

Chromatin accessibility has been linked with cell-type-selective regulatory elements[41], reflecting cell fate and maturity[42]. It has been shown that chromatin accessibility is increased during senescence[9,10]. To test whether senescence-associated lineage-inappropriate gene expression is unique to the EDC genes, we searched for similar H3K9me3 domains that encompass both H3K27ac and ATAC-seq peaks in proliferative and RIS IMR90 cells, using high confidence sets of H3K9me3, H3K27ac and ATAC-seq peaks (Materials and Methods).

While the number of H3K9me3 peaks were relatively stable with 763 common peaks (805 in proliferative cells; 874 in RIS cells[12]), we previously showed that the number of

ATAC-seq peaks (73,946 in proliferative cells; 238,269 in RIS cells) and H3K27ac peaks (95,464 in proliferative cells; 117,385 in RIS cells) increase overall during RIS in IMR90 cells[10]. Consistent with previous studies[43], H3K27ac peaks mostly coincided with ATAC-seq peaks (Extended Data Fig. 3a).

Using the information about these active marks as well as gene expression (RNA-seq data we previously published[20]), we characterized H3K9me3 peaks: 136 peaks (defined in the RIS condition) contained both de novo ATAC- and H3K27ac peaks ('permissive' H3K9me3 peaks). 110 of these peaks showed no induction of gene expression, whereas 26 peaks were accompanied by significant upregulation of gene expression, including the *LCE* genes. Thus, we grouped these H3K9me3 peaks as follows: group 1 (non-permissive; 627 peaks): H3K9me3 peaks with no de novo ATAC-seq and H3K27ac peaks, group 2 (110 peaks): permissive (both de novo ATAC-seq and H3K27ac peaks) without gene activation, and group 3 (26 peaks): permissive with gene activation (Fig. 4a, Extended Data Fig. 3b). The permissive H3K9me3 peaks (group 2+3), regardless of altered gene expression, appeared to be larger in width compared to the other non-permissive peaks (group 1) (Fig. 3b). However, group 3 H3K9me3 peaks were significantly higher in gene density and were less AT-rich than group 2 (Fig. 4c, d). Among repeat elements, group 3 peaks were enriched for short-interspersed nuclear elements (SINEs), which are often found in GC-rich regions and in gene promoters [44] (Fig. 4e, f, Extended Data Fig. 3c). As exemplified by the EDC locus mentioned above (Fig. 2a), group 3 H3K9me3 peaks exhibited the least pronounced reduction of Lamin B1 during RIS (Fig. 4g-i). Thus, permissive H3K9me3 peaks with aberrant gene induction (group 3) appear to have distinct features characterized by higher gene density with residual Lamin B1 association.

We next explored individual genes within group 3 H3K9me3 peaks (a list of genes included in each H3K9me3-peak group can be found in Supplementary Table 1). Close visual inspection revealed that some 'active regulatory regions' (i.e. co-occurring H3K27ac and ATAC-seq peaks) overlapped permissive H3K9me3 peaks either in regions with H3K9me3 signal or within small gaps in H3K9me3 in either the proliferative or RIS conditions (Extended Data Fig. 3d). Using stringent criteria (both gene bodies and promoters with de novo ATAC/H3K27ac peaks entirely overlapping H3K9me3 peaks and not gaps in the H3K9me3 ChIP-seq signal), we identified 8 genes in 6 loci (*PDPN*, *LCE2A*, *LCE2C*, *IVL*, *NLRP3*, *DCC*, *TMEM132B*, and *ZNF667*) among the 38 significantly up-regulated genes during RIS in group 3 H3K9me3 peaks (see highlights in Fig. 4b-i). Note, Involucrin (encoded by *IVL* within the EDC locus) was also detected in RIS fibroblasts (Fig. 1a). To address the orthotopic expression status for these aberrantly expressed genes in normal tissues, we compared our RNA-seq data[20] with publicly available RNA-seq data obtained from The Human Protein Atlas (HPA)[45]. The basal expression levels of these genes in IMR90 cells were mostly low and they tended to show 'tissue-enriched' expression patterns, according to the HPA criteria (Fig. 5a, Extended Data Fig. 4a). Thus, the H3K27ac/ATAC peaks established within H3K9me3 domains during senescence may mark aberrant inductions of lineage-inappropriate genes.

To test whether desilencing of these genes during senescence is commonly accompanied by chromatin decompaction, we performed multi-probe DNA FISH analysis for *NLRP3*, *DCC* and *TMEM132B* loci (BAC probe positions in Fig. 5b). Note, the H3K9me3-rich regions encompassing *PDPN* and *ZNF667* were too small (~ 200 kb and 150 kb, respectively) for the multi-probe FISH experiment, in which each BAC probe is ~200 kb long. Similar to the EDC locus, all loci tested were highly condensed

in proliferative IMR90 cells, but the FISH signal lengths were significantly extended in RIS cells (Fig. 5b-d, Extended Data Fig. 4b).

Decompaction of H3K9me3 domains correlates with expression of lineage-inappropriate but not lineage-appropriate genes during senescence

To further investigate whether heterochromatin decompaction during senescence is associated with lineage-inappropriate genes, we decided to take advantage of a RIS model in human keratinocytes, in which the EDC is a lineage appropriate gene cluster. We first profiled EDC gene expression in RIS keratinocytes and compared to the differentiation condition using RNA-seq. In contrast to RIS fibroblasts, the EDC genes were widely expressed in RIS keratinocytes, exhibiting a similar pattern to differentiated keratinocytes (Fig. 6a). This is consistent with previous studies showing that some EDC genes are induced in senescent keratinocytes, induced by oncogenic HRAS or oxidative stress[46,47]. Then, we examined the EDC condensation status. As a comparison, we chose the *DCC* locus, identified in this study as differentially expressed in RIS fibroblasts. *DCC* encodes a netrin 1 receptor, which is expressed mainly in testis, lung and brain[45]. In contrast to the EDC, the *DCC* locus was embedded in a H3K9me3 region in both IMR90 cells and undifferentiated keratinocytes, but not in hES cells (Fig. 5b), suggesting that *DCC* becomes stably heterochromatinized in both fibroblasts and keratinocytes during development, thus *DCC* is lineage-inappropriate in keratinocytes (Fig. 6b). We performed additional FISH experiments for the *DCC* and EDC loci in these three cell types: hES cells, fibroblasts (control and RIS conditions), and keratinocytes (control and RIS conditions). Similar to IMR90 fibroblasts, we found a significant decompaction of *DCC* in RIS keratinocytes (Fig. 6c). Consistently, the mRNA levels of *DCC* were modestly but significantly increased in the RIS keratinocytes accompanied with up-regulations of key SASP factors associated with senescence (Fig. 6d). Thus, the senescence-associated decompaction of lineage

413 inappropriate heterochromatic genes is not limited to fibroblasts. In contrast, there was
414 no substantial chromatin decompaction of the EDC in RIS keratinocytes (Fig. 6c). This
415 reinforces the link between aberrant expression of inappropriate genes and their
416 decompaction.
417
418 Importantly, the decompacted chromatin state of not only the EDC locus but also *DCC*
419 was not detected in hES cells (Fig. 6c), where the chromatin in general tends to be
420 more 'open', exemplified by the *DCC* region (Fig. 6b)[48,49]. Therefore, senescence-
421 associated decompaction of these heterochromatic loci is a distinct phenotype from
422 the 'open' chromatin configuration often seen in ES cells.
423 **Multiple mechanisms might be involved in decompaction of H3K9me3 regions**
424 In previous Hi-C studies, an increase in long-range interaction between H3K9me3
425 regions during senescence has been correlated with the detachment of the H3K9me3
426 regions from nuclear lamina, reduced interaction within the H3K9me3 regions, and
427 SAHF formation (clustering of the H3K9me3 regions)[25,33,50,51]. Consistently, in our
428 recently generated in situ Hi-C data in proliferative and RIS IMR90 cells[52], we observed
429 that increased chromatin contacts during RIS tended to be longer-range compared to
430 decreased contacts, which were mostly short distance (Extended Data Fig. 5a, b). We
431 further characterized RIS-associated dynamic chromatin interactions focusing on
432 H3K9me3 loci. In marked contrast to the features of H3K9me3 regions that are
433 potentially involved in SAHFs (i.e. increased inter-region contacts, decreased intra-
434 region contacts, and reduced contact to Lamin B1)[14,25,50], group 3 H3K9me3 peaks
435 exhibited the weakest interaction increase with other H3K9me3 peaks (Fig. 7a, b).
436 Interestingly, group 2 H3K9me3 peaks (permissive without increased gene
437 expression) showed more increased contacts than not only group 3 but also group 1.
438 The nature of group 2 H3K9me3 peaks and their involvement in higher-order
439 heterochromatin reorganization during RIS is unclear. Notably, Lamin B1 reduction in

group 2, similar to group 1, was significantly stronger than group 3 (Fig. 4i), thus group 2 H3K9me3 regions might play some role in heterochromatin reorganization.

We next examined the individual gene loci. For example, the EDC locus showed reduced interaction with other H3K9me3 peaks (Fig. 7c, '*LCE2*, *IVL*') but increased contacts within the locus (Fig. 7d). We reanalysed external Hi-C data generated in WI38 fibroblasts[25] and found a similar alteration in the EDC locus during RS (Fig. 7e). Hi-C mapping in human keratinocytes was recently published[53] and we reanalyzed the data to compare with senescent fibroblasts. In keratinocytes, new loop structures were established within the EDC locus during terminal differentiation (Fig. 7f, Extended Data Fig. 5c). We found new contacts in the EDC locus in RIS IMR90 and RS WI38 fibroblasts, resembling the loops identified in keratinocytes (Fig. 7d-f, arrows). Together with the FISH data (Fig. 2b, g), these data suggest that the EDC locus escaped from SAHFs and decompacted at the inter-SAHF region, where it gained active 3D regulatory interactions. In contrast, *NLRP3*, another gene that was derepressed from a H3K9me3 region, exhibited a highly distinct pattern. *NLRP3* is located near the border within the H3K9me3-rich topologically associating domain (TAD) in IMR90 fibroblasts (Fig. 7g). TADs are megabased-sized chromatin regions, within which genomic regions interact with each other with high frequency[54–57]. These proximal chromatin contacts around *NLRP3* were largely lost and thus the TAD structure was disrupted in fibroblasts (Fig. 7g, arrows). *NLRP3* is predominantly expressed in immune cells, such as macrophages, and the encoded protein NLRP3 is a component of the NLRP3-inflammasome, a critical mediator of innate immune responses[58,59]. In addition, the role of NLRP3 in inflammatory SASP in fibroblast senescence has been demonstrated[60]. We reanalyzed public Hi-C and ChIP-seq datasets in THP1 cells, a spontaneously immortalized human monocytic cell line. *NLRP3* was located within an open region in THP1 cells and the TAD disruption seen

in RIS fibroblasts was not detected during macrophage differentiation, which is accompanied by *NLRP3* upregulation (Fig. 7h)[61,62], reinforcing the functional relevance of the unique mechanism for senescence-specific activation of genes that are otherwise tightly silenced within H3K9me3 regions. Interestingly, we recently identified a similar TAD disruption, in which genes, best exemplified by *NRG1* (encoding a SASP factor), can escape from H3K27me3 repressive 3D environment during RIS[52]. Indeed, ChIP-seq data showed a reduction of H3K27me3 in this region (Extended Data Fig. 5d). Together, our data suggest that the mechanisms for decompaction of the lineage-inappropriate genes within H3K9me3 occupied regions might differ between gene loci.

A recent study reported induction of *Nlrp3* in synovial fibroblasts (SFs) in a mouse arthritis model[63]: SFs isolated from mouse paws exposed to a danger signal, monosodium urate crystals (MSU), twice (MSU/MSU), but not once (-/MSU), exhibited upregulation of *Nlrp3*. Such a delayed alteration in gene expression is reminiscent of a senescent-like phenotype. We reanalysed their RNA-seq data (Extended Data Fig. 5e). We confirmed significant upregulation of *Nlrp3* and cytokines that are associated with senescence. Moreover, MSU/MSU SFs exhibited senescence features and significant induction of several EDC genes, compared to -/MSU SFs. Note, rodents lack the *Lce2* sub-cluster, but instead they have an expansion of the *Lce1* genes[64]. Although epigenomic characterization is required for further interpretation, the result suggests EDC genes and *Nlrp3* can be expressed in senescent fibroblasts *in vivo*.

Discussion

In this work, we provide evidence that senescence is associated with disruption of the H3K9me3-mediated ‘lineage barrier’. Through this, specific heterochromatic genes, which are otherwise tightly silenced, can undergo ‘leaked’ expression. This process is accompanied by localized decompaction and increased accessibility of chromatin, without apparent loss of H3K9me3. We have shown that the same genes can be

induced via distinct mechanisms: the 'default' mechanism of orthotopic cell types and a senescent-associated mechanism in ectopic cell types. Notably, we demonstrate that the default mechanism appears to be dominant when those genes are induced in senescent orthotopic cells, therefore the initial cell type-specific chromatin states are perhaps critical for how these genes are derepressed in response to stress. Considering that decompaction of those gene loci was closely correlated with SAHF formation, it might be an integrated part of high-order chromatin structural reorganization, specifically occurring during senescence.

NLRP3-inflammasome has been implicated in aging and considered to be a therapeutic target against age-associated inflammatory diseases[65,66]. Targeting senescent cells is an emerging anti-aging therapeutics approach, including 'senolytics' (drugs that selectively kill senescent cells) or 'senomorphics' (drugs that suppress the SASP or any other adverse functionality of senescent cells)[67]. Our data suggest that NLRP3 might be a potential senomorphic target[59,60]. Currently it is not clear whether the other genes identified here are involved in the senescence phenotype. However, it was shown that *LCE1* is a downstream target of p53 in cancer cell lines and inhibits the arginine methyltransferase, PRMT5, which is often upregulated in various cancers and a potential therapeutic target[68]. PRMT5 was also shown to have anti-senescence activity in osteosarcoma cells[69]. It remains to be elucidated whether or not LCE2 inhibits PRMT5 in senescent fibroblasts. *DCC*, *ZNF667*, and *PDPN* have also been implicated in cancer and rheumatoid arthritis[70]. For example, Netrin 1 receptor, encoded by *DCC*, is involved in axon guidance and *DCC* has been identified as a tumor suppressor gene in ectopic tissues, including colon and rectum[71,72]. A similar aberrant derepression mechanism might apply in other pathological contexts, such as early tumorigenesis and chronic inflammation.

Materials and Methods

Cell culture

Human diploid fibroblasts (HDFs) (IMR90, WI38 and BJ) were cultured in phenol-red-free DMEM with 10% FBS under physiological 5% O_2, except for population doubling and colony formation assays, which were conducted in atmospheric O_2[19,37]. RIS was induced by retrovirus-mediated expression of either $HRAS^{G12V}$ or $HRAS^{G12V}$ fused to the estrogen receptor (ER) ligand-binding domain (ER:RAS) system in HDFs. Acute DNA damage (acDD) was induced by treating cells with 100 μM etoposide for 24 hours. To obtain DNA damage induced senescence (DDIS) cells, cells were treated with 100 μM etoposide for 48 hours and maintained for an additional five days in drug-free media. To generate RIS-bypassed cells, $HRAS^{G12V}$ and E1A (adenoviral oncoprotein) were co-transduced using the retroviral gene expression system (E1A+RAS)[18]. For population-doubling analysis, cells were counted and $2x10^6$ cells were plated on 10 cm plates every 3–4 days. For colony formation assays, 10,000 cells were plated on 35 mm plates and maintained for 30 days. Cells were fixed with 4% formaldehyde and stained with Crystal Violet (Sigma). Dye intensity was quantified as integrated density using "Fiji" image processing package.

Primary normal human keratinocytes were isolated from normal human skin. All procedures involving human subjects were approved by the ethics committee of the Ehime University Graduate School of Medicine, Toon, Ehime, Japan, and all subjects provided written informed consent. The keratinocytes were cultured in Epilife medium (Gibco) supplemented with human keratinocyte growth supplement (HKGS, Gibco) under 21% atmospheric O_2. To induce differentiation in culture, keratinocytes were treated with 500 nM phorbol 12-myristate 13-acetate (PMA, Sigma, P8139) for 48 h after cells became confluent. RIS was induced by lentivirus-mediated expression of $HRAS^{G12V}$ in human keratinocytes.

549

550 The WA09/H9 human embryonic stem cells were obtained from WiCell, and cultured
551 in TeSR-E8 (StemCell Technologies #05990) on Vitronectin-coated glass coverslips
552 (0.5 µg/cm^2; ThermoFisher Scientific, A14700) in an atmosphere of 5% O_2 & 5% CO_2
553 at 37°C for FISH experiments.

554

555 **Vectors**

556 The following retroviral vectors were used: pLNCX2 (Clontech) for ER:HRAS^{G12V};
557 pBabe-puro for HRAS^{G12V}; pWZL-hygro for HRAS^{G12V} and E1A[19], pMSCV-puro for
558 miR30 sh-p53 (target sequence 5'-CACTACAACTACATGTGTA-3')[18]. The following
559 lentiviral vectors were used: pRRL.Sin-18 lentiviral vector for miR30 shHMGA1 (target
560 sequence 5'-ATGAGACGAAATGCTGATGTAT-3') and miR30 control)[37], pLenti
561 HRAS^{G12V} (gift from Eric Campeau, Addgene plasmid # 22259).

562

563 **Antibodies**

564 Immunoblotting was performed as previously described[19]. The following antibodies
565 were used for immunoblotting: anti-beta-Actin (Sigma, A5441, 1:5000), anti-Cyclin A
566 (Sigma, C4170, 1:1000), anti-Involucrin (Sigma, I9018, 1:1000), anti-p16 (H-156,
567 Santa Cruz, sc-759, 1:500) and (G175-1239, BD Pharmingen, 554079, 1:500), anti-
568 p21 (F-5, Santa Cruz, sc-6246, 1:500), anti-p53 (DO-1, Sigma, P6874, 1:1000), anti-
569 C/EBPβ (C-19, Santa Cruz, sc-150, 1:500), anti-c-H-Ras (F235-1.7.1, Calbiochem,
570 OP23, 1:500), anti-E1A (Santa Cruz, sc-430, 1:2000), anti-HMGA1 (Active Motif,
571 39615, 1:2000). The anti-Pan-LCE2 antibody (1:1000) was raised in rabbits against a
572 synthetic peptide (RPRLFHRRRHQSPD), as previously described (note, this antigen
573 sequence perfectly matches human LCE2B, C and D, and differs by one amino acid
574 for LCE2A)[26]. The following antibodies were used for ChIP: anti-H3K9me3 (clone

CMA318), anti-H3K27me3 (clone CMA323), anti-H3K27ac (clone CMA309)[73], C/EBPβ (C-19, Santa Cruz, sc-150)[20].

qRT-PCR

RNA was prepared using the Qiagen RNeasy plus kit (Qiagen) according to the manufacturer's instructions and reverse-transcribed to cDNA using the reverse transcriptase Superscript III kit (Life Technologies). qRT-PCR was performed with SYBR Green Master Mix (Takara). Data were normalized to *ACTB* (encoding β-actin) expression. The following primers were used:

ACTB forward: 5'-CGAGCACAGAGCCTCGCCTT-3'

ACTB reverse: 5'-CATCATCCATGGTGAGCTGGCGG-3'

DCC forward: 5'-AGCAGGAAGAAGTCAGTCAGTG-3'

DCC reverse: 5'-CTGTGGGGATGGTTCTGCTT-3'

IL1A forward: 5'-AACCAGTGCTGCTGAAGGA-3'

IL1A reverse: 5'-TTCTTAGTGCCGTGAGTTTCC-3'

IL6 forward: 5'-TGAAAGCAGCAAAGAGGCACTG-3'

IL6 reverse: 5'-TGAATCCAGATTGGAAGCATCC-3'

IL8 forward: 5'-AAGGAAAACTGGGTGCAGAG-3'

IL8 reverse: 5'-ATTGCATCTGGCAACCCTAC-3'

LCE2A forward: 5'-GGACCTGTCCCAGAGTGATG-3'

LCE2A reverse: 5'-GATCCAGGATGGGCTCTTG-3'

LCE2B forward: 5'-GGTTGACTAAACTCTGCCAGG-3'

LCE2B reverse: 5'-CACTGGGGCAGGCATTTA-3'

LCE2C forward: 5'-CTTGGGACTGAATGGCCAAG-3'

LCE2C reverse: 5'-GACTTGCAATTGGGGTGTTAC-3'

LCE2D forward: 5'-CTGCAGAAGAGCTCTGGTACTG-3'

LCE2D reverse: 5'-CTCCATCAAGCACAAAGTTCTG-3'.

DNA FISH

DNA FISH was performed as previously described[14]. Cells were plated onto glass coverslips the day before fixation. Cells were pre-treated with digitonin/CSK buffer (150 mg/ml), then fixed in 4% paraformaldehyde. Cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS, soaked in liquid nitrogen, treated in 0.1M HCl and dehydrated in EtOH. Several genomic loci were observed by tiling the following BAC/PAC probes along the EDC locus: RP6-121F19 (or RP11-81P11), RP11-655C3, RP11-709L1, RP11-157C12, RP11-352B17, RP11-766D13 and RP11-157A11; OR locus: RP11-643N11, RP11-344I22, RP11-643G14 and RP11-1084O3, or *DCC* locus: RP11-25O3, RP11-108B6, RP11-1077M23, RP11-315H18, RP11-933N21 and RP11-186B13; *TMEM132B* locus: RP11-433N22, RP11-1139A9, RP11-728C18 and RP11-626N23; *NLRP3* locus: RP11-243D8, RP11-951B14, RP11-908P10, RP11-121O22 and RP11-248A15. Fluorescent directly labeled probes were generated using a Nick Translation kit (Abbott) and/or purchased from Empire Genomics. Confocal images were obtained using a Leica TCS SP5 microscope. The maximum width of multi-probed signals (any color) on the locus were measured on Leica Application Suite Advanced Fluorescence (LAS-AF) software (Leica, Mannheim). For 3D images, confocal Z-stack images were projected to show X-Y images. X-Z images were generated by re-slicing and projection of 3D reconstructed images using ImageJ. SADS were observed as previously described[15] using an oligo against the alpha-satellite repeat sequence (5'-CTTTTGATAGAGCAGTTTTGAAACACTCTTTTTGTAGAATCTGCAAGTGGATATTTGG-3').

Transcriptomic analysis

RNA-seq was performed as previously described[10]. RNA was purified from undifferentiated and differentiated human primary keratinocytes using the Qiagen

RNeasy plus kit according to the manufacturer's instructions. The quality was checked using the Bioanalyzer with an Agilent RNA 6000 nano kit (Agilent). Libraries were prepared from six biological replicates of each condition using TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's instructions and sequenced using the HiSeq-4000 and Novaseq-6000 platforms (Illumina). Low quality reads (quality score < 20) were removed from each sample using Cutadapt (1.10.0)[74]. Reads were mapped to the human reference genome (hg19) using the STAR aligner[75]. Read counting was carried out using Rsubread[76] and differentially expressed genes, comparing RIS and growing conditions, were identified using edgeR[77]. For heatmap analyses, raw counts were TMM normalized and converted to transcripts-per-million (TPM). The EDC locus was defined as Chr1:152,056,619-Chr1:153,234,364. The EDC gene list was retrieved from the UCSC Table Browser[78].

For microarray data analysis, data previously deposited in NCBI GEO as image corrected, quantile normalised and log2 transformed intensities were downloaded using the Bioconductor GEOquery package[79]. A linear model was fitted on the resulting values and log2-fold changes for each of the desired contrasts was computed using a simple empirical Bayes model with functions of the limma package[80].

To determine the tissue specificity of a gene, we used the Human Protein Atlas (HPA) resource (https://www.proteinatlas.org/)[45] and its associated criteria for defining the tissue enhanced/enriched status of gene (used for Fig. 5a). In order to estimate a tissue specificity score for each gene in different gene groups (such as in Extended Data Fig. 4a), we used the transcriptomic data from the HPA and computed the Tau score, which was shown previously[81] to be the best metric for computing tissue specificity. The score ranges from 0 to 1, with 0 indicating a ubiquitously expressed gene and 1 a highly tissue specific one.

656

657 **ChIP-seq and ATAC-seq analysis**

658 ChIP-seq and ATAC-seq were performed as previously described[10]. Low quality reads
659 (quality score < 20) were removed from each sample using Cutadapt (1.10.0)[74]. Reads
660 were mapped to the human reference genome (hg19) using BWA (v0.7.12)[82] and
661 reads mapping to the "blacklisted" regions identified by ENCODE[83] were removed from
662 further analysis. H3K27ac and ATAC-seq peaks were called with MACS2[84] and
663 H3K9me3 enriched domains were identified using EDD[85]. A high confidence peak set
664 was identified for data types and conditions separately keeping only those regions that
665 were present in at least two replicates. These intersections as well as the overlaps
666 between H3K27ac, H3K9me3 and ATAC-seq high confidence peak sets were then
667 identified using bedtools (v2.26.0)[86]. Average read coverage on the EDC locus was
668 summarized on 1kb long bins from THOR[87] normalized coverage files (TMM
669 normalization) using the deepTools package[88], which was also used to plot heatmaps
670 of the THOR normalized signal. The same THOR normalized coverage files were
671 loaded and visualized on the genome browser.

672

673 **Hi-C analysis**

674 Hi-C libraries were aligned using HiC-Pro[89] against the hg19 genome after initial QC
675 checks using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).
676 Significant differential interactions, as well as distance corrected ICE-normalized Hi-C
677 maps, were estimated using diffHiC[90]. Visualisation of distance-corrected and ICE
678 normalized[91] Hi-C matrices and associated annotation (arcs representing significant
679 interactions) was performed using HiCvizR[52].

680

681 **Data availability**

682 Human keratinocytes RNA-seq and ChIP-seq data and IMR90 ATAC-seq data (RIS)
683 and RNA-seq data generated for this study have been deposited at GEO (GSE130457).
684 Control ATAC-seq data were previously published (GSE103590)[10]. Microarray
685 datasets (Extended Data Fig. 1) were previously published (GSE53379 and
686 GSE59522)[18,19]. RNA-seq data for RIS/DDIS IMR90 cells are available at GSE72407[20].
687 Other senescence RNA-seq data[23,24] were downloaded from GSE61130[23],
688 GSE74238[7], GSE45833[24], GSE85082[92], GSE63577[93], GSE56293[94], GSE130306[25].
689 SFs RNA-seq were downloaded from GSE163749[63]. HPA RNA-seq data were
690 downloaded from The Human Protein Atlas (HPA) (https://www.proteinatlas.org) E-
691 MTAB-2836[45]. THP1 RNA-seq data were downloaded from GSE96800[62]. ChIP-seq for
692 H3K9m3, H3K27me3[12], H3K27ac[10], and Lamin B1[14] are available at GSE38448,
693 GSE103590, and GSE49341, respectively. ChIP-seq data for hES cells were
694 downloaded from ENCODE, GSE29611[83]. H3K27ac ChIP-seq and ATAC-seq data for
695 THP1 cells were downloaded from the Gene Expression Omnibus (GEO) accession
696 number GSE96800[62]. Hi-C data (IMR90 cells) can be found at GSE135090. Hi-C data
697 (human keratinocytes) were downloaded from GSE84660. Hi-C data (THP1 cells)
698 were downloaded from Sequence Read Archive accession number PRJNA385337[62].
699 Hi-C data (WI38 cells) were downloaded from GSE130306 [25].
700

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Competing interests

P.F. and S.S. are co-founders Enhanc3D Genomics Ltd. All other authors declare no competing interests.

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Figure Legends

Figure 1. Aberrant expression of lineage-inappropriate *LCE2* genes during senescence. (a) Comparative analysis of LCE2 expression by immunoblotting during fibroblast (IMR90) RIS (RAS-induced senescence) and keratinocyte (KC) differentiation (Diff). RIS was induced by retroviral expression of HRAS-G12V. Prolif, vector control proliferating cells. KC Diff was induced using PMA for 48h. Undiff, undifferentiated control. IVL, Involucrin, is an EDC-encoded KC Diff marker. Densitometry quantification was performed by ImageJ, normalized to βActin (n = 3). ***P* < 0.01,****P* < 0.001, xxxx test. **(b)** Representative genome browser images, centered around the EDC of the cells indicated. ER:HRAS-G12V expressing IMR90 cells were cultured with 4OHT (RIS) or without 4OHT (Prolif) for 6 days. ChIP-seq data were THOR-normalized. The region with the orange shading is magnified in the right-hand panel. **(c)** Averages of THOR-normalized read counts for H3K9me3 and H3K27me3 signals on the EDC locus of the cells indicated. ****P* < 0.001, Shapiro-Wilkes test. **(d)** Representative genome browser images of ATAC-seq at the EDC and OR loci. n = at least two. The same ChIP-seq data are used as in **b**.

Figure 2. Decompaction of EDC locus in senescence IMR90 cells. (a) Representative genome browser images of Lamin B1 ChIP-seq (n = 3) in proliferative and RIS IMR90 cells, with the location of BAC probes used in this study shown as green/purple blocks underneath. The same H3K9me3 ChIP-seq data as in Fig. 1 were used as the reference. **(b)** Confocal DNA-FISH images using multiple BAC probes tiled across the EDC locus as shown in **a**. **(c)** Quantification of the FISH signal length for the EDC locus; proliferating (Prolif), acute DNA damage (acDD), E1A and RAS expressing (E1A+RAS), RAS-induced senescence (RIS), DNA damage-induced senescence (DDIS), replicative senescence (RS) IMR90 cells; undifferentiated (Undiff) and differentiated (Diff) keratinocytes (KC). ****P* < 0.001, Mann-Whitney-Wilcoxon test (n = 150 alleles from 3 biological replicates). Bars are median with inter-quartile range.

966 **(d)** Time series analysis of EDC signal length following addition of 100 nM 4OHT to
967 ER:HRAS-G12V IMR90 cells. At least 150 alleles were counted from 3 biological
968 replicates. ***P < 0.001 (Mann-Whitney-Wilcoxon test). Bars are median with
969 interquartile range. **(e)** Representative immunoblotting of the time series experiments
970 as in **d**. *P < xxxxx (xxx test, n = 3) **(f)** Quantification of SAHF- and SADS-positive
971 nuclei of the time series experiments as in **d**. Representative images of SAHF and
972 SADS at d7 are shown. SADS are indicated by α-satellite targeted oligo hybridization
973 (red). Values are means +/- S.E.M. (n = 3). *** P < 0.001 compared with d0, one-way
974 ANOVA with Tukey's multiple comparisons test. **(g)** DNA-FISH using single BAC
975 probes for EDC and OR loci as depicted in Fig. 2a (green and purple bars underneath
976 top panel). Percentages of those loci colocalized with SAHF were measured. *** P <
977 0.001 unpaired t-test (n = 3).
978
979 **Figure 3. Decompaction of EDC locus and senescence effectors. (a)**
980 Quantification of SAHF-positive cells in RIS or proliferating (Vector) IMR90 cells with
981 shHMGA1 (+) or control miR30 vector (-). **(b)** Quantification of the FISH signal length
982 for the EDC locus in the indicated cells. **(c)** RNA-seq analysis in the indicated
983 comparison. **(d)** Immunoblotting for the indicated proteins. *P < 0.05, ***P < 0.001, (xxx
984 test, n = 3). **(e)** Left, immunoblotting for the indicated proteins in IMR90 cells expressing
985 control retroviral vector (Prolif), p16 (p16-Sen), and HRAS-G12V (RIS). Two
986 representative replicates are shown. Right, corresponding RNA-seq analysis, showing
987 log-fold change (logFC) in the indicated conditions compared to corresponding
988 proliferative controls. p16-Sen (n = 3), DDIS (n = 8), RIS (n = 8). **(f)** Quantification of
989 BrdU incorporation and SAHF-positive cells. **(g)** Quantification of the FISH signal
990 length for the EDC locus in the indicated cells. **(h)** RNA-seq analysis. Normalized
991 expression heatmap (log-counts per million) of EDC genes inp16-induced senescence
992 (p16-Sen), and DNA damage-induced senescence (DDIS) and RAS-induced
993 senescence (RIS) with corresponding proliferative controls. **(I - j)** Two representative

994 immunoblots (n ≥ 5) (i) and qPCR analysis (j) in indicated cells. ER:RAS IMR90 cells
995 expressing shp53 and shC/EBPβ with corresponding control vectors were treated with
996 4OHT (RAS) for 6 days. **(k)** IGV tracks of THOR-normalized ChIP-seq signal of p53,
997 C/EBPβ and H3K27ac in Proliferating (Prolif) and RIS samples. **(l)** Quantification of
998 the FISH signal length for the EDC locus in the indicated cells. ***P < 0.001, Mann-
999 Whitney-Wilcoxon test (n ≥ 150 alleles from 3 biological replicates). n.s., not significant.
1000
1001 **Figure 4. H3K9me3 peaks characterisation. (a)** Classification of H3K9me3 peaks
1002 into non-permissive (group 1) and permissive peaks (with de novo ATAC-seq and
1003 H3K27ac peaks) with (group 3) and without up-regulated genes (group 2). **(b-i)**
1004 Distribution of peak widths (Mb) **(b)**, gene density (per Mb) **(c)**, AT% **(d)**, SINE
1005 frequency **(e)**, Alu repeats frequency **(f)**, Lamin B1 THOR-normalized ChIP-seq signal
1006 in proliferating cells **(g)**, Lamin B1 THOR-normalized ChIP-seq signal in RIS cells **(h)**,
1007 differential LaminB1 signal between RIS and proliferative cells **(i)**. In group 3,
1008 H3K9me3-peaks associated with the genes of interest are highlighted **(b-i)**. All box
1009 plots correspond to the median, 25th to 75th percentiles, and the whiskers correspond
1010 to the 10th to 90th percentiles. Each pairwise comparison between groups was
1011 effectuated with a two-sided Student's t-test, ***P < 0.001, **P < 0.01, *P < 0.05. n.s.,
1012 not significant.
1013
1014 **Figure 5.** Aberrant expression of lineage-specific genes and chromatin decompaction
1015 during senescence. **(a)** Tissue-dependent expression pattern of RIS upregulated
1016 genes that have RIS-unique H3K27ac/ATAC-peaks (at TSS +/- 250 bp) within
1017 H3K9me3 domains. RNA-seq datasets from IMR90 for RIS and DDIS (GSE-72407)
1018 and Human Protein Atlas (HPA) RNA-seq for normal tissues (E-MTAB-2836) were
1019 used. TPM, Transcripts-Per-Million. **(b-d)** Chromatin states of three additional
1020 examples of upregulated genes from H3K9me3 regions during RIS. Representative

genome browser images **(b)**. Representative multiprobe DNA-FISH images for the *NLRP3* locus **(c)**. Either single or tow alternate colours were used in the probe set shown in **b** (green/purple blocks, underneath main panel). Measurement of FISH signals using the multiple BAC probes represented in **(d)**. ***P < 0.001, Mann-Whitney-Wilcoxon test (n ≥ 150 alleles from 3 biological replicates). Bars, median with interquartile range.

Figure 6. Distinct mode of expression of lineage-appropriate and lineage-inappropriate genes during RIS. **(a)** Heatmap of normalized expression values (xxxx) of the EDC genes from RNA-seq samples of proliferating and RAS-induced (n = 6) keratinocytes (KCs), as well as undifferentiated (n = 8) and differentiated (n = 9) KCs. **(b)** Genome browser images of H3K9me3 ChIP-seq of the EDC and *DCC* loci from the cells indicated. The human ES (hES) cell data are from ENCODE. Some of the IMR90 and human primary keratinocyte (KC) images were replotted for comparisons using the same datasets as in Fig. 1b (EDC) and Fig. 5b (*DCC*). **(c)** DNA-FISH analysis of the *DCC* and EDC loci in the cells indicated. ***P < 0.001, Mann-Whitney-Wilcoxon test (n ≥ 150 alleles from 3 biological replicates). Bars are median with inter-quartile range. **(d)** qRT-PCR for the indicated mRNA in vector control and RIS KCs. Error bars indicate mean +/- S.E.M. (n = 9). ***P < 0.001, *P < 0.05 paired t-test.

Figure 7. Differential 3D behaviour in proliferating and RIS cells of permissive H3K9me3 peaks. **(a)** Total H3K9me3-H3K9me3 interactions (connectivity) of the individual 200 kb bins (sum of the interactions with all other 200 kb bins within H3K9me3 peaks) within each group of H3K9me3 peaks. **(b)** Differential H3K9me3-H3K9me3 interaction between proliferative and RIS conditions in each group. Log-fold changes (logFC) of increased (top, green) and decreased interactions (bottom, blue) in each peak with all other H3K9me3 peaks are shown. Each pairwise comparison is

performed using two-sided Student's t-test with ***P < 0.001, **P < 0.01. n.s., not significant. Boxplots in **(a)** and **(b)** represent the median, 25th to 75th percentiles, whiskers: 10th to 90th percentiles. **(c)** Significant LogFC (green – positive, blue – negative) of all the Hi-C interactions involving each of the H3K9me3 peaks of interest within group 3 compared to the OR region (*OR10T2*) neighbouring the EDC (*LCE2*, *IVL*), as well as 30 other H3K9me3 peaks randomly chosen from group 1. **(d-f)** Hi-C contact maps at 40 kb resolution focused on the EDC locus in proliferating and RIS IMR90 cells (d), proliferating and replicative senescent WI38 cells (e) as well as in differentiating keratinocytes (induced by calcium) (f); the arrows indicate the formation of loops during senescence (fibroblasts) and differentiation (keratinocytes) at this locus. **(g-h)** Hi-C contact maps on the *NLRP3* locus at 40 kb resolution in proliferating and RIS IMR90 cells (g, the arrow indicates the interaction changes around the gene body of *NLRP3*) and THP1 monocytes and PMA-induced macrophages (h). Hi-C maps in THP1 cells are aligned with H3K27ac ChIP-seq, ATAC-seq, and RNA-seq data.

Extended Data Figure 1. Transcriptional profile of genes in the EDC locus. **(a)** Image corrected, quantile normalized and log2 transformed microarray intensity values were downloaded from the indicated NCBI GEO entries. Control, proliferative; RIS, HRAS-G12V-induced senescent; Qui, quiescent; DDIS, DNA damage-induced senescent; acDDR, acute DNA damage response to etoposide (24 or 48 hours); E1A+RAS, transformed/senescence-bypassed. Genes for which there is no available transcript probe on the microarray platform used are greyed out. **(b)** Meta-analysis of senescence-associated transcriptomic changes of genes in the EDC locus using RNA-seq datasets, downloaded from indicated NCBI GEO, comparing between different cellular phenotypes. The datasets were processed using the same analysis pipeline. Values for the indicated genes are shown for each of the senescent samples and their corresponding growing controls. Values are Transcripts-Per-Million (TPM). Genes are

ordered according to their position along Chr. 1. Genes for which there were no aligned reads in the specified RNA-seq runs are greyed out. **(c)** Immunoblot analysis of IMR90 cells stably expressing vector (-) or HRAS-G12V and control miR30 vector (-) or sh-p53 (+) for the proteins indicated. Arrow indicates non-specific bands. p21 is a p53-dependent cell cycle inhibitor that is upregulated during senescence. **(d-e)** IMR90 cells expressing ectopic EGFP-LCE2A and control vector were assessed for cumulative papulation doublings (d) and colony formation capacity (e) (n = 3). Error bars indicate mean +/- SD (two-sided paired t-test). The inset shows representative immunoblots of indicated protein (d).

Extended Data Figure 2. Correlation between EDC decompaction and SAHF formation. **(a)** 3D confocal images of DNA-FISH for the EDC locus in the cells indicated (see Fig. 2a for two alternate color probe designs). The perinuclear localization of the EDC locus was quantified on visual inspections by counting FISH signal, which attached at least 1 of the 7 tiled signals to the nuclear periphery (white arrowhead) (n > 100). **(b)** Representative images of DNA-FISH for the EDC and OR loci using the probes indicated and quantification of signal length (n = 100 alleles from 3 biological replicates). Note, for the EDC, 5 out of 7 probes (Fig. 2a) were used. **(c)** FISH signal length of EDC on SAHF-negative or positive RIS cells (n ≥ 100 alleles from 3 biological replicates). **(d)** LCE2 immunofluorescence signal on SAHF-negative or positive RIS cells (n = 3). **(e-h)** Quantification of SAHF-positive cells in the indicated conditions (e, g) and comparative analysis of LCE2 expression by immunoblotting in the conditions indicated (f, h). *P < 0.05, **P < 0.01, ***P < 0.001 one-way ANOVA with Tukey's multiple comparisons test (n = 3). **(i)** Quantification of FISH signal length in the conditions indicated for BJ and WI38 cells (n ≥ 150 alleles from 3 biological replicates). ***P < 0.001, Mann-Whitney-Wilcoxon test (bars are median with interquartile range) **(b, c, i)**.

Extended Data Figure 3. H3K9me3 exhibits increases in accessibility and H3K27ac ChIP-seq signal in RIS. **(a)** ATAC-seq and H3K27ac THOR-normalized signal in proliferative and RIS cells centred on three classes of ATAC-seq peaks: proliferative-specific, RIS-specific, and common between the two conditions. **(b)** Number of ATAC-seq (top) and H3K27ac (bottom) peaks across the three groups of H3K9me3 peaks defined as permissive (group 2 and 3) and non-permissive (group 1). The box plots correspond to the median, 25th to 75th percentiles, and the whiskers correspond to the 10th to 90th percentiles. **(c)** IGV tracks of genes of interest and position of SINE and Alu repeats (UCSC annotation) over their gene bodies. **(d)** Region surrounding the *SERPINA1* gene characterised by H3K9me3 ChIP-seq signal, as well as protruding H3K27ac and ATAC-seq peaks.

Extended Data Figure 4. Tissue specificity of genes in H3K9me3 peacks. **(a)** Tissue specificity score of the genes within the three groups of H3K9me3 peaks against all other genes. The Tau score ranges from 0 - ubiquitous expression across tissues to 1 - tissue specific; the 8 genes of interest in group 3 are highlighted. Significance testing was performed with two-sided Student's t-tests, ***$P < 0.001$, n.s., not significant. The box plots correspond to the median, 25th to 75th percentiles, and the whiskers correspond to the 10th to 90th percentiles. **(b)** *DCC* (left) and *TMEM132B* (right) genes represented by multiprobe DNA-FISH signals in the proliferative and RIS conditions. Probe sets are shown in Fig. 5b. Note, single color probe images are shown for *TMEM132B*.

Extended Data Figure 5. Three-dimensional interaction changes occurring during RIS. **(a)** 200 kb resolution Hi-C maps in proliferative and RIS cells as well as significant interaction changes between the two conditions, where green arcs represent increases and blue arcs represent decreases. **(b)** Significant H3K9me3-H3K9me3 interaction changes (200 kb bins): log-fold changes (logFC) against distance between the

interacting regions, dotted line corresponds to 10 Mb distance. **(c)** Hi-C interaction profiles at 40 kb resolution in proliferating (Prolif) and RIS IMR90 cells as well as differentiating keratinocytes at the EDC/OR loci. **(d)** Epigenetic profile of the *NLRP3* locus in terms of H3K9me3, LaminB1 and H3K27me3 THOR-normalized ChIP-seq signal in proliferating and RIS cells. **(e)** Normalized gene expression (log-counts per million) of pro-inflammatory genes, EDC genes and senescence-representative genes in -/MSU and MSU/MSU synovial fibroblasts. *Adjusted $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Supplementary Table 1. List of genes within the three types of H3K9me3 peaks determined. 38 genes significantly upregulated in group 3 are highlighted.