1   # HDAC4 controls senescence and aging by safeguarding the epigenetic identity

2   # and ensuring the genomic integrity

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20   **ABSTRACT**

21   The epigenome of senescent cells is characterized by a deep redistribution of H3K27 acetylation.

22   H3K27 is target of class IIa Histone Deacetylases (HDAC4, 5, 7, 9) as part of large repressive complexes.

23   We report here that, among class IIa HDACs, HDAC4 is post-transcriptionally downregulated during

24   senescence and aging. HDAC4 knock-out (KO) triggers premature senescence as a result of two waves of

25   biological events: the accumulation of replication stress (RS) and the expression of inflammatory genes. The

26   latter is achieved directly, through the activation of enhancers (TEs) and super-enhancers (SEs) that are

27   normally monitored by HDAC4, and indirectly, through the de-repression of repetitive elements of retroviral

28   origin (ERVs). The accumulation of DNA damage and the activation of the inflammatory signature influence

29   each other and integrate into a synergistic response required for senescence onset. Our work discloses the

30   key role played by HDAC4 in maintaining epigenome identity and genome integrity.

31

32   **INTRODUCTION**

33   Cellular senescence and aging are complex responses characterized by proliferative arrest and loss of

34   regenerative potential[1]. The senescence state is distinguished by a deep epigenetic reprogramming that

35   sculptures the chromatin to maintain cellular survival, arrest the cell-cycle and secrete apocrine and paracrine

36   factors in the presence of consistent DNA damage[2]. In turn, the haploinsufficiency of epigenetic and

37   metabolic regulators increases genome fragility, affects the DNA damage response (DDR) and predisposes

38   to senescence, cell death or malignant transformation[3–7].

39   H3K27ac/H3K27me3 ratio alterations were identified as the driving forces of premature senescence

40   and cancer[3,7–10]. The balanced action of acetyltransferases (HAT) of the SWI/SNF/p300 complex[11] and

41   HDACs of the Sin3, NuRD, CoREST, MiDAC and NCOR complexes[12] controls the acetylation status of

42   H3K27. Class IIa HDACs are catalytically inactive epigenetic readers, quickly recruited on H3K27ac

43   loci[13,14], where they monitor the acetylation status through the binding of class I HDACs[15].

44   Here we investigated the role played by class IIa HDACs in the regulation of senescence entrance.

45   The senescence phenotype induced by HDAC4 depletion was studied in detail to unveil the dual role of this

46   epigenetic regulator in preserving genome integrity and in safeguarding regulative elements controlling cell

47   fate.

48

49   **RESULTS**

50   **HDAC4 expression is silenced during cellular senescence and aging.**

51   The epigenetic reprogramming plays key roles in establishing cellular senescence and aging. In this

52   context the contribution of class IIa HDACs has been suggested[16,17], but not addressed in a comprehensive

53   manner. Important epigenetic modulators of senescence and aging are down-regulated during the progressive

54   cell cycle arrest[3,4,18,19]. For this reason, we evaluated class IIa HDACs expression in different models of

55   senescence and aging. HDAC4 and HDAC9, and to a lesser extent HDAC7, were progressively

56   downregulated in human IMR90 fibroblasts undergoing replicative senescence (Fig. 1a/b). As a model of

57 ageing, we compared the protein levels of class IIa HDACs in the dermis and in the liver of young (4

58 months) and old (25 months) mice. HDAC4 and HDAC9 levels decreased, both in aged dermis and liver,

59 whereas HDAC5 decreased only in old dermis (Fig. 1c). Therefore, telomers attrition in normal cells and

60 physiological aging similarly affected the protein levels of HDAC4 and HDAC9.

61 The delivery in BJ/*hTERT* of the strong oncogenes HRAS[G12V] or myrAKT1 triggers a premature

62 cell-cycle arrest named oncogene-induced senescence (OIS)[20,21]. In both models of OIS (Fig.S1a/b), the

63 permanent proliferation arrest is characterized by the post-transcriptional downregulation of HDAC4,

64 HDAC5 and HDAC7, but not of HDAC9 (Fig.1d, S1c,d,h). This trend was also observed in IMR90/*RAS*

65 cells (Fig. S1e).

66 Other oncogenes of viral origin, like E1A and its ΔC-fragment (1-143), are able to overcome OIS, by

67 targeting p16/Rb pathways[22]. HDAC4/5/9, but not HDAC7, were upregulated by the expression of E1AΔC

68 in BJ/*hTERT* (Fig.S1c). Moreover, the co-expression of E1A and RAS by-passed the senescent arrest and

69 recovered the protein levels of HDAC4 and HDAC5, but not of HDAC7 (Fig.S1c). Therefore, OIS and OIS

70 escape affect mainly HDAC4 and HDAC5 levels. HDAC4 and HDAC5 were decreased also during stress-

71 induced senescence (SISP) following H_2O_2 treatment and cytokines-induced senescence (Fig.S1f,g,i). In all

72 these conditions, class IIa HDACs are regulated at the post-transcriptional level, except for HDAC9, which

73 transcription is stimulated by oncogenes (Fig. S1c and Table S1).

74 Our preliminary screening identified HDAC4 as the class IIa HDAC member repressed in all tested

75 models of senescence and aging. Most of this downregulation is due to the ubiquitin-proteasome system

76 (UPS) mediated degradation. HDAC4 levels in senescent cells were restored after UPS, but not autophagy

77 inhibition (Fig. S2a/b). Accordingly, HDAC4 was highly poly-ubiquitylated in senescent cells (Fig. 1e). This

78 degradation requires GSK3β[23], as the treatment with LiCl (Fig. S2c) and the silencing of GSK3β restored

79 HDAC4 levels in senescent cells (Fig.1f).

80

81 **The depletion of HDAC4 triggers senescence in different cell types**

82 To investigate the role played by HDAC4 during senescence, we knocked-out HDAC4 in

83 BJ/*hTERT/Ras/E1A* cells, a standard model of OIS escape (Fig.1g and Fig.S2d/e). HDAC4 KO caused the

84 appearance of SA-β-gal positive cells (Fig.1h) and increased the expression of senescence markers

85 (*CDKN1A, IL1B, IGFBP7*) (Fig.S2f). The induction of senescence in these cells is unrelated to hTERT or

86 E1A levels (Fig. 1g). To confirm the anti-senescence effect of HDAC4, its expression was knocked-out also

87 in low grade leiomyosarcoma cells SK-LMS-1 (Fig.1i and Fig.S2d/e). HDAC4 depletion triggered cell-cycle

88 arrest (Fig.1j) and the appearance of SA-β-gal positive cells (Fig.1k and Fig.S2g). All KO clones failed to

89 grow in semisolid medium, a strong indication of malignancy suppression (Fig.S2h/i). These phenotypes

90 were reproducible in the 4 KO clones generated with 2 different sgRNAs (sg1: 76, 1231; sg2: 205, 1254),

91 while an intermediate phenotype was obtained in the heterozygous clone 275 (Fig. 1j). SK-LMS-1/*HDAC4^{-/-}*

92 cells were also characterized by a moderate induction of cell death (Fig.S2j).

93   $HDAC4^{-/-}$ cells were characterized by an altered nuclear morphology (Fig. 1l/m) and the accumulation of

94   γH2AX foci (Fig. 1n). The re-expression of a Cas9 resistant form of HDAC4 ($HDAC4^{PAM}$) rescued the

95   senescent phenotype in all these clones (Fig. S2k and Fig. 1o), stimulated the entering into the cell-cycle

96   (Fig. 1p), supported their growth in soft agar (Fig. S2l) and reduced the DNA damage levels (Fig. 1q).

97   Similarly, the tamoxifen inducible re-expression of $HDAC4$ in SK-LMS-1/$HDAC4^{-/-}$ cells (clone 66, +4-

98   OHT) (Fig. 1r) rescued every defect observed in the absence of HDAC4 (-4-OHT) (Fig.1r-v, S2m). As

99   further evidence of senescence induction, SK-LMS-1/$HDAC4^{-/-}$ were sensitive to the senolytic drug

100 navitoclax/ABT-263[24] (Fig. S3a) and lost the linker histone H1[25] and LMNB1[26] (Fig. S3b).

101   The premature senescence onset observed in $HDAC4^{-/-}$ cells was only partially dependent on MEF2

102 de-repression. In fact, the expression of a super-repressive mutant[27] of MEF2 partially restored the

103 proliferation of KO cells (Fig. S3c/d). The appearance of DNA damage and senescence was confirmed in

104 different cellular models (BJ-$TERT/LT$, BJ-$TERT/LT/RAS$ and in the melanoma cells WM115) after RNAi-

105 mediated silencing of $HDAC4$ (Fig. S3e/f).

106

107 The transcriptome of $HDAC4^{-/-}$ SK-LMS-1 cells is typical of senescent cells

108   The transcriptomes of four LMS $HDAC4^{-/-}$ clones were compared with two control clones

109 (expressing Cas9 or Cas9/sgRNA1 but in which the KO was not achieved) (Fig. S3g). By applying stringent

110 statistical criteria, we identified a minimal signature of 230 genes significantly modulated in all $HDAC4^{-/-}$

111 clones (Fig. 2a,S3g and Table S7). 142 out of 230 of these genes (62%) were induced after HDAC4 deletion

112 (Fig. 2b). An unbiased GSEA analysis identified a senescence geneset among the most enriched in $HDAC4^{-/-}$

113 cells (Fig. 2c). Moreover, senescence-associated secretory phenotype (SASP), Ras-induced senescence (RIS)

114 and NFKB1 target genes were all positively enriched in $HDAC4^{-/-}$ (Fig. 2c). Interestingly, in the case of RIS

115 the similarities arose not simply by the SASP but also from the activation of a common epigenetic

116 reprogramming (Fig. S3h).

117 Genome-wide levels of H3K27ac and H3K27me3 showed moderate and focused decreases of H3K27me3

118 and locus specific increases of H3K27ac, at 36h from HDAC4 depletion (Fig. 2d). Interestingly, the re-

119 acetylation observed in $HDAC4^{-/-}$ cells involved mainly intronic (18%) and intergenic regions (45%), as

120 similarly observed in other studies[14,28,29], while the demethylation occurred close to TSS (Fig.2e).

121 To unveil the contribution of NFKB in this senescent response, we inhibited its activity by introducing IKBα

122 S32A/S36A[30] (referred hereinafter as NFKBIA) in $HDAC4^{-/-}$ cells. NFKB1 blockage recovered senescence

123 only partially (Fig. 2f-h). In particular, while the expression of SASP genes ($IL1B, CXCL8$) strongly depends

124 on NFKB1, the anti-proliferative response ($CDKN1A, GADD45A, Fbox32/ATROGIN$) was rescued only by

125 HDAC4 (Fig. 2i and S3i). This was confirmed at chromatin level. 2% and 8% of the H3K27ac regions

126 hyper-acetylated in $HDAC4^{-/-}$ cells organize the chromatin respectively in: a) typical enhancers (TE) and b)

127 super-enhancers (SE), found activated during senescence (TESs and SESs) (Fig.S3j/k). The latter,

128 exemplified by the $IL1B$-$IL1A$-$IL37$ SES (Fig.2j), display high preference for NFKB, JUN/AP-1 and MEF2

129 binding (Table S2). The activation of typical enhancers, as well as the increased acetylation and decreased

130 methylation of H3K27 on gene promoters, can control the expression of negative regulators of cell
131 proliferation, as exemplified by the *CDKN1A* locus (Fig. 2k).

132

133 **HDAC4 depletion triggers senescence in melanoma cells.**

134 The best-characterized *in vivo* model of OIS is the human nevus[31]. Among the 18 HDACs, only
135 HDAC4 expression was significantly decreased in nevi and significantly increased in melanomas (Fig. S4a).
136 Moreover, high levels of HDAC4 negatively correlated with patients' survival (Fig. S4b). We therefore
137 knocked-out HDAC4 in A375 melanoma cells. Initially, we obtained only heterozygous clones, suggesting
138 an important role of HDAC4 in regulating cell fitness. Therefore, we generated Dox-inducible cell lines to
139 conditionally express HDAC4^{PAM} before its targeting. With this strategy, five *HDAC4$^{-/-}$* (320, 304, 401, 150,
140 1090) and two *HDAC4$^{+/-}$* clones (159, 317) were isolated (Fig. S4c). Similarly to sarcoma cells, HDAC4KO
141 caused the downregulation of LMNB1 (Fig. S4c). A time-course analysis in 304$^{-/-}$ cells evidenced that
142 HDAC4 loss triggered the rapid accumulation of DNA damage and the activation of TP53 (Fig. S4d). A pool
143 of genes up-regulated in SK-LMS-1/*HDAC4$^{-/-}$* cells turned out to be up-regulated also in A375 *HDAC4$^{-/-}$*
144 cells (Fig. S4e). Finally, removal of Dox-dependent HDAC4 expression elicited the acquisition of a
145 senescent-like phenotype (Fig. S4f-i). All these phenotypes were weaker in heterozygous clones and the re-
146 expression of HDAC4 rescued the normal phenotype (Fig. S4e-i).

147

148 **Loss of *Hdac4* accelerates senescence in non-transformed cells.**

149 Primary murine embryonic fibroblasts (MEFs) senescence rapidly when grown at atmospheric
150 oxygen, while the maintenance of hypoxic conditions preserves their proliferation[32]. The conditional (4-OHT
151 dependent) KO of *Hdac4* in MEFs under normoxia sped up the progressive increase in Cdkn2a/p16 levels
152 (Fig. S5a) and the accumulation of DNA damage (Fig. S5b). Similarly, with respect to wt cells, *Hdac4$^{-/-}$*
153 MEF earlier arrested proliferation (Fig. S5c,d) and switched on a senescence signature (Fig. S5e).

154 We confirmed that MEFs grown in 2% O$_2$ did not reach senescence, independently from the absence
155 or presence of HDAC4 (Fig. S5f/g). Curiously, after prolonged time of culture, *Hdac4$^{-/-}$* MEFs evidenced a
156 proliferative crisis from which they rapidly emerged (Fig. S5g).

157

158 **Loss of H1.2 and LMNB1 triggers a second wave of DNA damage in HDAC4$^{-/-}$ cells.**

159 To investigate the timely order of senescence appearance, we took advantage of the *HDAC4$^{-/-}$*
160 */HDAC4^{PAM}-ER* cells expressing H1.2-GFP as senescence sensor (Fig. S3b). Apparently, the loss of LMNB1
161 and the accumulation of DNA damage are concomitant events that are coupled to the loss of the linker
162 histone H1 (Fig. 3a). qRT-PCR analysis suggested that the modulation of IGF1 signaling and of the cell-
163 cycle *(CDKN1A)* are early events (Fig. 3b). Immunofluorescence analysis using different markers lead us to
164 define six major phenotypes as exemplified in figure 3c: i) cells positive for H1.2 (H1.2+); ii) cells with a
165 discontinuous (altered) LMNB1 ; iii) cells presenting cytosolic chromatin fragments (CCF^{+}); iv) cells with
166 multilobed nuclei; v) cells H1.2 negative (H1.2^{-}) with multilobed nuclei and vi) cells with DSBs (γH2AX^{+}).

167   This detailed analysis evidenced that DNA damage increased linearly in the first 48h after HDAC4 removal

168   and exponentially in the next 24h, when the loss of H1.2 and LMNB1 became consistent (Fig. 3d).

169

170   ***In vivo* definition of the early response to HDAC4 depletion.**

171   To gain more insight into the early events triggered by HDAC4 depletion, we introduced in *HDAC4*

172   *[/-]HDAC4[PAM]-ER* cells two reporters (H2B-GFP and Apple-TP53BP1), to monitor mitosis[33] and the

173   accumulation of DNA damage[34,35] *in vivo,* at single cell level. Unperturbed cells accumulate DNA lesions

174   during interphase, possibly during the proceeding through the S phase. Usually these lesions are resolved in

175   G2, before entering mitosis. After mitosis, cells emerge in G1 with few TP53BP1 positive foci defined as

176   nuclear bodies (TP53BP1-NBs) that could represent under-replicated DNA[35–37].

177   Cells were monitored over a period of 74 hours from 4-OHT removal/addition by *in vivo* time-lapse confocal

178   microscopy. During this time, 9 out of 10 cells re-expressing HDAC4 entered mitosis twice (Fig. 4). Cell

179   cycle length was constant in these cells (43.22±3.40 hours). The analysis of TP53BP1 dots evidenced the

180   accumulation of DSBs (Supplementary video S1), reflecting the generation of spontaneous DNA or

181   chromatin lesions[36]. Frequently, small TP53BP1 nuclear foci co-existed with larger TP53BP1-NBs, as

182   previously observed[35]. The accumulation of all TP53BP1 dots was quantified and represented as a heatmap

183   (Fig. 4 +4-OHT). The pattern of endogenous TP53BP1 distribution in NBs and in small foci was equal to

184   what observed with the fluorescent sensor (Fig.S6a,b,c).

185   HDAC4 removal impaired proliferation. In fact the analyzed cells, with two exceptions (13.2 and

186   19.1), did not enter the second mitosis. Moreover, *HDAC4^{-/-}* cells were characterized by the progressive

187   accumulation of TP53BP1 nuclear foci (Fig. 4 -4-OHT and supplementary movie S2) and by a marked

188   increase in the frequency and dimension of TP53BP1-NBs (Fig. S7). These TP53BP1-NBs were not always

189   symmetrically distributed among sister cells after mitosis (supplementary movie S2). In summary, the

190   absence of HDAC4 exacerbates the trend of DNA lesion accumulation, possibly occurring in late S phase

191   and G2.

192   To correlate the accumulation of pre-mitotic TP53BP1 nuclear dots with mitotic defects, we

193   measured the average number of all TP53BP1 dots for 100 minutes preceding the appearance of

194   chromosomes condensation (mitosis onset). In the presence of HDAC4 the number of TP53BP1 dots was

195   comparable among the analyzed cells (range of 0-3/cell) and the mitosis length was quite homogeneous

196   (100-150 min) (Fig. S6d). A similar behavior was observed during the second mitosis (Fig. S6d). In *HDAC4*

197   *[/-]* cells the number of TP53BP1 nuclear dots in G2 was increased, as well as the length of mitosis (Fig. S6d,f).

198   As a consequence of the unresolved chromosomal lesions that are transmitted to daughter cells,

199   TP53BP1-NBs re-emerged in G1[36]. In the presence of HDAC4, daughter cells emerged with few

200   symmetrical TP53BP1-NBs and this behavior was conserved in the succeeding mitosis (Fig. S6e). In absence

201   of HDAC4 the number of TP53BP1-NBs was increased and they were sometimes asymmetrically distributed

202   between daughter cells. The accumulation of macroscopic nuclear alterations (CCFs, ultrafine DNA bridges

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203 (UFBs), lobate nuclei and unproductive mitosis, Fig. S6g) could explain the mitotic delay observed in the
204 absence of HDAC4 (Fig. S6f).

205

206 **ROS and LMNB1 play a minor role in genomic instability of $HDAC4^{-/-}$ cells.**

207 Alterations in the nuclear lamina and increased ROS (reactive oxygen species) generation are two
208 possible sources of DNA damage[38,39], strongly correlated to senescence onset[40]. LMNB1-GFP was
209 overexpressed in SK-LMS-1/$HDAC4^{-/-}$/$HDAC4^{PAM}$-ER cells cultured in normoxia or in hypoxia to evaluate
210 their separate or joint contribution to HDAC4-mediated senescence. The overexpression of LMNB1 had a
211 partial effect on the appearance of senescence (Fig. 5a) and, while it did not reduce the total number of DNA
212 CCFs, it reduced the appearance of naked (with a defective LMNB1 envelope) CCFs (Fig. 5b). Interestingly,
213 naked CCFs were frequently positive for γH2AX (arrows and arrowheads Fig. 5b). Accordingly, in LMNB1-
214 GFP cells the increase of γH2AX signal, observed upon HDAC4 depletion, was slightly reduced (Fig. 5d).
215 Growing the same cells in hypoxia did not affect the accumulation of DNA damage and the appearance of
216 senescence (Fig. 5c/d/e). However, hypoxia modestly potentiated the effects of LMNB1 re-expression in
217 escaping senescence (Fig. 5e). As previously reported[41], LMNB1 re-expression almost completely
218 suppressed SASP, whereas growing cells in hypoxia showed only a modest effect on the expression of
219 *GADD45A* and *CXCL8* (Fig. 5f).

220

221 **DNA lesions occurring during replication accumulate in the absence of HDAC4.**

222 DNA lesions frequently arose during DNA replication. Several intracellular and extracellular
223 conditions can trigger RS and forks stalling or collapsing that lead to DSBs[42]. Damaged replication forks are
224 marked by ATR-mediated hyperphosphorylation of ssDNA-bound RPA32 and by the monoubiquitylation of
225 PCNA. These PTMs are instrumental for the recruitment of repair factors and Polη[43,44]. Starting from 12h
226 after the switch-off of $HDAC4^{PAM}$-ER expression, $HDAC4^{-/-}$ cells accumulated Ub-PCNA and phosphorylated
227 (S4/S8) RPA32, which became more evident after 48 hours. These accumulations were paired to the
228 induction of DSBs (Fig. 6a). Most of this DNA damage is due to RS. In fact, inhibition of cell cycle
229 progression, achieved through the CDK4 inhibitor Palbociclib, reduced PCNA ubiquitylation, almost
230 abolished RPA32 phosphorylation and reduced the increase in DSBs (Fig 6b), similarly to HDAC4 re-
231 expression (Fig. 6c). Low doses of aphidicolin (APH) or camptothecin (CPT) can trigger RS[45,46]. Depletion
232 of HDAC4 showed additive effects to APH or CPT for DSBs accumulation (Fig. 6d-e). Similarly, the
233 recovery after the APH block was delayed by the absence of HDAC4 (Fig. 6f). Overall, these experiments
234 indicate RS as an important source of DNA damage in $HDAC4^{-/-}$ cells.

235 $HDAC4^{-/-}$ cells did not show evident marks of epigenetic stress that could lead to
236 transcription/replication conflicts[47–49] in correspondence of genomic common fragile sites (CFSs)[50] or early
237 replicating fragile sites (ERFSs)[51]. Indeed, some epigenetic alterations fall in CFSs (5.5% with altered
238 H3K27ac and 7.4% with altered H3K27me3) (Fig.6g/h) and, even if they correlate well with the expression
239 of the associated genes (ρac(H3K27ac KO/wt)=0.37, ρme(H3K27me3 KO/wt)= -0.32) (Fig.6i), only 16% of

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240 HDAC4-regulated genes (13% up-regulated, 20% down-regulated) fall in CFSs, that cover 15% of the
241 genome. In conclusion, the source of RS that characterizes $HDAC4^{-/-}$ cells cannot be ascribed to abrupt
242 epigenetic and transcriptional changes at ERFSs and CFSs.

243

244 **HDAC4 depletion causes the activation of ERV transcripts.**

245   Epigenetic perturbations elicit the transcription of ERVs[52,53] during cellular senescence, aging and
246 cancer[54,55]. Demethylating agents and HDACi trigger the expression of ERVs[52,54]. All tested ERVs, with the
247 exception of *ERVW1* that contains a coding gene (*SYCY1*), were induced in $HDAC4^{-/-}$ cells (Fig. 6j).
248 Similarly, the IFN response was switched on (Fig. 6j), as a consequence of dsRNAs accumulations (Fig. 6k).
249 The time-course analysis confirmed the up-regulation of ERVs and of the IFN response. The levels of *ERV9*
250 and *ERVFc2* peaked at 60h from HDAC4 removal and then decreased, probably because of an OAS1-
251 dependent degradation (Fig. 7a). The same pattern of ERVs was upregulated during replicative senescence in
252 human fibroblasts (Fig. 7b). ERVs and the IFN response were induced in IMR90-*E1A/RAS* transformed cells
253 after HDAC4 KO (Fig. S8a), in dermis and liver of aged mice (Fig. S8b/c), in $HDAC4^{-/-}$ A375 cells (Fig.
254 S8e). ERVs up-regulation was not due to Cas9 activity or nuclear lamina dismantling, as they were
255 unperturbed by the KO of unrelated genes (Fig. S8d) or by the re-expression of LMNB1 in $HDAC4^{-/-}$ SK-
256 LMS-1 cells (Fig. S8f). Interestingly, in MCF10A cells the KO of HDAC7, the most abundant class IIa
257 HDAC, induced ERVs expression and this up-regulation was increased in the presence of RAS (Fig. S8g).

258   Importantly, the exogenous delivery of dsRNAs species immunopurified from $HDAC4^{-/-}$ cells
259 triggered senescence and cell death in SK-LMS-1 cells (Fig. 7c-e). In $HDAC4^{-/-}$ cells, ERVs are
260 characterized by a depletion of H3K27me3 and by an increase in the H3K27ac/H3K27me3 ratio (Fig. 7f).
261 The strong demethylation of K27 of H3 wrapping the genomic DNA of ERVs represents the second most
262 pronounced epigenetic alteration related to HDAC4 removal after the activation of TESs and SESs (Fig. 7g-
263 j).

264

265 **A MAVS/ERV dependent pathway sustains HDAC4-dependent senescence**

266   To better define the contribution of ERVs and of the DDR in this senescence response, we
267 suppressed these pathways by taking advantage of dominant negative (DN) versions of MAVS
268 (MAVSΔCARD)[56] and TP53 (TP53[R175H])[57]. MAVS was selected since MDA5-RIG1-MAVS recognizes the
269 dsRNA shape of ERV transcripts and triggers the activation of a NFKB/IFN/TNF antiviral responses, which
270 sustain senescence[58,59].

271 The expression of TP53-DN and of MAVS-DN in $HDAC4^{-/-}$ cells reduced SA-β-gal positivity more
272 efficiently than MEF2-DN (Fig. 8a,h). The impact on counteracting the DDR showed a gradient of efficiency
273 with HDAC4>MAVS-DN>TP53-DN>MEF2-DN (Fig. 8b). The effect of MAVS-DN on DNA damage was
274 unexpected. Hence, we evaluated this suppressive effect in relation to the RS using the CDK4i. Figure 8c
275 shows that MAVS-DN did not completely suppress DNA damage and a stronger effect was observed with
276 the co-expression of HDAC4. As expected, the level of DNA damage was further dropped after the block of

277 the cell cycle operated by CDK4i. This result demonstrates that dsRNA/MAVS are secondary sources of RS

278 that add to the primary and earlier generation, triggered by HDAC4 depletion. The senescence rescue of

279 TP53-DN and MAVS-DN was confirmed by LMNB1 expression (Fig. 8d/e). The role of MAVS in this

280 senescent response was verified by silencing its expression and that of RIG1, using RNAi (Fig. 8f). Next, we

281 evaluated the influences on a pattern of genes (ERVs, SASP, IFNs and cell cycle genes) defining the

282 senescent signature (Fig.8g/h). Re-expression of HDAC4 efficiently rescued all observed alterations.

283 MAVS-DN impacted on SASP and IFN genes, but only partially on cell cycle genes. TP53-DN was

284 ineffective in suppressing ERVs and some SASP genes but effective for the IFN response. LMNB1,

285 NFKBIA and MEF2-DN partially impacted on the senescent signature and mainly at the level of SASP

286 expression (Fig. 8g/h).

287 We confirmed these observations in melanoma A375 cells. MAVS-DN and TP53-DN (more strongly than in

288 SK-LMS-1) inhibited the HDAC4-dependent senescence, rescued LMNB1 levels (Fig. 8i/j) and modulated

289 the senescent signature (Fig. 8k) similarly to what observed in SK-LMS-1 cells.

290

HDAC4 binds and control the acetylation status of super-enhancers

292 Removal of HDAC4 triggers several dysfunctions including the induction of RS, SEs and the

293 activation of ERVs, which ultimately lead to senescence. Importantly, HDAC4 was still able to repress the

294 senescent signature when CD4Ki was used to induce senescence and cell-cycle arrest with only minimal RS

295 (Fig. 8l). We therefore hypothesized that HDAC4 could act as a safeguard of cell fate by repressing

296 senescent genes. HDAC4 ChIP-seq profile evidenced that HDAC4 binding was highly enriched at the level

297 of specific SE, mainly those activated in senescence (SES) (Fig. 8m). Even though this property is partially

298 shared with the other class IIa HDACs, it particularly marks HDAC4 (Fig. 8m). By binding these SE,

299 possibly through the engagement of transcription factors previously associated with SE like BACH2[60] and

300 AP-1[3] (Fig.8n) and/or MEF2[61] (Table S3), HDAC4 could regulate the expression of BRD4-dependent

301 inflammatory genes (8o,p, Table S4; ρac(H3K27ac KO/wt)=0.35 p=0.02 in BRD4 associated[3] SESs,

302 ρac=0.04 p=0.86 in SESs not associated to BRD4). This response is reinforced and integrated by the H3K27

303 demethylation and subsequent de-repression of ERVs, which engages about 10% of HDAC4 peaks (Fig.

304 8m). In conclusion, HDAC4 has a dual role in regulating senescence, by controlling the replication stress and

305 by repressing directly (SE) or indirectly (through ERV) the inflammatory response.

306

DISCUSSION

308 We have identified HDAC4 as the class IIa HDAC member downregulated in all the tested models

309 of senescence and aging. GSK3β[23] supervises the UPS-mediated degradation of HDAC4 during senescence.

310 HDAC4 depletion forces the senescence entrance in transformed and pre-transformed cells. These responses

311 stem from the activation of two early events: DNA damage and inflammation. $HDAC4^{-/-}$ cells become more

312 susceptible to RS. Whether this is due to increased replication fork stalling and collapsing or decreased fork

313 restarting[62] is presently unknown and deserves further studies. HDAC4-regulated genes falling in CFSs and

314 ERFs underwent normal epigenetic control. This makes it unlikely that a) replication/transcription conflicts[47]

315 arising in these sites or b) other influences of RNA Pol-II on replication origin firing[48,49] could be at the

316 origin of HDAC4-dependent RS. Interestingly, class I HDACs were found to bind nascent DNA and protect

317 from RS without altering the acetylation of the histones proximal to the fork[63,64].

318   HDAC4 depletion elicits H3K27 hyper-acetylation and activation of TE and SE[3,7] and the H3K27

319 de-methylation and de-repression of repetitive elements of retroviral origin (ERVs)[52,55]. The activation of

320 these two complementary epigenetic mechanisms confirms that class IIa HDACs are pivotal nodes for the

321 formation of high molecular weight complexes with deacetylase and methyltransferase activities[65,66]. One

322 third of HDAC4 peaks supervises directly the chromatin remodeling of these elements and promotes the RS-

323 independent activation of a pro-inflammatory signaling. Licensing of SE is emerging as a key aspect of the

324 senescence response[3,7,60,67]. The activation of this pathway could increase the genomic fragility due to

325 HDAC4 depletion, as demonstrated in other studies[68,69]. The dismantling of the nuclear lamina and the loss

326 of the linker H1 can further increase transcriptional dysregulation and DNA damage.

327   The complex circuit of events that orbit around HDAC4 demonstrate the centrality of the role played

328 by epigenetic regulators in preserving the correct expression and the integrity of the genome.

329

337

338 **AUTHOR CONTRIBUTIONS**

339 CB conceived the project. EDG and CB designed and analyzed all experiments. EDG generated all knocked-

340 out cell lines, prepared the libraries for ChIP-seq and performed all gene expression studies. HP completed

341 RNAi experiments and supported EDG in immunoblot experiments. EDG, ED and RF performed

342 bioinformatics analysis. LR performed some western blotting. CB did the confocal microscopy and time-

343 lapse analysis. EDG, HP and CB analyzed the time-lapse experiments. EDG, VC, HP, AR, VM generated

344 cell lines used in the study. EDG, ED and CB interpreted data. CB designed the figures with the help of

345 EDG, ED and RP. EDG and CB wrote the paper.

346

347 **COMPETING INTERESTS STATEMENT**

348 The authors declare no competing interests.

349

REFERENCES

1. Ermolaeva, M., Neri, F., Ori, A. & Rudolph, K. L. Cellular and epigenetic drivers of stem cell ageing. *Nat. Rev. Mol. Cell Biol.* **19**, 594–610 (2018).

2. McHugh, D. & Gil, J. Senescence and aging: Causes, consequences, and therapeutic avenues. *J. Cell Biol.* **217**, 65–77 (2018).

3. Tasdemir, N. *et al.* BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. *Cancer Discov.* **6**, 612–629 (2016).

4. Ito, T., Teo, Y. V., Evans, S. A., Neretti, N. & Sedivy, J. M. Regulation of Cellular Senescence by Polycomb Chromatin Modifiers through Distinct DNA Damage- and Histone Methylation-Dependent Pathways. *Cell Rep.* **22**, 3480–3492 (2018).

5. Wakita, M. *et al.* A BET family protein degrader provokes senolysis by targeting NHEJ and autophagy in senescent cells. *Nat. Commun.* **11**, 1935 (2020).

6. Di Micco, R. *et al.* Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638–642 (2006).

7. Sen, P. *et al.* Histone Acetyltransferase p300 Induces De Novo Super-Enhancers to Drive Cellular Senescence. *Mol. Cell* **73**, 684-698.e8 (2019).

8. Kaneda, A. *et al.* Activation of Bmp2-Smad1 Signal and Its Regulation by Coordinated Alteration of H3K27 Trimethylation in Ras-Induced Senescence. *PLoS Genet.* **7**, e1002359 (2011).

9. Piunti, A. *et al.* Therapeutic targeting of polycomb and BET bromodomain proteins in diffuse intrinsic pontine gliomas. *Nat. Med.* **23**, 493–500 (2017).

10. Cruickshanks, H. A. *et al.* Senescent cells harbour features of the cancer epigenome. *Nat. Cell Biol.* **15**, 1495–1506 (2013).

11. Alver, B. H. *et al.* The SWI/SNF chromatin remodelling complex is required for maintenance of lineage specific enhancers. *Nat. Commun.* **8**, 14648 (2017).

12. Joshi, P. *et al.* The functional interactome landscape of the human histone deacetylase family. *Mol. Syst. Biol.* **9**, 672 (2013).

13. Stasevich, T. J. *et al.* Regulation of RNA polymerase II activation by histone acetylation in single living cells. *Nature* **516**, 272–275 (2014).

14. Di Giorgio, E. *et al.* Different class IIa HDACs repressive complexes regulate specific epigenetic responses related to cell survival in leiomyosarcoma cells. *Nucleic Acids Res.* **48**, 646–664 (2020).

15. Di Giorgio, E. & Brancolini, C. Regulation of class IIa HDAC activities: it is not only matter of subcellular localization. *Epigenomics* **8**, 251–269 (2016).

16. Marampon, F. *et al.* HDAC4 and HDAC6 sustain DNA double strand break repair and stem-like phenotype by promoting radioresistance in glioblastoma cells. *Cancer Lett.* **397**, 1–11 (2017).

17. Mason, D. X., Jackson, T. J. & Lin, A. W. Molecular signature of oncogenic ras-induced senescence. *Oncogene* **23**, 9238–9246 (2004).

18. Chen, J. *et al.* Cathepsin Cleavage of Sirtuin 1 in Endothelial Progenitor Cells Mediates Stress-

11

387   Induced Premature Senescence. *Am. J. Pathol.* **180**, 973–983 (2012).

388 19. Zirkel, A. *et al.* HMGB2 Loss upon Senescence Entry Disrupts Genomic Organization and Induces

389   CTCF Clustering across Cell Types. *Mol. Cell* **70**, 730-744.e6 (2018).

390 20. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras Provokes

391   Premature Cell Senescence Associated with Accumulation of p53 and p16INK4a. *Cell* **88**, 593–602

392   (1997).

393 21. Kennedy, A. L. *et al.* Activation of the PIK3CA/AKT Pathway Suppresses Senescence Induced by an

394   Activated RAS Oncogene to Promote Tumorigenesis. *Mol. Cell* **42**, 36–49 (2011).

395 22. Deng, Q. *et al.* The Ability of E1A to Rescue ras -Induced Premature Senescence and Confer

396   Transformation Relies on Inactivation of Both p300/CBP and Rb Family Proteins. *Cancer Res.* **65**,

397   8298–8307 (2005).

398 23. Cernotta, N., Clocchiatti, A., Florean, C. & Brancolini, C. Ubiquitin-dependent degradation of

399   HDAC4, a new regulator of random cell motility. *Mol. Biol. Cell* **22**, 278–289 (2011).

400 24. Inoue-Yamauchi, A. *et al.* Targeting the differential addiction to anti-apoptotic BCL-2 family for

401   cancer therapy. *Nat. Commun.* **8**, 16078 (2017).

402 25. Funayama, R., Saito, M., Tanobe, H. & Ishikawa, F. Loss of linker histone H1 in cellular senescence.

403   *J. Cell Biol.* **175**, 869–880 (2006).

404 26. Freund, A., Laberge, R.-M., Demaria, M. & Campisi, J. Lamin B1 loss is a senescence-associated

405   biomarker. *Mol. Biol. Cell* **23**, 2066–2075 (2012).

406 27. Di Giorgio, E., Hancock, W. W. & Brancolini, C. MEF2 and the tumorigenic process, hic sunt leones.

407   *Biochim. Biophys. Acta - Rev. Cancer* **1870**, 261–273 (2018).

408 28. Cutano, V. *et al.* HDAC7-mediated control of tumour microenvironment maintains proliferative and

409   stemness competence of human mammary epithelial cells. *Mol. Oncol.* **13**, 1651–1668 (2019).

410 29. Azagra, A. *et al.* In vivo conditional deletion of HDAC7 reveals its requirement to establish proper B

411   lymphocyte identity and development. *J. Exp. Med.* **213**, 2591–2601 (2016).

412 30. Wood, K. M., Roff, M. & Hay, R. T. Defective IκBα in Hodgkin cell lines with constitutively active

413   NF-κB. *Oncogene* **16**, 2131–2139 (1998).

414 31. Michaloglou, C. *et al.* BRAFE600-associated senescence-like cell cycle arrest of human naevi.

415   *Nature* **436**, 720–724 (2005).

416 32. Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts.

417   *Nat. Cell Biol.* **5**, 741–747 (2003).

418 33. Kanda, T., Sullivan, K. F. & Wahl, G. M. Histone–GFP fusion protein enables sensitive analysis of

419   chromosome dynamics in living mammalian cells. *Curr. Biol.* **8**, 377–385 (1998).

420 34. Feringa, F. M. *et al.* Persistent repair intermediates induce senescence. *Nat. Commun.* **9**, 3923 (2018).

421 35. Spies, J. *et al.* 53BP1 nuclear bodies enforce replication timing at under-replicated DNA to limit

422   heritable DNA damage. *Nat. Cell Biol.* **21**, 487–497 (2019).

423 36. Lukas, C. *et al.* 53BP1 nuclear bodies form around DNA lesions generated by mitotic transmission of

424   chromosomes under replication stress. *Nat. Cell Biol.* **13**, 243–253 (2011).

425   37.   Moreno, A. *et al.* Unreplicated DNA remaining from unperturbed S phases passes through mitosis for

426   resolution in daughter cells. *Proc. Natl. Acad. Sci.* **113**, E5757–E5764 (2016).

427   38.   Graziano, S., Kreienkamp, R., Coll-Bonfill, N. & Gonzalo, S. Causes and consequences of genomic

428   instability in laminopathies: Replication stress and interferon response. *Nucleus* **9**, 289–306 (2018).

429   39.   Panieri, E. & Santoro, M. M. ROS homeostasis and metabolism: a dangerous liason in cancer cells.

430   *Cell Death Dis.* **7**, e2253–e2253 (2016).

431   40.   Paluvai, H., Di Giorgio, E. & Brancolini, C. The Histone Code of Senescence. *Cells* **9**, 466 (2020).

432   41.   Shah, P. P. *et al.* Lamin B1 depletion in senescent cells triggers large-scale changes in gene

433   expression and the chromatin landscape. *Genes Dev.* **27**, 1787–1799 (2013).

434   42.   Técher, H., Koundrioukoff, S., Nicolas, A. & Debatisse, M. The impact of replication stress on

435   replication dynamics and DNA damage in vertebrate cells. *Nat. Rev. Genet.* **18**, 535–550 (2017).

436   43.   Olson, E., Nievera, C. J., Klimovich, V., Fanning, E. & Wu, X. RPA2 Is a Direct Downstream Target

437   for ATR to Regulate the S-phase Checkpoint. *J. Biol. Chem.* **281**, 39517–39533 (2006).

438   44.   Kannouche, P. L., Wing, J. & Lehmann, A. R. Interaction of Human DNA Polymerase η with

439   Monoubiquitinated PCNA. *Mol. Cell* **14**, 491–500 (2004).

440   45.   Mazouzi, A. *et al.* A Comprehensive Analysis of the Dynamic Response to Aphidicolin-Mediated

441   Replication Stress Uncovers Targets for ATM and ATMIN. *Cell Rep.* **15**, 893–908 (2016).

442   46.   Puddu, F. *et al.* Chromatin determinants impart camptothecin sensitivity. *EMBO Rep.* **18**, 1000–1012

443   (2017).

444   47.   Sanchez, A. *et al.* Transcription-replication conflicts as a source of common fragile site instability

445   caused by BMI1-RNF2 deficiency. *PLOS Genet.* **16**, e1008524 (2020).

446   48.   Brison, O. *et al.* Transcription-mediated organization of the replication initiation program across large

447   genes sets common fragile sites genome-wide. *Nat. Commun.* **10**, 5693 (2019).

448   49.   Blin, M. *et al.* Transcription-dependent regulation of replication dynamics modulates genome

449   stability. *Nat. Struct. Mol. Biol.* **26**, 58–66 (2019).

450   50.   Fungtammasan, A., Walsh, E., Chiaromonte, F., Eckert, K. A. & Makova, K. D. A genome-wide

451   analysis of common fragile sites: What features determine chromosomal instability in the human

452   genome? *Genome Res.* **22**, 993–1005 (2012).

453   51.   Barlow, J. H. *et al.* Identification of Early Replicating Fragile Sites that Contribute to Genome

454   Instability. *Cell* **152**, 620–632 (2013).

455   52.   Chiappinelli, K. B. *et al.* Inhibiting DNA Methylation Causes an Interferon Response in Cancer via

456   dsRNA Including Endogenous Retroviruses. *Cell* **162**, 974–986 (2015).

457   53.   Yu, Y. C.-Y. *et al.* Transient DNMT3L Expression Reinforces Chromatin Surveillance to Halt

458   Senescence Progression in Mouse Embryonic Fibroblast. *Front. Cell Dev. Biol.* **8**, (2020).

459   54.   Topper, M. J. *et al.* Epigenetic Therapy Ties MYC Depletion to Reversing Immune Evasion and

460   Treating Lung Cancer. *Cell* **171**, 1284-1300.e21 (2017).

461   55.   Colombo, A. R., Elias, H. K. & Ramsingh, G. Senescence induction universally activates

462   transposable element expression. *Cell Cycle* **17**, 1846–1857 (2018).

463   56.   Qi, N. *et al.* Multiple truncated isoforms of MAVS prevent its spontaneous aggregation in antiviral

464   innate immune signalling. *Nat. Commun.* **8**, 15676 (2017).

465   57.   Paluvai, H., Di Giorgio, E. & Brancolini, C. Unscheduled HDAC4 repressive activity in human

466   fibroblasts triggers TP53-dependent senescence and favors cell transformation. *Mol. Oncol.* **12**,

467   2165–2181 (2018).

468   58.   Gao, L. *et al.* Bone Marrow-Derived Mesenchymal Stem Cells From Patients With Systemic Lupus

469   Erythematosus Have a Senescence-Associated Secretory Phenotype Mediated by a Mitochondrial

470   Antiviral Signaling Protein-Interferon-β Feedback Loop. *Arthritis Rheumatol.* **69**, 1623–1635 (2017).

471   59.   Ghosh, R., Roy, S. & Franco, S. PARP1 depletion induces RIG-I-dependent signaling in human

472   cancer cells. *PLoS One* **13**, e0194611 (2018).

473   60.   Vahedi, G. *et al.* Super-enhancers delineate disease-associated regulatory nodes in T cells. *Nature*

474   **520**, 558–562 (2015).

475   61.   Jin, Y. *et al.* Active enhancer and chromatin accessibility landscapes chart the regulatory network of

476   primary multiple myeloma. *Blood* **131**, 2138–2150 (2018).

477   62.   Zeman, M. K. & Cimprich, K. A. Causes and consequences of replication stress. *Nat. Cell Biol.* **16**,

478   2–9 (2014).

479   63.   Wells, C. E. *et al.* Inhibition of Histone Deacetylase 3 Causes Replication Stress in Cutaneous T Cell

480   Lymphoma. *PLoS One* **8**, e68915 (2013).

481   64.   Kehrli, K. *et al.* Class I Histone Deacetylase HDAC1 and WRN RECQ Helicase Contribute

482   Additively to Protect Replication Forks upon Hydroxyurea-induced Arrest. *J. Biol. Chem.* **291**,

483   24487–24503 (2016).

484   65.   Cao, C. *et al.* Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific

485   demethylase 1 (LSD1) promotes breast cancer progression. *Oncogene* **36**, 133–145 (2017).

486   66.   Di Giorgio, E. *et al.* The co-existence of transcriptional activator and transcriptional repressor MEF2

487   complexes influences tumor aggressiveness. *PLOS Genet.* **13**, e1006752 (2017).

488   67.   Schäfer, A. *et al.* Impaired DNA demethylation of C/EBP sites causes premature aging. *Genes Dev.*

489   **32**, 742–762 (2018).

490   68.   Hazan, I., Monin, J., Bouwman, B. A. M., Crosetto, N. & Aqeilan, R. I. Activation of Oncogenic

491   Super-Enhancers Is Coupled with DNA Repair by RAD51. *Cell Rep.* **29**, 560-572.e4 (2019).

492   69.   Chang, Y.-H. & Dubnau, J. The Gypsy Endogenous Retrovirus Drives Non-Cell-Autonomous

493   Propagation in a Drosophila TDP-43 Model of Neurodegeneration. *Curr. Biol.* **29**, 3135-3152.e4

494   (2019).

495   70.   Cersosimo, U. *et al.* Synthesis, Characterization, and Optimization for in Vivo Delivery of a

496   Nonselective Isopeptidase Inhibitor as New Antineoplastic Agent. *J. Med. Chem.* **58**, 1691–1704

497   (2015).

498 71. Marzagalli, M., Casati, L., Moretti, R. M., Montagnani Marelli, M. & Limonta, P. Estrogen Receptor
499 β Agonists Differentially Affect the Growth of Human Melanoma Cell Lines. *PLoS One* **10**,
500 e0134396 (2015).
501 72. Clocchiatti, A. *et al.* The MEF2-HDAC axis controls proliferation of mammary epithelial cells and
502 acini formation in vitro. *J. Cell Sci.* **128**, 3961–3976 (2015).
503 73. Ciotti, S. *et al.* GSK3β is a key regulator of the ROS-dependent necrotic death induced by the
504 quinone DMNQ. *Cell Death Dis.* **11**, 2 (2020).
505 74. Potthoff, M. J. *et al.* Histone deacetylase degradation andMEF2 activation promote the formation of
506 slow-twitch myofibers. *J. Clin. Invest.* **117**, 2459–2467 (2007).
507 75. Nam, Y.-J. *et al.* Induction of diverse cardiac cell types by reprogramming fibroblasts with cardiac
508 transcription factors. *Development* **141**, 4267–4278 (2014).
509 76. Di Giorgio, E., Gagliostro, E., Clocchiatti, A. & Brancolini, C. The Control Operated by the Cell
510 Cycle Machinery on MEF2 Stability Contributes to the Downregulation of CDKN1A and Entry into
511 S Phase. *Mol. Cell. Biol.* **35**, 1633–1647 (2015).
512 77. Peruzzo, P. *et al.* Transformation by different oncogenes relies on specific metabolic adaptations. *Cell
513 Cycle* **15**, 2656–2668 (2016).
514 78. Paroni, G. *et al.* Caspase-dependent regulation of histone deacetylase 4 nuclear-cytoplasmic shuttling
515 promotes apoptosis. *Mol Biol Cell* **15**, 2804–2818 (2004).
516 79. Muellera, S. *et al.* RNAi-mediated immunity provides strong protection against the negative-strand
517 RNA vesicular stomatitis virus in drosophila. *Proc. Natl. Acad. Sci. U. S. A.* (2010).
518 doi:10.1073/pnas.1014378107
519 80. Lasry, A. & Ben-Neriah, Y. Senescence-associated inflammatory responses: Aging and cancer
520 perspectives. *Trends in Immunology* **36**, 217–228 (2015).
521 81. Cerami, E. *et al.* The cBio Cancer Genomics Portal: An open platform for exploring
522 multidimensional cancer genomics data. *Cancer Discov.* **2**, 401–404 (2012).
523 82. Jiang, Y. *et al.* SEdb: A comprehensive human super-enhancer database. *Nucleic Acids Res.* (2019).
524 doi:10.1093/nar/gky1025
525 83. Paces, J. HERVd: the Human Endogenous RetroViruses Database: update. *Nucleic Acids Res.* **32**,
526 50D – 50 (2004).
527 84. Kumar, R. *et al.* HumCFS: a database of fragile sites in human chromosomes. *BMC Genomics* **19**,
528 985 (2019).
529 85. Eisenberg, E. & Levanon, E. Y. Human housekeeping genes, revisited. *Trends Genet.* **29**, 569–574
530 (2013).
531 86. Whyte, W. A. *et al.* Master Transcription Factors and Mediator Establish Super-Enhancers at Key
532 Cell Identity Genes. *Cell* **153**, 307–319 (2013).
533 87. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features.
534 *Bioinformatics* **26**, 841–842 (2010).

535   88.   Bailey, T. L., Johnson, J., Grant, C. E. & Noble, W. S. The MEME Suite. *Nucleic Acids Res.* **43**,

536   W39–W49 (2015).

537

538   **METHODS**

539   **Cell culture and reagents.**

540   BJ/hTERT and IMR90 cells (previously characterized[57]), SK-LMS-1 (*TP53*[wt/G245S]) and SK-UT-1

541   leiomyosarcoma cells (previously characterized[14]), HEK293T, LinXE, Ampho Phoenix and MEF

542   *HDAC4*[loxp/loxp] cells were cultured as previously described[14] in 10% FBS DMEM (Euroclone). A375[70]

543   *(TP53*[wt/wt]*)* and WM115[71] *(TP53*[wt/wt]*)* melanoma cells were grown in RPMI, MCF10A were grown as

544   previously described[72]. MCF10A/*HDAC7*^{-/-}, U87MG/*GSK3β*^{-/-}, SK-UT-1/*HDAC4*^{-/-} and *HDAC9*^{-/-} were

545   previously described[14,28,73]. For the conditioning of BJ/hTERT cells, the medium obtained from

546   BJ/*hTERT/HRAS*[G12V] or *HYGRO* cells cultured in 60mm plates for 8 days, was filtered and diluted 1:1 with

547   fresh medium and used to treat cells twice for 96h, with a change after 48h. For the experiments performed

548   in Hypoxia, cells were grown in hypoxic chambers at 37°C, 5% CO_2, 2% O_2 (Baker Ruskinn). The following

549   chemicals were used: 250nM 4-OHT (Sigma-Aldrich), 1µM Doxycycline (Sigma-Aldrich), 1µM MG132

550   (Sigma-Aldrich), 10µM Chloroquine (Sigma-Aldrich), 0.4% Trypan Blue (Sigma-Aldrich), 200µM H_2O_2

551   (Sigma-Aldrich), 1µM PD0332991 (Sigma-Aldrich), 500nM Aphidicolin (Sigma-Aldrich), 3.125µm

552   Camptothecin (Enzo Life Sciences), 20µM Etoposide (Enzo Life Sciences), 100nM ABT-263

553   (Clinisciences).

554

555   **Generation and culture of *Hdac4*^{fl/fl} and *Hdac4*^{-/-} murine embryonic fibroblasts**

556   *Hdac4*^{fl/fl} mice were previously described[74]. MEFs were generated following standard procedures[75] from 13.5

557   days-old embryos. Single cell suspensions were expanded in DMEM/10% FBS in hypoxia. $5*10^6$ cells were

558   retrovirally infected to express Cre-ER. Not infected cells were removed from culture by puromycin

559   (2µg/ml, Sigma-Aldrich) selection. The recombination was achieved through the treatment for 48h with 4-

560   OHT (Sigma-Aldrich). At the end of this incubation, half of the culture was kept in normoxia and half in

561   hypoxia. At each splitting the total number of cells was counted (Countess II, LifeTechnologies) and the

562   doubling time (dt) was calculated as previously described[76].

563   **Plasmid construction, transfection, retroviral and lentiviral infection, silencing**

564   pLENTI-*CRISPR/V2* (Plasmid #52961), p*SpCas9*(BB)-2A-*GFP*(PX458) (Plasmid #48138), p*SpCas9*(BB)-

565   2A-*Puro* (PX459) (Plasmid #62988), pCW-*Cas9* (Plasmid #50661), pCW57/Hygro-MCS1-2A-MCS2

566   (Plasmid #80922), *Apple-53BP1trunc* (Plasmid #69531), *pEGFP-N1*/H2B-GFP (Plasmid #11680), pBabe-

567   *Puro-IKBalpha* (NFKBIA) S32A/S36A (Plasmid #15291), MSCV-*CreERT2-Puro* (Plasmid #22776) were

568   obtained from Addgene. pLKO-Puro *shHDAC4* 1 (TRCN0000314667) and 2 (TRCN000004832) were

569   obtained from Sigma-Aldrich. pWZL-*Hygro-HDAC4*[PAM] (V31L or P16A), pWZL-*Hygro-HDAC4*[PAM]-*ER*

570   pCW-*Hygro- HDAC4*[PAM] were obtained by sub-cloning a mutagenized HDAC4 (QuikChange Site-Directed

571   Mutagenesis Kit, Agilent) into the linearized empty backbones by a restriction-based approach. *Apple-*

16

572 *53BP1trunc* and *H2B-GFP* were sub-cloned respectively in pBABE-*Zeo* and pWZL-*Neo*, NFKBIA-

573 S32A/S36A into pWZL-*Neo-GFP*. pWZL-*Neo-MCS1-2A-MCS2* was obtained by sub-cloning the MCS of

574 pCW57 *Hygro MCS1-2A-MCS2* into pWZL-*Neo* through a recombination-based approach. The generated

575 plasmid was used as acceptor vector for the cloning of *HRAS^{G12V}* (*Nhe*I/*Sal*I) and *E1A*/1-143 (*Mlu*I/*Bgl*II-

576 *Bam*HI) to generate pWZL-*Neo-HRAS/G12V*-2A-*E1A 1-143*. pWZL-*Neo/GFP-LMNB1*, pWZL-*Neo-*

577 *MAVSDN*(ΔCARD 1-100), pBABE-Z*eo-MAVSDN* and pWZL-*Neo/H1.2-GFP* were obtained by amplifying

578 the relative cDNAs from IMR90 cells. pWZL-*Hygro-MEF2/ENG*, pWZL-*Hygro-HRAS^{G12V}*, pBABE-*Puro-*

579 *HRAS^{G12V}* and pBABE-*Puro-myrAKT1* were previously described[57]. pCW-Puro-*HRAS^{G12V}* and *myrAKT1*

580 were obtained by sucloning. pLKO-*Hygro* plasmid expressing the same shRNAs were obtained by oligo

581 cloning. All the generated plasmids were checked by restriction and sequencing. The primers used for

582 cloning are listed in Table S5. Transfections, viral infections and siRNA delivery were done as previously

583 described[28,77]. The following siRNAs (148 pmol) were used: HDAC4

584 (CCACCGGAAUCUGAACCACUGCAUU, Invitrogen Stealth), MAVS 1

585 (CCACCUUGAUGCCUGUGAA), MAVS 2 (CAGAGGAGAAUGAGUAUAA), RIG-1

586 (AAUUCAUCAGAGAUAGUCA).

587

588 **CRISPR/Cas9 Genome editing**

589 SpCas9 was stably transduced to generate SK-LMS1 *HDAC4$^{-/-}$* clones (76,1231,205,1254,66), *HDAC4$^{+/-}$*

590 clone 275 and BJ/E1A-RAS *HDAC4$^{-/-}$*. SpCas9 was transiently transfected (Lipofectamine 2000,

591 LifeTechnologies) to generate SK-LMS-1 *HDAC4$^{-/-}$* clones (635, 273, 274, 275, 277, 279), A375 *HDAC4$^{-/-}$*

592 clones (304, 401, 150, 1090) and *HDAC4$^{+/-}$* clones (317, 159). A Cas9 resistant HDAC4 (PAM mutated,

593 using a strategy previously described[28]), was stably expressed prior to the KO (in SK-LMS-1 clones

594 273,274,275, 277, 279) or continuously re-expressed in a 4-OHT dependent (SK-LMS-1 clone 66) or DOX

595 dependent (A375 clones 304, 401, 150, 1090) manner. The sgRNA used are listed in Table S5. Monoclonal

596 cultures were generated by seeding n=1 (SK-LMS-1 and A375), n=3 (BJ/E1A-RAS) cells in each well of 96-

597 well plates (Sarstedt). The successful generation of KO clones was screened by immunoblotting and

598 confirmed by Sanger sequencing.

599 **Immunofluorescence and immunoblotting**

600 Cells were fixed with 3% paraformaldehyde and permeabilized with 0.3% Triton X-100. The secondary

601 antibodies were Alexa Fluor 488-, 546- or 633-conjugated anti-mouse and anti-rabbit secondary antibodies

602 (Molecular Probes). Actin was labelled with phalloidin-AF546 or AF-660 (Molecular Probes). For the

603 intracellular staining of dsRNA, the permeabilization step was performed for 5' with 0.5% Triton X-100.

604 ICC blocking solution (3% w/v BSA (Sigma-Aldrich), 3% v/v goat serum (Abcam), 0.02% v/v Tween-20 in

605 PBS) was applied for 1h to block nonspecific binding and for the incubation at 37°C for 1h with 250 ng J2

606 antibody (Scicons). For S phase analysis, cells were grown for 3 h with 50 μM Bromodeoxyuridine (BrdU).

607 After fixation, coverslips were treated with HCl (1% and 2%), quenched with Borate and processed for

17

608 immunofluorescence. Cells were imaged with a confocal microscope Leica AOBS SP8 or with Leica

609 AF6000 LX. Nuclei were stained with Hoechst 33258 or DAPI (Sigma-Aldrich).

610 Cell lysates after SDS-PAGE and immunoblotting on nitrocellulose (Whatman) were incubated with primary

611 antibodies. HPR-conjugated secondary antibodies were obtained from Cell Signalling and blots were

612 developed with Super Signal West Dura (Thermo Fisher Scientific). Primary and secondary antibodies were

613 removed by using Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific), according to

614 manufacturer. Unless otherwise indicated, all the immunoblot figures were representative of at least two

615 biological replicates. The primary and secondary antibodies used in this work are listed in Table S6. Images

616 represent maximum intensity projections of 3D image stacks and were adjusted for brightness and contrast

617 for optimal visualization.

618 **Time-lapse video microscopy**

619 SK-LMS-1 and SK-LMS-1/*HDAC4*^{-/-}/*HDAC4*[PAM]-*ER* cells engineered to express H2B-GFP and Apple-

620 53BP1 trunc or H2B-GFP alone were seeded on fibronectin coated 35mm Glass bottom dishes (MatTek) at

621 low density ($0.3*10^5$ cells). After 24h, medium was refreshed and 4-OHT was added in HDAC4 re-

622 expressing cells. 6h later, the dishes were housed in the live cell imaging chamber of a Leica AOBS SP8

623 confocal microscopy, maintained in a humidified atmosphere at 37°C and 5%CO_2 and imaged every 10

624 minutes for 74h under four dimensions. 5 z-stacks were collected for each time-point. Laser power, exposure

625 time, pinhole aperture and acquisition intervals were chosen appropriately to minimize toxicity and

626 bleaching.

627 **Proteomics and transcriptomics from *in vivo* murine aging models**

628 C57BL/6J female mice were obtained from Shared Ageing Research Models (ShARM, UK). Tissues

629 explanted from 4 months (128 days) and 26 months (774 days) old mice were snap-frozen in liquid nitrogen.

630 For protein lysates generation, subsections of the liver and of the skin were grinded into a powder with a

631 pestle and lysed for 1h at 4°C respectively with 400 and 200µl RIPA lysis buffer for 10mg of tissue. 4x

632 Laemli sample buffer was added to the clarified lysates and after boiling the samples were loaded on

633 SDS/PAGE gels. For RNA extraction, 1ml Tri Reagent (Molecular Research Center) was added to 10mg of

634 smashed tissues. After 1h incubation at 4°C, RNA was recovered by phenol-chloroform extraction/ethanol

635 precipitation and resuspended in 20 µL RNase-free water.

636 **Immunoprecipitation**

637 Cells were lysed for 10' into hypotonic lysis buffer (20mM Tris-HCl pH7.4, 10mM KCl, 10mM $MgCl_2$, 1%

638 Triton X-100, 10% glycerol, 50mM Iac, 1 mM phenylmethylsulphonylfluoride, 5 mM NaF, 1 mM Na_3VO_4),

639 supplemented with protease inhibitors and 10µM MG-132 and 10µM G5. Lysates were incubated for 5 h

640 with 1µg anti-HDAC4[78] or rabbit IgG and for 1h with 30µl slurry protein A (GE). After 4 washes, the

641 immunocomplexes were reversed with 2x Laemli sample buffer, boiled, resolved by SDS-PAGE and

642 subjected to western-blotting. 1/100 of total lysate has been collected as input.

643 **dsRNA immunoprecipitation**

18

644   Total RNA was extracted with Tri Reagent (Molecular Research Center) from a pellet of 35*10^6 SK-LMS-1

645   HDAC4 KO cells. IP was performed O/N in Polysomal Lysis Buffer[79] in the presence of 10μg J2 antibody

646   (Scicons). 50μl slurry protein A (GE) was added and incubated in continuous rotation at 4°C for 4h. After 5

647   washes of the collected immunocomplexes, RNA was recovered by phenol-chloroform extraction/ethanol

648   precipitation and resuspended in 20μL RNase-free water. The precipitation was repeated until a final amount

649   of 1.6 μg of purified RNA was reached. 800ng were treated for 20' at 37°C with 50u ShortCut Rnase III

650   (NEB), in the digestion buffer supplied by the manufacturer. The digestion was stopped with 10x EDTA.

651   The remaining 800ng were treated in the same manner but without the addition of RNAse III. 30pmoles of

652   purified dsRNA treated or not with RNAseIII were transfected in recipient cells, by using 15μl

653   Lipofectamine 3000 (LifeTechnologies) and 250μl Optimem (Gibco). The enrichment of dsRNA in the

654   preparation was evaluated by qPCR and expressed as % of enrichment over input.

655   **SA-β-gal assay**

656   Cells seeded on coverslips in 12-well plates were fixed for 5' (PBS 2% formaldehyde/0.2% glutaraldehyde),

657   washed twice with 0.9% NaCl and stained for 16h at 37°C with staining solution: 40 mM citric acid/Na

658   phosphate buffer, 5 mM K4[Fe(CN)6]3H2O, 5 mM K3[Fe(CN)6], 150 mM sodium chloride, 2 mM

659   magnesium chloride and 1 mg/mL X-gal (Panreact Applichem). Images were acquired with Leica LD bright

660   field optical microscope.

661   **Transformation assay**

662   Soft agar assay was performed as previously described[57,77]. Briefly, a total of 0.8*10^5 cells were seeded in

663   0.3% top agar/DMEM layer above a 0.6% agar/DMEM basement. Fresh medium was added twice/week.

664   After 15 days of culture the supernatant was discarded and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

665   diphenyltetrazolium bromide] staining (0.5mg/ml in PBS) was applied for 2h. Images were acquired with a

666   Leica DN6000 microscope. Foci were automatically counted with Clono Counter.

667   **RNA extraction and quantitative qRT-PCR**

668   Cells were lysed using Tri Reagent (Molecular Research Center). 1.0 μg of total RNA was DNAseI treated

669   (NEB #T2010) and retro-transcribed by using 100 units of M-MLV Reverse transcriptase (Life

670   Technologies) in the presence of 1.6 μM oligo(dT) (Sigma-Aldrich) and 4 μM Random hexamers

671   (Euroclone). qRT-PCRs were performed using SYBR green technology (KAPA Biosystems). Data were

672   analyzed by comparative threshold cycle (delta delta Ct) using *HPRT* and *GAPDH* or *ACTB* and *GAPDH* as

673   normalizer. The primers used for qRT-PCR are listed in Table S5.

674   **RNA array expression and data analysis**

675   Total RNA was purified with Quick-RNA Miniprep (ZymoResearch), amplified according to the

676   specifications of the Illumina TotalPrep RNA Amplification Kit (Ambion) and hybridized on Illumina

677   whole-genome HumanHT-12 v 4.0 chip (Illumina). Acquisition and data analysis were performed as

678   previously described[14]. Principal component analysis (PCA) was performed by using R function prcomp.

679   Differentially expressed genes (DEGs) were called accordingly to the following criteria: |fold change|>2 and

19

680   p adj.<0.05. The list of the DEGs is provided as Table S7. GSEA analysis in Fig.2 was performed as

681   previously described[14]. The transcripts defining the "NFκβ", "SASP"[80] and "RIS up-regulated genes" gene

682   sets are listed in Table S8. Gene list enrichment in Table S4 was performed by interrogating MSigDB

683   collections (BP,C6,CGP,H,MF) with the transcripts associated to promoters (with 2kb from TSS), TEs or

684   SEs bound by HDAC4; the obtained enrichments were considered significant for p and FDR <0.05 and if at

685   least three Gene Sets fall in the same category. For the expression levels and Kaplan-Meier analysis of

686   TCGA Skin Cutaneous Melanoma samples, data were retrieved from CBioPortal[81] and expressed as z-score.

687   Z-scores > |1.75| were selected as cut-off. For bioinformatics analysis in Fig.2, 7, S3 and S4, the following

688   GEO datasets were analyzed: GSE38410, GSE74324, GSE40349, GSE3189, GSE78138, GSE45276,

689   GSE36640, GSE40349, GSE132569.

690

691   **ChIP, library construction, ChIP-seq and NGS data analysis**

692   Chromatin was obtained from SK-LMS-1 cells, 36h after or not HDAC4 removal, and immunoprecipitated

693   with 2 µg of anti-H3K27ac, 3µg of anti-H3K27me3, 4µg of anti-LMNB1 and 4µg of anti-HDAC4 antibodies

694   or control IgG, as previously described[14]. Three independent biological replicates were pulled according to

695   BLUEPRINT requirements and 5 ng of total DNA were used to prepare ChIP-seq libraries, according to

696   TruSeq ChIP Sample Preparation guide (Illumina). Libraries were sequenced on the Illumina HiSeq 2000

697   sequencer. The ShortRead R/Bioconductor package was used to evaluate the quality of sequencing reads and

698   Bowtie 2 was used to align them to NCBI *GRCh38* human genome reference. Peak calling was performed

699   against input sequences using HOMER for HDAC4 ChIP ("factor" mode) and MACS2 for H3K27ac and

700   H3K27me3 ("sharp" mode and "broad" mode, respectively); gene annotations were performed as previously

701   described[14]. gplots, biomaRt and Gviz R/Bioconductor packages and the deepTools suite were used to

702   generate peak heatmaps and for the visualization of genomic loci. The H3K27ac and H3K27me3 enriched

703   genomic regions between HDAC4 KO and wt were called according to $\sum_{k=peak\ summit}^{\pm 15000} f(k), where\ f(k) =$

704     $\frac{enrichment\ KO}{enrichment\ wt}$. |log$_2$(Fc)|≥1 was used as cut-off. The following databases were consulted for the dissection of

705   the ChIP-seq peaks as reported in Fig.6g/h, 7g/h, 8m, S3j/k: SEdb[82] for super-enhancers, HERVd[83] for

706   endogenous retroviruses, HumCFS[84] and the refinement[50] for common fragile sites; ERFs[51] and HK exons[85].

707   sample_01_066_SE of smooth muscle SE was used as lineage reference. "SES", defined according to the

708   ROSE algorithm[86], represents the SEs activated during senescence. SES is the union of the SE identified

709   during OIS[3] and replicative senescence[7] and is provided as Table S9. Liftover tool was used to convert

710   genome coordinates between assemblies and to remap homologous sequences between genomes. The

711   bedtools toolset (--intersect option)[87] was used to identify overlaps of at least one nucleotide. The

712   investigated classes of genomic elements have been considered as separated, not redundant and not

713   overlapping. The enrichment has been calculated with respect to the genome coverage of each genomic

714   element (length of female haploid human genome: 3184709445 nucleotides). Known and novel motif

715   discovery was performed using the MEME-ChIP tool from the MEME Suite[88]. The following parameters

716   were used: -ccut 0; -order 1; -meme-maxsize 100000000; -meme-minsites 2; -meme-maxsites 100; -meme-

717   minw 6; -meme-maxw 10; -meme-nmotifs 10; -meme-mod anr; -dreme-e 0.05; -centrimo-score 5.0; -

718   centrimo-ethresh 10. The identified enriched motifs were compared to the Jolma2013,

719   JASPAR2018_CORE_vertebrates_non_redundant and uniprobe_mouse databases for annotation. Enrichr

720   (TRRUST) (http://amp.pharm.mssm.edu/Enrichr/) was used for the motif enrichment analysis in Table S2.

Statistics

722   For experimental data, Student t-test was employed. Mann–Whitney test was applied when normality could

723   not be assumed. $p<0.05$ was chosen as statistical limit of significance. For comparisons between more than

724   two samples, the Anova test was applied coupled to Kruskal–Wallis and Dunn's Multiple Comparison Test.

725   For correlation between two variables, Pearson correlation or Spearman correlation were calculated for

726   normal or non normal distributions, respectively. Excel and GraphPad Prism were used for routineer

727   analysis, R/Bioconductor packages for large data analysis and heatmap generation. We marked with

728   *$p<0.05$, **$p<0.01$, ***$p<0.001$. Unless otherwise indicated, all the data in the figures were represented as

729   arithmetic means ± the standard deviations from at least three independent experiments.

Data availability

731   Raw data corresponding to ChIP-seq experiments are uploaded with GEO accession GSE149644.

732   For reviewers, to access the data, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149644

733   Enter token clyvsuiwrlqfrgn into the box.

734

FIGURES LEGENDS

736   **Figure 1. HDAC4 is dysregulated during senescence and aging and is required for senescence escape.**

737   **a.** Immunoblot analysis in IMR90 cells undergoing replicative senescence. Actin was used as loading

738   control. **b.** Microscopic images of SA-β-gal stained IMR90 cells (scale bar 50 µm). **c.** Immunoblot analysis

739   in tissue-derived lysates obtained from C57BL/6J female mice sacrificed at 128 (young) and 774 (old) days

740   of age. Actin was used as loading control. **d.** Immunoblot analysis in BJ/*hTERT* expressing the indicated

741   transgenes for the indicated time. Vimentin was the loading control. **e.** Cellular lysates obtained in BJ/*hTERT*

742   expressing for 8 days the indicated transgenes and treated or not for 8h with MG132 were

743   immunoprecipitated using anti-HDAC4 and immunoblotted with the indicated antibodies. **f.** Immunoblot

744   analysis in BJ/*hTERT* cells expressing HRAS[G12V] and silenced for GSK3β, as indicated. **g.** Immunoblot

745   analysis in BJ/*hTERT/E1A/RAS/HDAC4*^{+/+} or [-/-] cells, as indicated. Actin was used as loading control. **h.**

746   Analysis of the senescent cells as scored after SA-β-gal staining. Mean ± SD; n = 3. **i.** Immunoblot analysis

747   in SK-LMS-1/*HDAC4*^{+/+} or [-/-] as indicated. **j.** Cell-proliferation curve of the indicated *HDAC4*[+/+, +/-, -/-] SK-

748   LMS-1 cells. Mean ± SD; n = 4. **k.** Analysis of the senescent cells as scored after SA-β-gal staining. Mean ±

749   SD; n = 4. **l.** Representative image of normal and altered DAPI-stained nuclei observed in SK-LMS-1

750   *HDAC4*^{-/-} cells (scale bar 10 µm). **m-n.** Analysis of the % of cells displaying altered nuclei or γH2AX foci

751   (>5). Etoposide was a control (2h, 20µM). Mean ± SD; n = 4. **o-p-q.** Analysis of SA-β-gal (o), BrdU (p) and

752   γH2AX (q) positivity in wt or HDAC4 KO cells expressing HYGRO[R] (clone 635) or HDAC4[PAM]. Mean ±

753 SD; n = 4. The significance is relative to clone 635. **r.** Immunoblot analysis in SK-LMS-1 wt or KO (clone

754 66) cells re-expressing a tamoxifen inducible *HDAC4^{PAM}-ER*. Arrowheads point to HDAC4 cleavage

755 products observed in HDAC4-ER expressing cells. Actin was the loading control. **s-v.** Analysis of SA-β-gal

756 (s), BrdU (t), nuclear alteration (u) and γH2AX (v) positivity in the indicated cells. Mean ± SD; n = 4.

757 **Figure 2. HDAC4 depletion causes a senescence-like transcriptional and epigenetic reprogramming. a.**

758 Venn diagrams showing the number of transcripts differently regulated in SK-LMS-1/*HDAC4$^{-/-}$* cells

759 generated by using two different sgRNAs (green and red). Differentially expressed genes (DEGs) were

760 selected based on |fold change| >2 and p <0.05. **b.** Heat-map of the absolute expression levels of the DEGs in

761 the indicated clones and biological replicates hierarchically clustered accordingly to average linkage. Blue

762 shades intensity is proportional to transcripts abundance. **c.** GSEA plots displaying the NES obtained by

763 interrogating the transcriptome of HDAC4 wt and KO cells with the indicated gene sets. **d.** (Upper) Heat-

764 map of the 97831 H3K27ac and 139200 H3K27me3 enriched peaks in the indicated SK-LMS-1 cells;

765 (Lower) Heat-map of the 4441 H3K27ac peaks displaying a FPKM (KO/wt)>2 and of the 1205 H3K27me3

766 peaks displaying a FPKM (wt/KO)>2 (FC>2), in a region of ±15Kb around peak summit. **e.** Genomic

767 distribution of all the H3K27ac and H3K27me3-enriched peaks (top panel), or only of those associated with

768 a FC>2, as indicated and shown in Fig. 2d. **f-g-h.** Analysis of BrdU (f), SA-β-gal (g) and γH2AX (h)

769 positivity in wt or HDAC4 KO cells expressing HYGRO^{R} (clone 635) or GFP-NFKBIA (clones 55 and 70).

770 Mean ± SD; n = 4. The significance is relative to clone 635. **i.** mRNA expression levels of the indicated

771 genes in the indicated SK-LMS-1 clones. Mean ± SD; n = 4. The significance is relative to clone 635. **j-k.**

772 Detailed view of the H3K27ac (green) and H3K27me3 (light-blue) tracks at the *IL1B* (j) and *CDKN1A* (k)

773 loci in SK-LMS-1/*HDAC4$^{+/+}$* and *$^{-/-}$*, as indicated. Red boxes highlight the H3K27ac regions significantly

774 hyper-acetylated in *HDAC4$^{-/-}$*, that correspond to a super-enhancer in the case of *IL1B* (SE_02_17300084)

775 and to an intronic region previously associated to class IIa HDACs binding for *CDKN1A*. In the latter case a

776 strong demethylation in *HDAC4$^{-/-}$* affects the whole locus.

777 **Figure 3. Time-course morphological and transcriptional alteration induced by HDAC4 depletion. a.**

778 Immunoblot analysis of LMNB1, γH2AX and H1.2-GFP in SK-LMS-1/*HDAC4$^{-/-}$* cells re-expressing

779 *HDAC4^{PAM}-ER*. Cells were harvested at the indicated time after 4-OHT removal. SMC3 was used as loading

780 control. **b.** mRNA expression levels of the indicated genes. Mean ± SD; n = 4. **c.** Representative images of

781 the combination of cellular phenotypes observed after the depletion of HDAC4 in SK-LMS-1 cells (scale bar

782 10 μm). **d.** Quantification of the time-course accumulation of the phenotypes represented in Fig. 3C in the

783 indicated cells. Mean ± SD; n = 5. At least 200 individual cells were evaluated in each biological replicate.

784 **Figure 4. HDAC4 depletion causes the rapid accumulation of TP53BP1 foci and bodies in G2 and the**

785 **subsequent mitotic slowdown and impairment.** Heatmap representing the quantification of the TP53BP1

786 foci/bodies in SK-LMS-1/*HDAC4$^{-/-}$*/*HDAC4^{PAM}-ER*, during 74h of analysis starting from 6h after 4-OHT

787 removal (time "0"), as indicated. The intensity of the red is proportional to the number of TP53BP1 spots. 10

788 and 9 starting cells were analyzed respectively for the +4-OHT and the -4-OHT conditions.

22

789 **Figure 5. LMNB1 re-expression and hypoxic growing conditions have minimal and complementary**
790 **effects on the senescence induced by HDAC4 loss in SK-LMS-1 cells. a.** Analysis of the % of SK-LMS-
791 1/*HDAC4$^{-/-}$* cells, grown in normoxia, expressing H1.2-GFP or GFP-LMNB1, as indicated, and re-expressing
792 (+4-OHT) or not (-4-OHT) *HDAC4^{PAM}-ER*, displaying positivity for SA-β-gal or the accumulation of CCFs
793 and naked CCFs. Mean ± SD; n = 4. The significance is relative to H1.2-GFP re-expressing cells. **b.**
794 Representative confocal picture of SK-LMS-1/*HDAC4$^{-/-}$* cells, expressing GFP-LMNB1 and immunostained
795 for DNA (Hoechst, blue), LMNA (red) and γH2AX (violet). Arrows point to naked CCFs, arrowheads to
796 LMNB1+ CCFs. **c-d.** Immunoblot analysis of HIF-1α, γH2AX, GFP-LMNB1 and H1.2-GFP (anti-GFP
797 antibody) in SK-LMS-1/*HDAC4$^{-/-}$* cells, re-expressing (+4-OHT) or not (-4-OHT) *HDAC4^{PAM}-ER* and
798 expressing the indicated transgenes. Lysates were generated after 4d of culture in normoxia or in hypoxia, as
799 indicated. **e.** Analysis of the SA-β-gal positivity in the cells described in Fig. 5D. The significance is relative
800 to the same cells grown in normoxia. Mean ± SD; n = 3. **f.** mRNA expression levels of the indicated genes in
801 SK-LMS-1 cells generated and maintained as described in Fig. 5D. The levels are relative to wt cells grown
802 in normoxia (considered as 1). The significance is relative to SK-LMS-1/*HDAC4$^{-/-}$* cells grown in normoxia.
803 Mean ± SD; n = 3.

804 **Figure 6. HDAC4 depletion causes the accumulation of replication stress and of cytoplasmic dsRNAs**
805 **of retroviral origin. a.** Immunoblot analysis in SK-LMS-1/*HDAC4$^{-/-}$* cells re-expressing *HDAC4^{PAM}-ER*.
806 Cells were harvested at the indicated time after 4-OHT wash out. Actin was used as loading control. **b.**
807 Immunoblot analysis in SK-LMS-1/*HDAC4$^{-/-}$* cells re-expressing *HDAC4^{PAM}-ER*. 4-OHT was removed 6h
808 before the treatment with CDK4i (1μM), as indicated. Actin was used as loading control. **c.** Analysis of
809 BrdU, SA-β-gal, TP53BP1 foci and γH2AX positivity in the indicated cells treated as in Fig. 6B. Mean ±
810 SD; n = 4. The significance is relative to untreated cells. **d-e.** Time-course immunoblot analysis in SK-LMS-
811 1/*HDAC4$^{-/-}$* cells re-expressing or not *HDAC4^{PAM}-ER* (48h) and treated for the indicated time with 500nM
812 Aphidicolin (APH) or 3.125μm Camptothecin (CPT). Actin was used as loading control. Densitometric
813 analysis of γH2AX/Actin ratio is provided. **f.** Immunoblot analysis on the same cells described in Fig. 6D,
814 harvested at the indicated time after the release from 1h APH treatment. Densitometric analysis of
815 γH2AX/Actin ratio is provided. **g.** Histogram representing the percentage of hyper-acetylated (green bar) or
816 demethylated (light blue) H3K27 peaks in SK-LMS-1/*HDAC4$^{-/-}$* cells and falling in the indicated genomic
817 elements or displaying the indicated epigenomic features. The genome coverage of each element is indicated
818 by gray bars. **h.** Histogram representing the enrichment of each element described in Fig. 6g in respect to the
819 expected distribution calculated according to the genome coverage. **i.** Histogram of 10 CFSs associated to the
820 transcripts (median of the associated transcripts, indicated as RNA (KO/wt)) more up-regulated (red) or
821 down-regulated (blue) in SK-LMS-1/*HDAC4$^{-/-}$* in respect to *HDAC4$^{+/+}$* cells. The correlation between the
822 RNA (KO/wt) and H3K27ac/me3 levels and the gene length are indicated. **j.** Histogram representing the
823 RNA levels of the indicated genes/ERVs in SK-LMS-1/*HDAC4$^{-/-}$/HDAC4^{PAM}-ER* cells, at 72h from last 4-
824 OHT treatment (+4-OHT) or wash-out (-4-OHT). Mean ± SD; n = 4. The significance is relative to wt cells.
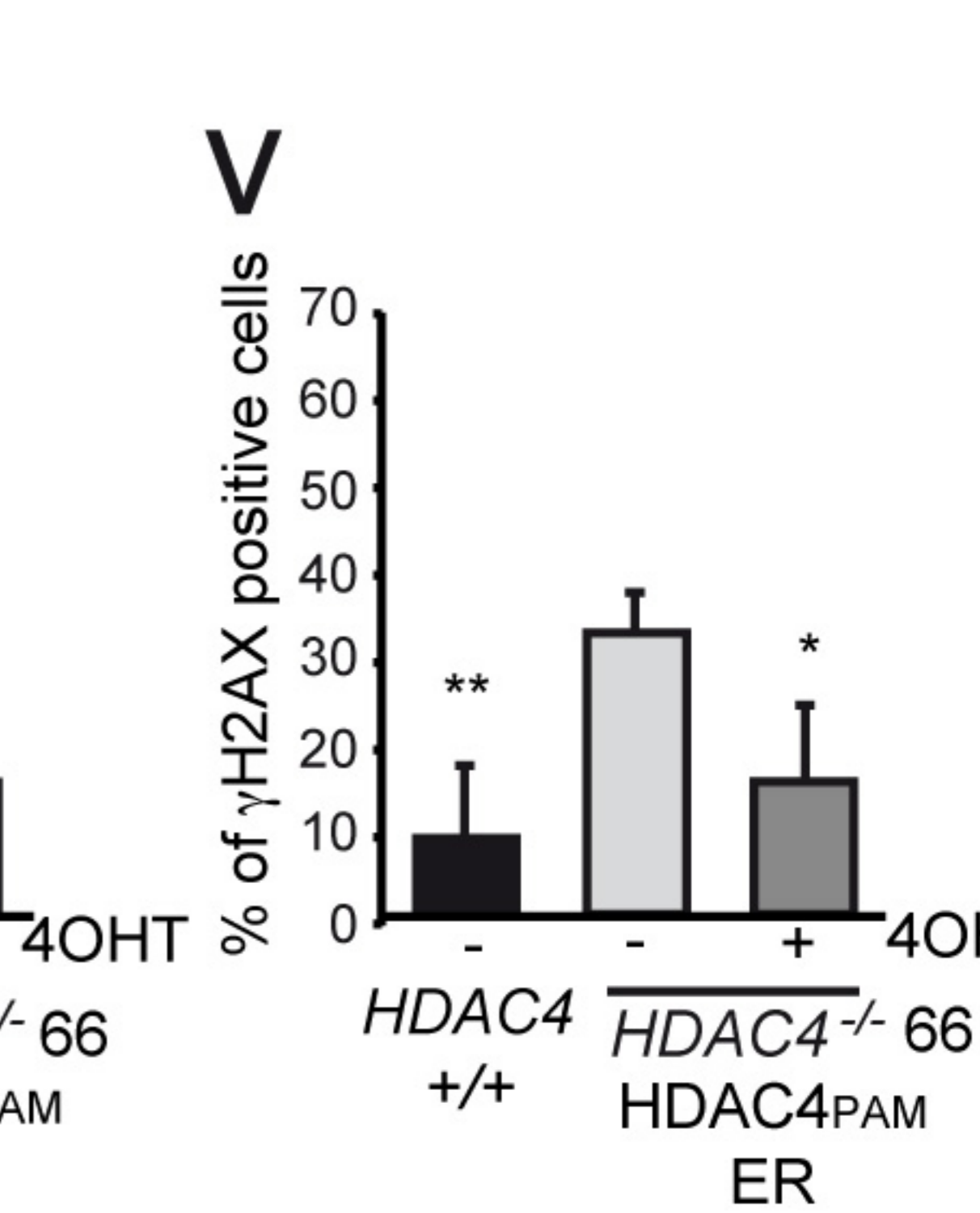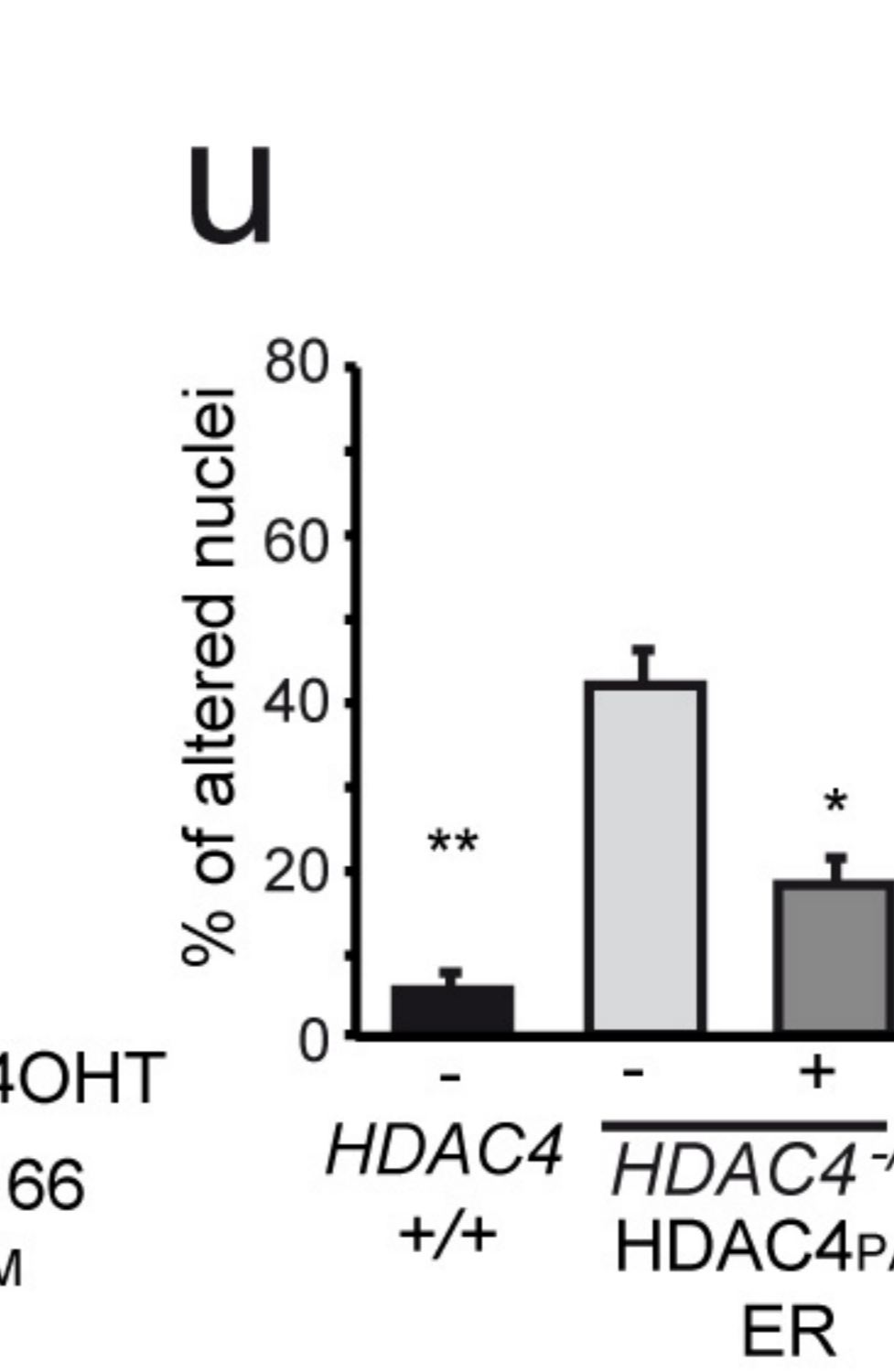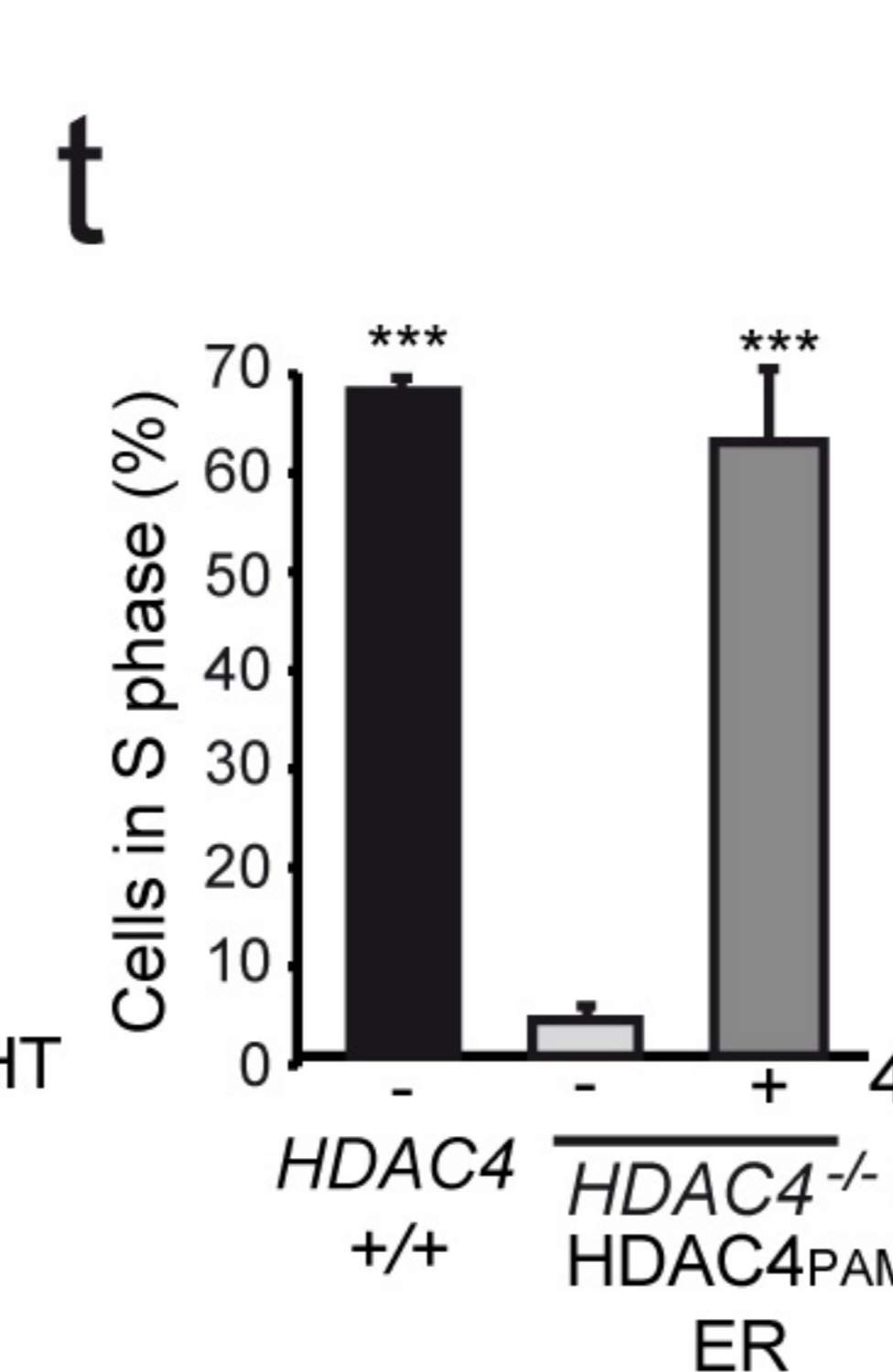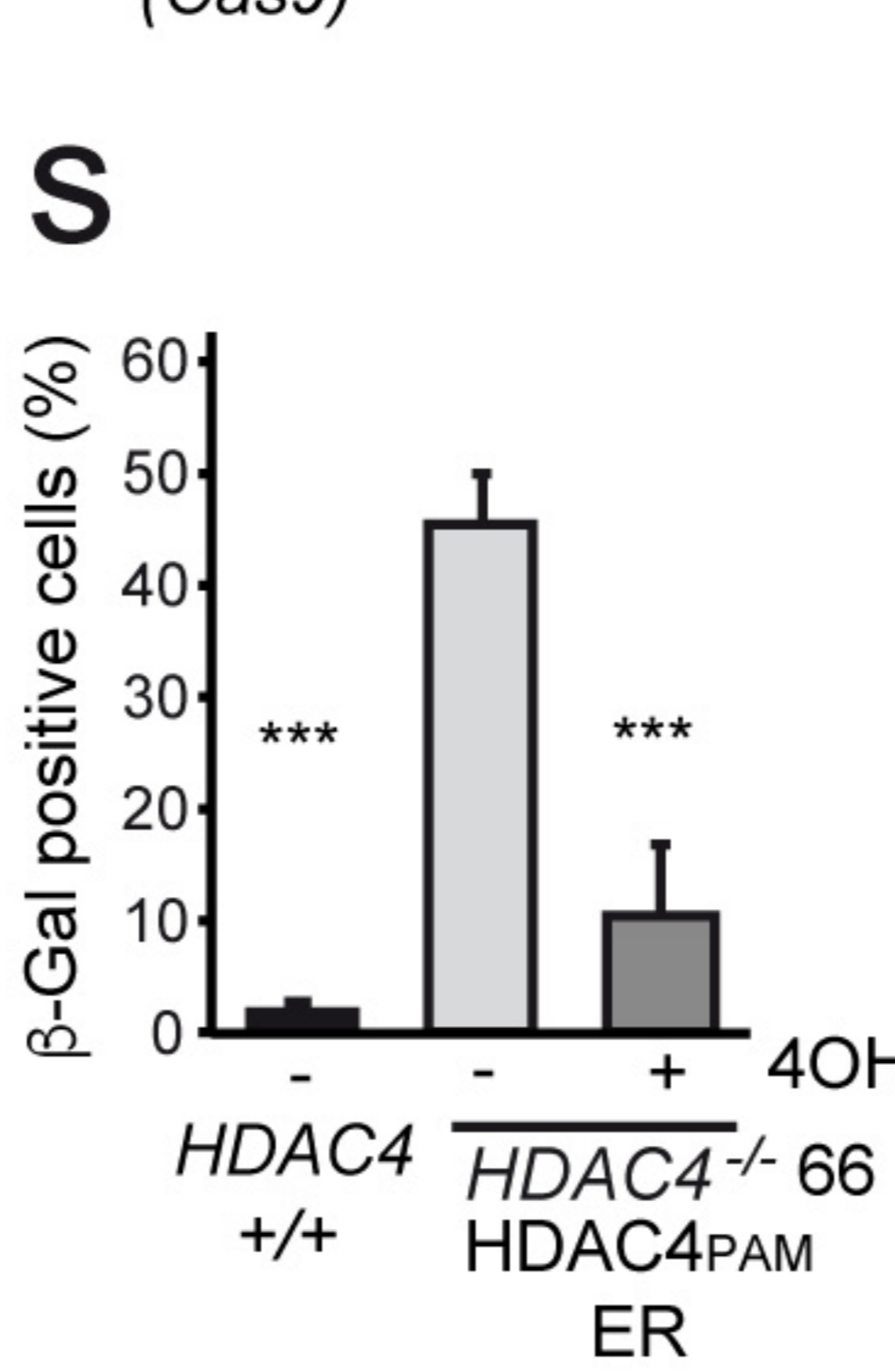
23

825   **k.** Representative confocal pictures of the indicated SK-LMS-1 cells at 48h from 4-OHT removal/addition.

826   Immunofluorescence was performed to visualize dsRNAs (red) and nuclei (blue). Scale bar 10μM.

827   **Figure 7. The epigenetic stress induced by HDAC4 depletion causes the accumulation of ERVs and**

828   **triggers the IFN-response. a-b.** mRNA expression levels of the indicated genes, in SK-LMS-1/*HDAC4$^{-/-}$*

829   /*HDAC4^{PAM}-ER* re-expressing cells harvested at the indicated time after HDAC4 depletion (a) and in IMR90

830   cells harvested at the indicated cellular splittings (b). Mean ± SD; n = 3. The significance is relative to time 0

831   (a) or to split 12 (b). **c.** Quantification of the enrichment over input of the indicated species of RNAs in the

832   preparation of dsRNAs used to treat the cells in Fig. 7d/e. **d-e.** Analysis of SA-β-gal (c) and Trypan blue (d)

833   positivity in SK-LMS-1 cells transfected for 72h with 30pmoles of double-stranded enriched RNAs obtained

834   from *HDAC4$^{-/-}$* cells, and pre-digested or not for 30' with 50u RNAseIII. **f.** Heat-map of the top (1%)

835   H3K27ac and H3K27me3 enriched peaks in the indicated SK-LMS-1 cells in a 30kb region around an ERV;

836   each row represents the same ERV in wt and KO cells. **g.** Histogram representing the percentage of hyper-

837   acetylated (green bar) or demethylated (light blue) H3K27 peaks in SK-LMS-1/*HDAC4$^{-/-}$* cells and falling in

838   ERVs. The genome coverage of each element is indicated by gray bars. **h.** Histogram representing the

839   enrichment of hyper-acetylated or demethylated ERVs as explained in Fig. 7g in respect to the expected

840   distribution calculated according to the genome coverage. **i-j.** Detailed view of H3K27ac (green) and

841   H3K27me3 (light-blue) tracks at two ERVs rich regions on Chr16 (g) and Chr17 (hH). Regions H3K27

842   hyper-acetylated (g) or demethylated (h) in SK-LMS-1/*HDAC4$^{-/-}$* in respect to the wt are indicated.

843   **Figure 8. A senescence signature triggered by the activation of RIG-1/MAVS pathway or by the**

844   **HDAC4-dependent de-repression and activation of SE sustains the premature senescence entrance. a.**

845   Microscopic images of SA-β-gal stained SK-LMS-1/*HDAC4$^{-/-}$* cells re-expressing HDAC4 (+4-OHT) or the

846   indicated transgenes, at 72h from HDAC4 depletion. Scale bar 50 μm. **b.** Immunoblot analysis in the cells

847   described in Fig.8a. Densitometric analysis of γH2AX/Actin ratio is provided. n=3. **c.** Immunoblot analysis

848   in the indicated cells treated as in fig. 6b. **d-e.** Immunoblot analysis in the indicated cells harvested after 72h

849   of HDAC4 depletion. Actin was the loading control. **f.** Analysis of SA-β positivity in wt or *HDAC4$^{-/-}$* SK-

850   LMS-1 cells transfected with the indicated siRNAs. Mean ± SD; n =3. **g.** Heat-map reporting the expression

851   levels (log2 fold change relative to wt cells) of the indicated genes and ERVs in SK-LMS-1/*HDAC4$^{-/-}$* cells

852   expressing the indicated transgenes as described in Fig. 8b. **h.** Heat-map reporting the % of the indicated SK-

853   LMS-1 cells displaying positivity to γH2AX, CCF, SA-β-gal and BrdU. **i.** Analysis of SA-β-gal positivity in

854   wt or *HDAC4$^{-/-}$* A375 cells re-expressing the indicated transgenes, 5d after HDAC4 removal. Mean ± SD; n

855   =3. The significance is relative to *HDAC4$^{-/-}$*-Neo cells. **j.** Immunoblot analysis in the indicated cells

856   harvested as in Fig. 8i. SMC3 was the loading control. **k.** Heat-map reporting the expression levels (log2 fold

857   change relative to wt cells) of the indicated genes and ERVs (left) and SA-β-gal positivity (right) in the same

858   A375 cells described in fig. 8i. **l.** Heat-map reporting the expression levels (log2 fold change relative to

859   DMSO treated *HDAC4^{PAM}-ER* cells) of the indicated genes and ERVs, in the same SK-LMS-1 cells

860   described in fig. 6b. **m.** Histogram representing the percentage of class IIa HDACs bound peaks in the

861   indicated cells and falling in the described genomic/epigenomic elements. The genome coverage is indicated

862   by gray bars. **n.** Motif analysis of 93 HDAC4-gained SESs for putative transcription factor binding sites.

863   Motifs with p-value<0.5*10^{-4} were selected. **o-p.** Detailed view of 2 representative SESs directly bound by

864   HDAC4 in SK-LMS-1 wt cells in correspondence to H3K27ac-defined SEs.

865

Replicative Senescence in IMR90 cells

a Passage: 12 13 27 28 37 44 — HDAC4, HDAC5, HDAC7, HDAC9, LMNB1, Actin. SA-β gal (%): 2.4 2.4 33.1 36.3 63.8 89.5

b P12, P27, P37, P44

c Liver / Dermis, Young 1 2 / Old 1 2 — HDAC4, HDAC5, HDAC7, HDAC9, LMNB1, CDKN1A, p53, Actin

d Day 2 / Day 8, GFP Ras Akt1 / GFP Ras Akt1 — HDAC4, HDAC5, HDAC7, HDAC9, γH2AX, RAS, AKT1, Vimentin. SA-β gal (%): 5.1 36.7 14.2 4.3 48.1 68.2

e BJ-TERT/GFP, BJ-TERT/AKT1, BJ-TERT/RAS — IPαIgs, IPαHDAC4, MG132, Ubiquitin, HDAC4

f +DOX 8d, siRNA 24h, split 48h — BJ-TERT pcwRAS, siGSK3β, DOX — HDAC4, GSK3β, RAS, ACTIN. SA-β gal (%): 6.4 7.5 84.2 80.1. BrdU (%): 24.5 29.2 1.9 2.5

g BJ-TERT/Ras/E1A, HDAC4+/+, HDAC4-/- — HDAC4, E1A, Actin

h BJ-TERT/Ras/E1A, β-Gal positive cells (%): HDAC4+/+, HDAC4-/-

i SK-LMS-1, HDAC4+/+ (Cas9, Control), HDAC4-/- (76, 1231, 205, 1254) — HDAC4, ACTIN

j SK-LMS-1, Cells x10.000 vs Days from seeding

k β-Gal positive cells (%): Cas9, Control, 76, 1231, 205, 1254

l HDAC4-/-

m % of altered nuclei: Untr., Etop., 1231, 205

n % of γH2AX positive cells: Untr., Etop., 1231, 205

o HDAC4, β-Gal positive cells (%): HYGRO +/+, -/- 635, HDAC4PAM -/- 273, 274, 275, 277, 279

p HDAC4, Cells in S phase (%): HYGRO +/+, -/- 635, HDAC4PAM -/- 275, 279

q HDAC4, % of γH2AX positive cells: HYGRO +/+, -/- 635, HDAC4PAM -/- 275, 279

r HDAC4PAM ER +/+, -/- 66 / HDAC4 — HDAC4-ER, γH2AX, LMNB1, Actin, 4OHT

s β-Gal positive cells (%): HDAC4+/+, HDAC4-/- 66 HDAC4PAM ER, 4OHT

t Cells in S phase (%): HDAC4+/+, HDAC4-/- 66 HDAC4PAM ER, 4OHT

u % of altered nuclei: HDAC4+/+, HDAC4-/- 66 HDAC4PAM ER, 4OHT

v % of γH2AX positive cells: HDAC4+/+, HDAC4-/- 66 HDAC4PAM ER, 4OHT

a

GDEs HDAC4-/-

HDAC4-/-
Clone 76
Clone 1231

39 · 230 · 66

HDAC4-/-
Clone 205
Clone 1254

c

FRIDMAN SENESCENCE UP

NES = -2.1
p < 0.001

Enrichment score

HDAC4+/+ · HDAC4 -/-

NFKB TARGETS GILMORE

NES = -2.1
p < 0.001

Enrichment score

HDAC4+/+ · HDAC4 -/-

SASP TARGETS

NES = -2.0
p < 0.001

Enrichment score

HDAC4+/+ · HDAC4 -/-

RIS TARGETS UP

NES = -2.9
p < 0.001

Enrichment score

HDAC4+/+ · HDAC4 -/-

b

a b   a b   a b   a b   a b   a b
Cas9 Control   76   1231   205   1254
HDAC4+/+   HDAC4-/-

d

H3K27ac   GLOBAL   H3K27me3
HDAC4-/- HDAC4+/+   HDAC4-/- HDAC4+/+

H3K27ac   FC>2   H3K27me3
HDAC4-/- HDAC4+/+   HDAC4-/- HDAC4+/+

e

GLOBAL

H3K27ac   H3K27me3

FC>2

H3K27ac   H3K27me3

- Promoter <1kb
- Promoter 1-2kb
- Promoter 2-3kb
- 5' UTR
- 3' UTR
- 1st Exon
- Other Exon
- 1st Intron
- Other Intron
- Downstream (<300)
- Distal Intergenic

f

Cells in S phase (%)

HDAC4 +/+   -/-   -/-   -/-
              635 55   70
        HYGRO   NFKBIA

g

β-Gal positive cells (%)

HDAC4 +/+   -/-   -/-   -/-
              635 55   70
        HYGRO   NFKBIA

h

% of γH2AX positive cells

HDAC4 +/+   -/-   -/-   -/-
              635 55   70
        HYGRO   NFKBIA

i

IL1B

mRNA relative levels

HDAC4 +/+   -/-   -/-   -/-   -/-   -/-
              635 55   70   275 279
            NFKBIA   HDAC4PAM

CXCL8

mRNA relative levels

HDAC4 +/+   -/-   -/-   -/-   -/-   -/-
              635 55   70   275 279
            NFKBIA   HDAC4PAM

CDKN1A

mRNA relative levels

HDAC4 +/+   -/-   -/-   -/-   -/-   -/-
              635 55   70   275 279
            NFKBIA   HDAC4PAM

Fbxo32/ATROGIN1

mRNA relative levels

HDAC4 +/+   -/-   -/-   -/-   -/-   -/-
              635 55   70   275 279
            NFKBIA   HDAC4PAM

GADD45A

mRNA relative levels

HDAC4 +/+   -/-   -/-   -/-   -/-   -/-
              635 55   70   275 279
            NFKBIA   HDAC4PAM

j

IL1B: chr2:112750000-112950000

H3K27ac
HDAC4 +/+
-/-

H3K27me3
+/+
-/-

CKAP2L   IL1A   AC079753.2   XIAPP3   IL37   CDK8P2
IL1B   AC079753.1
112.8   112.85   112.9

k

CKN1A: chr6:36500000-36800000

H3K27ac
HDAC4 +/+
-/-

H3K27me3
+/+
-/-

STK38   SRSF3   MIR3925   AL127115.1   RAB44   Z85996.1   CPNES
RNU1-88BP   PANDAR   CPNES
LAP3P2   Z85996.2
CDKN1A   DINOL
36.55   36.60   36.65   36.70   36.75

a

| -4OHT | +4OHT | p53-DN | MEF2-ENG | MAVS-DN |

b SKLMS1 HDAC4-/-

WT | Neo | MAVS-DN | TP53-DN | MEF2-ENG | HDAC4

γH2AX — -14
Actin — -45 -36
1   5.8   1.4   1.8   2.4   1.2

c SKLMS1
HDAC4⁻/⁻ 66 HDAC4_PAM ER

CDK4i: - + - +
4OHT: + + - -
MAVS-DN: + + + +

γH2AX — -14
MAVS-DN FLAG — -56 -45
Actin — -45

d SKLMS1
+/+ -/- +/+ HDAC4
- + + - 4OHT
- + + + TP53-DN

TP53 — -56 -45
LMNB1 — -66 -56
Actin — -45 -36

e SKLMS1

WT | HDAC4-/-
- - + MAVS-DN

MAVS-DN FLAG — -56 -45
LMNB1 — -66
Actin — -45

f SKLMS1

WT | Control | RIG1 | MAVS siRNA
HDAC4-/-

g SKLMS1 HDAC4-/-

Columns: ERV9 ERVW1 ERVFc2 ERVFb1 OAS1 IFI6 DDX58 IFIH1 IRF2 IRF7 IL1B CXCL8 IGFBP7 FBXO32 CDKN1A GADD45A IFNB FOXO1 FOXO4 GAPDH

Rows: LMNB1, MAVS-DN, TP53-DN, MEF2-ENG, NFKBIA, HDAC4, Neo

+4.0 log(2) 0 -4.0

h

Columns: γH2AX CCF β-Gal BrdU
Rows: LMNB1, MAVS-DN, TP53-DN, MEF2-ENG, NFKBIA, HDAC4, Neo

% 60 ... 0

i A375

WT | Neo (304 401) | MAVS DN (304 401) | TP53 DN (304) | HDAC4 (304 401)
HDAC4-/-

j A375 HDAC4-/-

WT | Neo | HDAC4 | TP53-DN | MAVS-DN

LMNB1 — -66 -56
γH2AX — -14
MAVS-DN FLAG — -56 -45
TP53 — -56 -45
SMC3 — -116

k A375 HDAC4-/-

Columns: ERV9 ERVW1 ERVFc2 ERVFb1 OAS1 IFI6 DDX58 IFIH1 IRF2 IRF7 IFNB IL1B CXCL8 IGFBP7 FBXO32 CDKN1A GADD45A GAPDH | β-Gal

Rows: HDAC4 (401 304), TP53-DN (304), MAVS-DN (401 304), Neo (401 304)

+4.0 log(2) 0 -4.0   60 ... 0 %

l

DMSO | CDK4i
4OHT: - - +

Rows: ERV9, ERVW1, ERVFc2, ERVFb1, OAS1, IFI6, DDX58, IFIH1, IRF2, IRF7, IFNB, IL1B, CXCL8, IGFBP7, FBXO32, CDKN1A, GADD45A, GAPDH

+4.0 log(2) 0 -4.0

m

Legend: HDAC4 in SKLMS1, HDAC4 in SKUT1, HDAC9 in SKUT1, HDAC7 in MCF10A, Genome distribution

X-axis: CFSs, ERFSs, ERVs, SEMs, SESs, TESs, HK
Y-axis: Peaks (%)

n Motifs in HDAC4 bound SESs (n=93) | TFs

TGACTCA — FOS-JUN, BACH2, FOS2L-JUN E-value 1.1e-026
TGACTAA — BATF-JUN, Mafb DBD 2, FOS E-value 2.4e-008

o *AKR1E2 chr10: 4,600,000-4,900,000: SE_02_17300332*

H3K27ac +/+ (400)
H3K27ac -/- (400)
HDAC4 +/+ (60)
H3K27me3 +/+ (60)
H3K27me3 -/- (60)

4.7 mb — 4.8 mb — 4.9 mb

p *SEMA7A chr15: 74,336,000-74,553,000: SE_02_17300871*

H3K27ac +/+ (400)
H3K27ac -/- (400)
HDAC4 +/+ (60)
H3K27me3 +/+ (60)
H3K27me3 -/- (60)

74.35 mb — 74.4 mb — 74.45 mb — 74.5 mb — 74.55 mb