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CCM2 deficient endothelial cells undergo a ROCKs dependent reprogramming into senescence associated secretory phenotype.

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1 **Abstract:**

2 Cerebral Cavernous Malformation (CCM) is a cerebrovascular disease in which stacks of
3 dilated haemorrhagic capillaries form focally in the brain. Whether and how defective
4 mechanotransduction, cellular mosaicism and inflammation interplay to sustain the progression
5 of CCM disease is unknown. Here, we reveal that CCM1- and CCM2-silenced endothelial cells
6 expanded *in vitro* enter into senescence-associated secretory phenotype (SASP) that they use
7 to invade the extracellular matrix and attract surrounding wild-type endothelial and immune
8 cells. Further, we demonstrate that this SASP is driven by the cytoskeletal, molecular and
9 transcriptomic disorders provoked by ROCKs dysfunctions. By this, we propose that CCM2
10 and ROCKs could be parts of a scaffold controlling senescence, bringing new insights into the
11 emerging field of the control of aging by cellular mechanics. These *in vitro* findings reconcile
12 the known dysregulated traits of CCM2-deficient endothelial cells into a unique endothelial
13 fate. Based on these *in vitro* results, we propose that a SASP could link the increased ROCK-
14 dependent cell contractility in CCM2-deficient ECs with microenvironment remodelling and
15 long-range chemo-attraction of endothelial and immune cells.

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18

19 **Introduction:**

20 Cerebral Cavernous malformations (CCM) are stacks of overgrown, dilated and haemorrhagic
21 venous capillaries formed by a unique layer of poorly joined endothelial cells (ECs) without
22 intervening cerebral mural cells(1). Loss-of-function mutations on 3 genes (*CCM1/KRIT*,
23 *CCM2/Malcavernin*, *CCM3/PDCD10*) are associated with the familial form of the disease(2,3).
24 *CCM1* or *CCM2*-associated disease develops later in life than *CCM3* which is a more aggressive
25 form of the disease(4,5).

26 CCM lesions expand with time and they become infiltrated by immune cells that sustain a
27 chronic inflammatory response(6,7). Intriguingly, CCM lesions are composed of a mosaic of
28 mutant and wild-type ECs (8–11). Malinverno and colleagues have further shown that the
29 majority of ECs bordering large mature ccm3 caverns are actually wild-type ECs that have been
30 attracted to the lesion site at least in part by mutant ECs(11). Other studies have reported that
31 *CCM* mutant ECs secrete metalloproteases(12–14) or cytokines(15), over-produce ROS(16),
32 present defective autophagy(17) or that they undergo an endothelial to mesenchymal transition
33 (EndMT)(18). Moreover, loss of CCM proteins activates β 1 integrin (19,20), p38 MAPK(21),
34 ERK5-KLF2/4(20,22,23) and TLR4 signaling pathways(24). However, how these various
35 dysregulations interplay to generate CCM lesions is not well known.

36 A remarkable feature of CCM lesions is their peculiar mechanical microenvironment. Indeed,
37 ECs in CCM lesions experience disturbed forces coming from stagnant blood flow(25) on their
38 luminal side and increased ECM stiffness upon matrix remodelling on their basal side(19).
39 Increased RhoA/ROCK-dependent intracellular tension (19) is a conserved feature of *CCM*
40 mutant ECs in humans and animal models (13,26–28). ROCK overactivation stimulates the
41 polymerization of a contractile acto-myosin cytoskeleton that shifts the tensional homeostasis
42 between cell-cell and cell-extracellular matrix (ECM) adhesions. We previously showed that
43 the endothelial tensional homeostasis is actually under the control of the coupled activities of
44 the two ROCK isoforms(29). The molecular scaffold formed by the association of CCM1 and
45 CCM2 recruits ROCK2 to VE-cadherin complexes to promote the polymerization of a cortical
46 acto-myosin cytoskeleton supporting cell-cell junctions. At the same time, this CCM/ROCK2
47 complex keeps ROCK1 kinase activity low thereby limiting the adhesion of the cell to the ECM.
48 When the CCM1/2 complex is lost, ROCK2 delocalizes from VE-cadherin while ROCK1 gets
49 over activated and promotes the polymerization of numerous β 1 integrin-anchored acto-myosin
50 stress fibers that most likely tear the cell-cell junctions apart(29). Importantly, it is yet unknown
51 whether, beyond their role on the architecture of the endothelium, ROCKs are also involved in
52 the control of gene expression downstream of CCM2.

53 The mechanical defects play a primary role in the development of the disease. Inhibition of both
54 ROCKs with chemical inhibitors, among which fasudil, blocks the genesis and maturation of
55 CCM lesions in animal models(30–32). However, the toxicity of these drugs precludes their
56 use in patients. It is therefore critical to find new therapies targeting specific downstream
57 pathways. Toward this goal, we need to find a mechanistic explanation that could integrate all
58 the different dysregulated traits of *CCM* mutant ECs.

59 Here, we reveal that the transcriptome of CCM2-silenced ECs cultured *in vitro* presents a
60 signature of Senescence-Associated Secretory Phenotype (SASP). Cellular senescence
61 contributes to a wide variety of human age-related pathologies, including cancer, fibrosis,
62 cardiovascular diseases, or neurological disorders(33). We demonstrate that CCM2-silenced
63 ECs indeed enter into premature senescence and acquire degradative and invasive skills.
64 Moreover, CCM2-deficient ECs gain paracrine functions through secreted factors that are able
65 to attract *in vitro* wild-type ECs and immune cells. Remarkably, we show that this SASP is a
66 triggered by dysfunctional ROCK1 and ROCK2 and by increased ECs contractility. This
67 unexpected endothelial fate transition triggered by the loss of CCM2 reconciles all the known
68 dysregulated features of CCM2-deficient ECs. These features, if conserved *in vivo*, could
69 support the mosaicism of the CCM lesions and their inflammatory state.

70

71

72 **Results:**

73 **1) The loss of CCM2 turns on a SASP transcriptomic program in endothelial cells.**

74 KRIT and CCM2 proteins interact with each other to form a molecular scaffold(34). We
75 previously showed that, owing to the stabilizing effect of CCM2 on KRIT, both proteins are
76 lost when CCM2 is silenced(19). We therefore chose to silence CCM2 in order to deplete the
77 entire KRIT/CCM2 complex. To study the gene expression program of a pure population of
78 ECs depleted for CCM2, we performed RNA sequencing on monolayers of human umbilical
79 vein endothelial cells (HUVECs) after two consecutive rounds of transfection with CCM2
80 targeting siRNA (siCCM2) or with non-targeting siRNA (siNT). CCM2 silencing was of 86%
81 (figS1A) as reported in Lisowska et al., 2018(29). A total number of 2057 differentially
82 expressed genes (DEGs) (fold change [FC] ≥ 2 ; $P < 0.05$, FDR corrected using Benjamini
83 Hochberg method (35)) were identified in siCCM2-treated compared to siNT-treated
84 HUVECs. Among these DEGs, 1316 upregulated while 739 were downregulated (Table S1).

85

86 To investigate the cellular functions altered by the loss of CCM2, we performed a Gene
87 Ontology analysis on these DEGs using cellular component (Fig 1A) and biological process
88 (Fig 1B) annotations (Table S2). Up-regulated genes were associated with the plasma
89 membrane, secretory vesicles, extracellular matrix and focal adhesions (Fig 1A). They related
90 to ECM organization, cell adhesion and migration, secretion, inflammatory response to
91 cytokines and calcium homeostasis (Fig1B). Down-regulated genes were associated with the
92 nuclear part of the cell, chromosomes, chromatin, the mitotic spindle pole, kinetochores and
93 microtubules (Fig 1A). They relate to DNA replication, recombination and repair, chromosome
94 segregation, microtubule-dependent movements and cell cycle progression (Fig 1B). A
95 schematic representation of the enriched pathways found by a Reactome analysis in siCCM2
96 HUVEC is given in Figure 1C. In fact, these upregulated and downregulated functions (Fig 1B,
97 C) evoke a striking unique cellular state; a senescence-associated secretory phenotype (SASP).
98 This phenotype defines the ability of cell-cycle-arrested cells to secrete pro-inflammatory
99 cytokines, chemokines, growth factors and proteases giving rise to ECM remodelling and to the
100 stimulation of neighbour cells proliferation and invasiveness(36).

101

102 To test the hypothesis that a SASP transcriptomic program is turned on in siCCM2 HUVECs,
103 we searched for specific transcriptomic signatures of senescence and SASP in the literature
104 corresponding to gene sets enriched in senescent cells among which fibroblasts and endothelial
105 cells(37–40). These gene sets relate to up- and down-regulated genes (Table S3). We then
106 searched for an enrichment in these gene sets in the CCM2-depleted ECs transcriptome using
107 Gene Set Enrichment Analysis (GSEA). We confirmed that these premature replicative
108 senescence and SASP signatures are significantly enriched in the CCM2-silenced transcriptome
109 (Fig1D, Fig S2). Overall, these functional analyses of the transcriptome suggest that ECs
110 undergo a SASP when CCM2 is lost.

111 **2) CCM2- and KRIT-depleted ECs display hallmarks of SASP.**

112 Since a SASP transcriptomic program is turned on upon the loss of CCM2, we next sought for
113 features of premature senescence in these cells. We looked for different hallmarks, as the
114 combination of multiple traits is required to ascertain senescence(41). HUVECs were analysed
115 at passage 4 when siNT cells are still proliferative and healthy. In addition to flattening and
116 elongating upon the production of transversal stress fibers (Fig S5B), CCM2-depleted ECs
117 expressed almost a 3-fold increase in lysosomal senescence-associated β -galactosidase (SA- β -
118 gal) activity, the historical marker of senescence (Fig 2A, 2B). In addition, their nuclei
119 displayed senescence-associated heterochromatin foci (SAHF) as revealed by spots in DAPI
120 and HIRA staining (Fig 2C, 2D) and their area was increased (Fig 2E). As senescence leads to
121 cell cycle arrest, we looked at the expression level of the cell cycle inhibitors CDKNs. Among
122 them, p21/CIP1 and p15/INK4b were 3-fold upregulated (Fig 2F). On the contrary, cyclin-
123 dependent kinase 1 (CDK1), its regulator CKS1B and cyclin 2A (CCN2A) as well as the
124 transcription factor E2F1, a driver of S phase entry, were all dramatically downregulated (Fig
125 S3). Using BrdU incorporation followed by cytometry analysis, we detected a 2-fold reduction
126 in the percentage of cells in S phase and an accumulation in G1 indicative of a defect in the
127 G1/S transition of the cell cycle (Fig 2G). This translated into a significantly lowered rate of
128 proliferation of the ECs population as shown by impedance measurements (Fig 2H), and a two-
129 fold lowered percentage of cells positive for the proliferative marker Ki67 (Fig 2I, 2J). Overall,
130 the combination of all these traits confirms that the loss of CCM2 indeed induced premature
131 senescence in HUVECs.

132 Senescent cells secrete paracrine factors that can promote tumor development *in vivo* by
133 engaging deleterious inflammatory responses and malignant phenotypes such as proliferation
134 and invasiveness (42). We found that siCCM2 treated HUVECs overexpressed ECM
135 remodelling mediators including matrix proteins, metalloproteases of the MMP and ADAM
136 families, the plasminogen activator uPA and cross-linking enzymes (Fig 2K). Moreover, they
137 overexpress cytokines and inflammatory chemokines among which IL-1A and B, IL8, CXCL1,
138 2, CCL20 and EREG that are hallmarks of SASP (43) (Fig 2K).

139 Having shown that the loss of CCM2 leads to SASP in HUVECs, we wondered whether this
140 would also be conserved in cerebral microvascular ECs (HBMVECs), an *in vitro* cellular model
141 more closely related to CCM. We also wondered whether this SASP would be a common
142 feature of ECs depleted for KRIT and CCM3. Therefore, each of these proteins was depleted
143 in HUVECs and HBMVECs (Fig S1B) and the appearance of marks of senescence was
144 analysed. Firstly, results in HBMVECs paralleled that in HUVECs (Fig 3) confirming that the
145 loss of CCM2 also led to senescence in brain microvascular ECs. Moreover, KRIT-depleted
146 HUVECs or HBMVECs displayed the same senescent phenotype as CCM2-depleted HUVECs
147 or HBMVECs. We observed an increase in cells expressing SA- β -gal activity (Fig 3A-D) and
148 HIRA nuclear foci (Fig 3E-H), and a decrease in Ki67-positive cells (Fig 3I-L). Moreover, their
149 proliferation rate was consistently reduced (Fig 3M-N). Interestingly, CCM3-depleted
150 HUVECs or HBMVECs did not display marks of senescence and behaved as control cells in
151 these assays in good agreement with CCM3 distinct role in ECs biology and onset of the
152 disease(44). They did not show increased SA- β gal activity (Fig 3A-D), nor HIRA nuclear foci
153 (Fig 3E-H). They had a normal level of Ki67 positive cells (Fig 3I-L) and they did not

154 proliferate differently from control cells (Fig 3M-N). Therefore, consistently with their strong
155 association and regulation of common signaling pathways (19,44), the loss of KRIT and CCM2
156 similarly lead to premature *in vitro* cellular senescence of brain microvascular ECs.

157 **3) ROCK2 controls the SASP transcriptomic program of CCM2-depleted ECs**

158 ROCK-dependent perturbations in the mechanotransduction of ECs have a major role in the
159 genesis and progression of CCM lesions. Inhibition of ROCK is sufficient to block the
160 formation and the maturation of CCM lesions (30–32). We previously showed that the CCM1/2
161 complex is a scaffold recruiting ROCK2 at VE-cadherin complexes thereby limiting ROCK1
162 kinase activity to maintain the tensional homeostasis between cell-cell and cell-ECM adhesions.
163 This coordination between ROCK1 and ROCK2 activities preserve the integrity of the
164 endothelial monolayer(29). However, it is yet unknown whether ROCKs are also involved in
165 the control of gene expression downstream of CCM2 and in particular in the regulation of this
166 SASP transcriptomic program. Hence, we analysed the contribution of ROCK1 and ROCK2 by
167 performing RNA sequencing on monolayers of CCM2-silenced HUVECs that were
168 additionally silenced for ROCK1 or ROCK2 or HUVECs silenced only for ROCK1 or ROCK2
169 (Fig S1A) in the same set of experiments as that shown in figure 1. We have previously shown
170 that the additional depletion of ROCK1 but not ROCK2 restores the morphological defects of
171 the CCM2-deficient HUVECs and their permeability barrier(29). Similar phenotypes were
172 observed in HBMVECs (Fig S5A). Strikingly, 65% of the DEGs with $\log_2FC > 1$ had their
173 expression significantly returned toward the control level by the additional silencing of ROCKs
174 (Fig 4A, Table S4). The silencing of ROCK2 had a stronger restoring effect than that of ROCK1
175 on the number of restored genes (Fig 4A, Table S4) and their level of expression (Fig 4B).
176 Importantly, silencing of ROCK2 alone had overall an opposite effect to that of CCM2 on gene
177 expression (Fig 4B), suggesting that ROCK2 acquires a gain of transcriptional function when
178 CCM2 is lost.

179 We then studied the restoring effect of ROCK1 or ROCK2 silencing on the biological functions
180 perturbed by CCM2 depletion. Table S5 gives the Gene Ontology analysis of the biological
181 functions of the DEGs restored by ROCK1 and ROCK2 (common DEGs) or restored
182 specifically by ROCK1 or ROCK2. ROCK2 had a broad restoring effect on all the biological
183 functions dysregulated upon the loss of CCM2. Figure 4C shows the heatmap of the expression
184 of DEGs associated with GO biological processes found enriched in siCCM2 cells. The
185 expression of DEGs related to the down-regulated nuclear functions in siCCM2 HUVECs was
186 fully restored by ROCK2 depletion while ROCK1 depletion had only a partial effect. In
187 addition, up-regulated peri-membrane functions were rescued by the silencing of ROCK2 to a
188 higher extent than that of ROCK1 (Fig 4C). Going further, we focused on the effect of ROCKs
189 on the signatures of senescence or SASP found in siCCM2 transcriptome. While these
190 signatures of senescence were still present upon ROCK1 depletion, they were not anymore
191 significantly enriched in HUVECs doubly silenced for CCM2 and ROCK2 (Fig 4D, Fig S2).

192 Overall, our transcriptomic data reveal that, beyond their role on the cytoskeletal homeostasis
193 of the endothelial monolayer, ROCK2 and to a lesser extent ROCK1 has a massive effect on

194 the genes regulated by CCM2. These genes are involved in a transcriptomic program supporting
195 the onset of SASP when CCM2 is lost.

196 **4) ROCKs dysfunctions induce premature senescence in CCM2-depleted ECs.**

197 Having shown that ROCKs mediate the expression of genes involved in SASP, we next asked
198 whether dysfunctional ROCKs played a causal role in the entry into SASP of CCM2-depleted
199 ECs. Additional silencing of ROCK1 or ROCK2 in HUVECs and HBMVECs was similarly
200 efficient in preventing the appearance of most of the features of senescence tested i.e. SA- β -gal
201 activity, HIRA-positive SAHF and restored normal level of Ki67+ cells (Fig 5A, B, C, F, Fig
202 S6). However, ROCK2 silencing was more efficient in preventing the accumulation of
203 HUVECs in the G1 phase of the cell cycle (Fig 5E). This effect of ROCK2 silencing is
204 consistent with its higher efficiency in lowering the expression of p21/CIP1 and p15/INK4b
205 (Fig 5D) and in restoring the gene expression of down-regulated cyclins and cyclin dependent
206 kinases (Fig S3). Moreover, the silencing of ROCK2 and to a lesser extent that of ROCK1
207 lowered the expression of SASP factors, i.e. ECM remodelers or inflammatory chemokines (Fig
208 5G, H).

209 Our next goal was then to know whether the cytoskeletal defects provoked by dysregulated
210 ROCK1 and ROCK2 play a direct role in the premature senescence of CCM2-depleted
211 HUVECs. To answer this question, we treated siCCM2 HUVECs with blebbistatin, an inhibitor
212 of myosin II that blocks cell contractility or with Y-27632, an inhibitor of ROCK1 and 2 kinase
213 activities. Both treatments inhibited the production of transversal actin stress fibers found in
214 siCCM2 HUVECs and siCCM2 HBMVECs and restored a more cortical actin rim alike the one
215 observed in control HUVECs (Fig S5A, B). Interestingly, these treatments inhibited all the
216 senescent traits studied above, i.e. high SA- β -gal activity, HIRA-positive SAHF and
217 accumulation in G1 phase of the cell cycle and restored normal level of Ki67+ cells (Fig 5A,
218 B, C, E, F; Fig S6). These results support the fact that increased contractility is involved in the
219 premature senescence of CCM2-depleted ECs. Noticeably, blebbistatin and Y-27632 had no
220 effect on siNT HUVECs in these assays (Fig S5C, D, and E).

221 Overall, these data reveal that dysregulated ROCK1 and ROCK2 functions together with
222 increased cell contractility lead CCM2-depleted ECs to enter into a premature senescence.

223

224 **5) ROCK1 mediates ECM degradation and supports invasiveness of CCM2-depleted ECs** 225 **and neighbouring WT ECs.**

226 Cancer cells undergoing SASP can promote tumour development through a juxtacrine effect on
227 their microenvironment by secreting matrix metalloproteinases (MMPs) and ECM remodeling
228 enzymes that facilitate tumour cell invasiveness and metastasis(42). Similar to cancer
229 progression, the formation of CCM lesions could result from a SASP-dependent invasion of the
230 brain tissue by ECs. Consistently, MMPs have been found around CCM lesions in human(14)
231 and in mouse(13), or zebrafish models(22) and their upregulation plays a role in CCM defects
232 (12,20). To know whether upregulated expression of SASP factors confers invasive skills to
233 siCCM2 HUVECs, we tested the ability of these cells to degrade ECM and invade a 3D matrix.
234 To visualize the degradation of the ECM, siRNA transfected HUVECs or HBMVECs were

235 cultured overnight on fluorescent gelatin. siNT ECs barely degraded the gelatin as expected for
236 differentiated ECs (Fig 6A, B; Fig S7A, B). Conversely and consistently with their new SASP
237 expression program, siCCM2 ECs degraded the gelatin through scratch zones appearing dark
238 in the fluorescently labelled layer (Fig 6A; FigS7A). The linear shapes of these scratch zones
239 suggested that they were produced under focal adhesions. They were dependent on the activity
240 of MMPs as demonstrated by their complete disappearance upon MMP inhibitor GM-6001
241 treatment (Fig 6A, B). Together with blocking the mechanosensitive assembly of focal
242 adhesions (Fig S5F), Y-27632 and blebbistatin blocked the degradation of gelatin (Fig 6A, B;
243 Fig S7A, B). We previously showed that additional silencing of ROCK1, but not of ROCK2,
244 limits excessive focal adhesion formation in siCCM2 ECs(29). Accordingly, only the additional
245 depletion of ROCK1 inhibited ECM degradation by *siCCM2* ECs (Fig 6A, B; Fig S7). Overall,
246 these data show that ROCK1-dependent increase in cell contractility and in cell-ECM adhesive
247 sites together with the overexpression of MMPs are responsible for the acquisition of ECM
248 degradative skills by *siCCM2* ECs.

249 We next tested whether these senescent ECs had acquired invasive skills. Consistently with
250 Maddaluno et al. (18), these cells expressed mesenchymal markers suggestive of an invasive
251 phenotype (Fig S8). Interestingly, the expression of the EndMT markers were returned toward
252 siNT level by the additional silencing of ROCKs (Fig S8). To actually measure their 3D
253 invasiveness, GFP-expressing siRNA treated HUVECs were plated on 3D-degradable
254 polyethylene glycol gels (PEG). Invasive sprouts were imaged after 18 hours and the maximum
255 invasion distance was quantified. siCCM2 HUVECs invaded the 3D gel twice as deep as siNT
256 HUVECs (Fig 6D). Moreover, upon silencing of CCM2, the leading cells consistently detached
257 from the multicellular sprouts and migrated independently (Fig 6C). Additional silencing of
258 ROCK1 or blebbistatin treatment reduced the invasiveness of siCCM2 HUVECs to the level of
259 siNT HUVECs and blocked the detachment of the leading EC whereas silencing of ROCK2
260 enhanced them (Fig 6C, D). This promotion of invasiveness upon ROCK2 additional depletion
261 is consistent with their increased traction forces recorded in a previous work(29).

262
263 CCM lesions are mosaics of mutant and WT ECs as shown in human and murine lesions(9–
264 11). Moreover, it has been shown that CCM3 KO ECs can attract WT ECs *in vivo* in the brain
265 vasculature and *in vitro* on mixed monolayers(11). Therefore, we asked whether senescent
266 siCCM2 HUVECs could also stimulate the sprouting of WT HUVECs when mixed together on
267 the surface of 3D-PEG gels. Strikingly, we observed that RFP-expressing WT HUVECs
268 invaded the gels twice as deep when they were mixed with GFP-expressing siCCM2 HUVECs
269 as when mixed with GFP-expressing siNT HUVECs (Fig 6E, F). Interestingly, this increased
270 invasion was significantly reduced when WT HUVECs were mixed with siCCM2+siROCK1
271 HUVECs but remained unchanged when they were mixed with siCCM2+siROCK2 HUVECs
272 (Fig 6E, F).

273 Altogether, our results show that senescent siCCM2 HUVECs have acquired a ROCK1-
274 dependent capacity to invade the ECM and to sustain the invasion by WT HUVECs.

275

276 **6) ROCK2 mediates the expression of paracrine factors by CCM2-depleted ECs that**
277 **chemo-attract WT ECs and immune cells.**

278 Apart from remodelling their surrounding ECM, senescent cells secrete paracrine factors that
279 have been shown to promote invasiveness of neighbouring cells and engage deleterious
280 inflammatory responses (42). A chronic inflammation is observed at the site of human and
281 murine CCM lesions through the recruitment of activated lymphocytes and monocytes(45). Our
282 transcriptome analysis showed the upregulation of around 30 cytokines upon CCM2 loss that
283 returned towards control level by ROCKs silencing (Fig 5H). These inflammatory cytokines
284 have a broad spectrum of targets among immune cells and ECs and bear different roles on cell
285 proliferation, differentiation and chemoattraction as summarized in table S6.

286 We first tested whether these secreted factors would have a long distance paracrine effect
287 allowing the recruitment of WT ECs. We measured the capacity of a media conditioned by
288 siCCM2 HUVECs to chemo-attract serum-starved WT HUVECs through a layer of Matrigel in
289 a modified Boyden chamber measuring the impedance of cells. Remarkably, siCCM2-
290 conditioned media significantly increased the speed of invasion of WT HUVECs through the
291 Matrigel showing that chemo-attractive factors secreted by siCCM2 HUVECs could also attract
292 ECs (Fig 6G). Interestingly, this effect was inhibited by ROCK2 but not ROCK1 additional
293 silencing (Fig 6G). Therefore, siCCM2 HUVECs produce paracrine factors that attract WT ECs
294 in a ROCK2-dependent manner.

295 We then tested whether the loss of CCM2 leads to the secretion of chemo-attractive cues for
296 immune cells. Thus, we measured the capacity of media conditioned by siCCM2 HUVECs or
297 HBMVECs to chemo-attract IMAC, an immortalized macrophage cell line in a modified
298 Boyden chamber as above. Almost no transmigration of macrophages was observed in the case
299 of siNT-conditioned media (Fig 6H; Fig S7C). Strikingly, siCCM2-conditioned media
300 provoked a rapid transmigration of the macrophages suggesting that siCCM2 ECs had secreted
301 chemo-attractive factors that siNT HUVECs had not (Fig 6H; Fig S7C). Importantly, this
302 chemo-attraction was inhibited when ROCK2 but not ROCK1 was additionally silenced (Fig
303 6H; Fig S7C). Therefore, siCCM2 ECs secrete paracrine factors that can attract immune cells
304 such as macrophages. Moreover, these chemo-attraction skills depend on ROCK2.

305 Overall, these results demonstrate for the first time to our knowledge that, when CCM2 is lost,
306 ECs cultured *in vitro* undergo a SASP that is driven by the cytoskeletal and molecular disorders
307 provoked by ROCKs dysfunctions. A major consequence of this SASP is a profound ROCKs-
308 dependent remodelling of the microenvironment leading to the recruitment of wild-type ECs
309 and immune cells.

310 **Discussion**

311 Many cellular pathways are dysregulated in CCM-deficient ECs and it has been difficult to
312 propose a mechanistic model that could take into account all these aspects. In these cells,
313 transition in cellular fate(18) upon morphological and cytoskeletal changes during the loss of
314 cell-cell junctions(19,29,46) are associated with overproduction of ROS(16), decreased
315 autophagy(17), secretion of metalloproteases(12–14) or cytokines(15) and increased
316 integrin(19,20), p38(21), MEKK3/KLF2(20,22,23) and TLR4(24) signaling. The first
317 breakthrough of this current study is to show, for the first time to our knowledge, that CCM2-
318 deficient ECs engage *in vitro* towards a Senescence-Associated Secretory Phenotype (SASP)
319 (Fig 7). Multiple senescent traits were validated such as a transcriptomic SASP signature (Fig
320 1D), high lysosomal SA- β -galactosidase activity, an upregulation of CDK inhibitors, a cell
321 cycle blockage in G1 phase, the presence of SAHF along with the secretion of chemo-attractant
322 factors (Fig 2). Moreover, we show that the functional consequence of this SASP is an acquired
323 ability of CCM2-deficient ECs to invade the ECM and recruit wild-type ECs and immune cells
324 (Fig 6 G-H). Whereas the absence of *in vivo* data is a limitation to our work, our studies on pure
325 and mixed populations of CCM2-depleted and WT ECs have brought up new hypotheses on
326 the mechanisms underlying the mosaicism of the CCM lesions by proposing the juxtacrine (Fig
327 6E) and paracrine (Fig 6F, G) attraction of WT ECs and immune cells by mutant ECs. These
328 hypotheses will have to be tested *in vivo*.

329

330 The SASP is characterized by cell growth arrest, widespread changes in chromatin organization
331 and gene expression(36). These changes also include the secretion of numerous pro-
332 inflammatory cytokines, chemokines, growth factors and proteases(43). Acute senescence is
333 beneficial in development, tissue regeneration or cancer through the clearance of senescent cells
334 by the immune system(47). Chronic senescence triggers chronic inflammation that can on the
335 contrary favour age-related diseases including cancer, fibrosis, cardiovascular diseases, type 2
336 diabetes, osteoarthritis or neurological disorders(33). Indeed, the long-term secreted pro-
337 inflammatory factors promote cell proliferation, microenvironment remodelling, angiogenesis
338 and inflammation in a paracrine manner. Remarkably, decreased autophagy(48), ROS
339 overproduction(49), P38 MAPK(50), KLF2/4(51,52) and TLR4(53) signaling pathways have
340 all been involved in the induction of the SASP either through regulation of CDK inhibitors or
341 through the NF κ B or C/EBP β -driven expression of cytokines. Therefore, we propose that each
342 of the dysregulated features of CCM-deficient ECs represents a different facet of the same
343 cellular state. Future research should help reconstructing the complex chronology of the
344 different events in the framework of this SASP. This could help identifying key therapeutic
345 targets for either single or combinatorial drug treatments to block at their root the defective
346 molecular pathways involved in the CCM2 disease. Senolytic drugs and drugs targeting the
347 SASP *per se* are currently under pre-clinical trials for cancer therapy or other age-related
348 diseases(33). They could be tested to prevent the formation and expansion of CCM lesions. A
349 recent study strongly supports that premature aging of the neurovascular system could be the
350 cause of CCM disease by showing that aging and CCM brains share common dysregulated
351 features including impaired endothelial barrier function, inflammation, 320 DEGs, plasma
352 molecules and imaging biomarkers (54).

353

354 A third hit corresponding to an oncogenic mutation on PI3K was recently detected in human
355 CCM lesions that adds to the somatic loss-of-function mutation on the *CCM* genes (55).
356 Interestingly, cellular senescence is a well-known response to oncogenic stress which arrests
357 the aberrant proliferation of the cancerous cell(36). The expansion of ECs through several
358 passages in our *in vitro* experiments may have mimicked the oncogenic stress occurring *in vivo*.
359 Importantly, it has allowed the discovery of the increased sensitivity of KRIT- and CCM2-
360 depleted ECs to senescence. Differently from KRIT and CCM2, the loss of CCM3 was actually
361 shown to preserve primary ECs from replicative senescence *in vitro*(56). We found that CCM3
362 depletion does not at least increase the sensitivity of the ECs to replicative senescence (Fig 3).
363 These differences in sensitivity to senescence may explain the different time courses of CCM
364 diseases. Premature senescence of KRIT- or CCM2-deficient ECs after a first phase of clonal
365 proliferation may lead to the progressive maturation of lesions through recruitment of WT ECs
366 and to the late onset of the CCM1/2 disease. Differently, a more prolonged phase of clonal
367 proliferation of CCM3-deficient ECs may trigger the formation of aggressive lesions at early
368 age. Using the confetti reporter system in *Ccm1/2* mouse models made compatible with SA- β -
369 galactosidase staining would help testing the occurrence of a SASP *in vivo*, its time course and
370 allow the comparison with the *Ccm3* model.

371

372 The second major breakthrough of this study is that dysregulated ROCKs are involved in the
373 onset of this SASP in our *in vitro* endothelial models. The fact that cellular mechanical defects
374 can provoke senescence is an emerging area of investigations(57). We previously showed that
375 upon the loss of CCM1/2, a vicious cycle sets up between aberrant ECM remodelling and
376 increased intracellular contractility which breaks the permeability barrier of the
377 endothelium(19). Moreover, we demonstrated that placing WT ECs on ECM remodelled by
378 mutant ECs provoke CCM-like morphological defects in these cells(19). Going deeper into
379 mechanistic investigations, we showed that upon the loss of CCM2, ROCK2 is delocalized
380 from VE-cadherin junctions while ROCK1 gets over activated, enhancing the polymerization
381 of β 1 integrin-anchored stress fibers(29). Here we uncover for the first time that, beyond their
382 role in the disruption of the endothelial monolayer architecture, dysregulated ROCKs are
383 instrumental in the *in vitro* transition of CCM2-depleted ECs towards a SASP (Fig 7).

384

385 ROCK2 controls the expression of a very significant fraction of the genes dysregulated upon
386 the loss of CCM2 among which genes involved in senescence, cell cycle and paracrine SASP
387 factors. The tendency to opposite expression profiles in absence of CCM2 or ROCK2 suggests
388 that ROCK2 could gain a broad transcriptional regulatory function when its scaffold CCM2 is
389 lost (Fig 4B). In fact, ROCK2 has already been involved in cellular senescence(58). In this
390 study, the loss of the two ROCK isoforms led to senescence of MEF and was due to the
391 downregulation of CDK1, CKS1 and cyclin2A. Intriguingly, we observe a similar
392 downregulation of these genes in HUVECs when CCM2 is lost (Fig S3), but contrary to this
393 other study, their expression is rescued by the additional silencing of ROCK2. This suggests
394 that the presence or absence of CCM2 has could have a strong influence on the transcriptional
395 activity of ROCK2. KRIT is found at cell-cell junctions and shuttles between the cytoplasm
396 and the nucleus(59,60). We previously showed that it acts as a scaffold to recruit ROCK2 at

397 VE-cadherin dependent-Adherens junctions(29). ROCK2 has also been shown to translocate to
398 the nucleus(61,62). It is therefore possible that KRIT and ROCK2 shuttle together and that
399 KRIT loss impairs ROCK2 nuclear localization. Future mechanistic studies will be necessary
400 to understand whether and how ROCKs interplay with the CCM complex to modulate ROCK2
401 nucleocytoplasmic shuttling and its regulatory functions on gene expression.

402

403 We previously showed that ROCK1 overactivation allows CCM2-depleted HUVECs to exert
404 stronger traction forces on the ECM (19,29). Here we show that ROCK1 enables the
405 mechanosensitive degradation and invasion of the ECM by the senescent CCM2-depleted ECs
406 and by neighbouring WT ECs. Since ECs pull on their microenvironment to invade it(63), it is
407 likely that these stronger forces are responsible for enhanced invasion. Further investigations
408 will help to better understand the molecular events at play. Like ROCK2, ROCK1 affects gene
409 expression downstream of CCM2 though to a lesser extent. Moreover, inhibition of the myosin
410 motor or silencing of ROCK1 is enough to block senescence. These data suggest that cross talks
411 may exist between the contractility of the acto-myosin cytoskeleton and the transcription of
412 genes involved in SASP. Indeed, mechanosensitive transcription factors and/or epigenetic
413 factors could have their activity affected by the CCM/ROCKs-dependent increase in acto-
414 myosin contractility. As such, it is known that F-actin polymerization controls the shuttling of
415 MRTF to the nucleus and that ROCK2 is required for YAP nuclear localization (64,65).
416 Moreover, ROCK2 binds to and phosphorylates p300 increasing its acetyltransferase
417 activity(61). The mechanosensitive transcription factors KLF2 and KLF4 have a causative role
418 in the pathogenesis of the CCM disease(13,20,23). Intriguingly, KLF2/4 transcriptional
419 activities are modified by acetylation by p300(66,67) and the anti-inflammatory function of
420 KLF2 requires p300(66). The regulation of p300 by ROCK2 may control in turn KLF2/4 anti-
421 inflammatory activities. Future investigations should unravel how ROCK1 and ROCK2
422 cooperate to regulate chromatin organization and gene expression through the control of
423 mechanosensitive transcription machineries.

424

425 Overall, this study demonstrates that CCM2-deficient ECs undergo a SASP *in vitro* that is
426 driven by the cytoskeletal, transcriptional and molecular disorders provoked by ROCKs
427 dysfunctions. Remarkably, this discovery unifies all the known dysregulated traits of CCM1/2-
428 deficient ECs into a unique cellular state that could, if validated *in vivo*, trigger the remodelling
429 of a microenvironment favourable for the chronic recruitment of wild-type ECs and immune
430 cells to the site of the lesion. These *in vitro* observations open new avenues for mechanistic *in*
431 *vivo* studies on the dynamics and penetrance of the CCM disease as well as for exploring new
432 therapies.

433 **Material and Methods**

434 **Cell culture and transfections:**

435 Pooled HUVECs (human umbilical vein endothelial cells) and HBMVECs (human brain
436 microvascular endothelial cells) were obtained from Lonza and Pelobiotech respectively. Upon
437 reception, HUVECs at passage 0 and HBMVECs at passage 2 were expanded over 2 and 1
438 passage respectively in collagen 1 (from rat tail, BD) coated flasks in complete EGM-2 medium
439 (HUVECs) or EGM-2MV (HBMVECs) supplemented with 100 U/ml penicillin and 100 µg/ml
440 streptomycin at 37°C in a 5% CO₂ - 3% O₂ humidified chamber. Cells were transfected twice
441 at 24 h-interval with 30 nM siRNA and Lipofectamine RNAi max (Life Technologies, ref.
442 13778-150) according to the manufacturer's instructions in 37°C in a regular 5% CO₂ in a
443 humidified chamber. For double transfections, 30 nM of each siRNA duplexes (Dharmacon
444 smartpool ON-TARGET plus Thermo Scientific) was used; Non-targeting siRNA #1, CCM2;
445 ref. L-014728-01), KRIT; ref. L-003825-00, CCM3; ref. L-004436-00, ROCK1; ref. L-003536-
446 00 and ROCK2; ref. L-004610-00. When required, Blebbistatin, Y-27632 and GM6001 were
447 used at 10 µM final. IMAC (SV40 immortalized mouse bone marrow derived macrophages,
448 gift from B. Py, CIRI, Lyon) were cultured in DMEM supplemented with 10% foetal calf
449 serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂.

450 **RNA sequencing and differential analysis:**

451 One million of siRNA transfected HUVECs were seeded at confluence the day after the second
452 round of transfection in wells of 6-well plates coated with collagen 1 (from rat tail, BD) and
453 cultured for 48h in complete media at 37°C, 5% CO₂. Three independent experiments were
454 performed, each with 6 experimental conditions: siNT, siCCM2, siCCM2+siROCK1,
455 siCCM2+siROCK2, siROCK1 and siROCK2. Total RNA were extracted from HUVECs using
456 the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions.
457 cDNA libraries were prepared with the TruSeq Stranded mRNA Sample Preparation (Illumina)
458 and sequenced on a HiSeq 2500 Illumina platform using single-end 50 basepair reads at the
459 MGX facility (Montpellier). Fastq files were aligned using STAR (2.5.2b) on UCSC hg19
460 genome. The contents of the annotation directories were downloaded from UCSC on: July 17,
461 2015. Bam files were counted using htseq-count (0.11.2.) with option -t exon -f bam -r pos -s
462 reverse -m intersection-strict -nonunique none. Differential analyses were performed with
463 SARTools (1.4.1) (68) using DESeq2 (1.12.3) (69) and default options. P-values were adjusted
464 with Benjamini-Hochberg procedure(35) set for 5% of FDR. Heatmaps using a correlation
465 matrix and boxplots were obtained with custom R script (3.3.0). The raw sequencing data used
466 in this study are available in the National Center for Biotechnology Information's Gene
467 Expression Omnibus (GEO) database and are accessible through GEO series accession number
468 (pending).

469

470 **Data Availability:** The raw data (FastQ files) and processed data (count files) are deposited in
471 the Gene Expression Omnibus database under ID code GEO: GSE165406, token
472 gnujmwcdjohhkb (for reviewers). All data supporting the findings of this study are available
473 from the corresponding author on reasonable request.

474

475 **Bioinformatics analyses:**

476 Gene ontology analyses on DEGs in siCCM2 condition (fold change [FC] ≥ 2 ; $P < 0.05$ with
477 FDR corrected using Benjamini Hochberg method (35)) were made with PANTHER version
478 15,0 Released 2020-02-14 using slim cellular components and biological processes. Enriched
479 pathways analyses were conducted with Reactome. DEGs ($P < 0.05$, 5% FDR) rescued by
480 additional silencing of ROCK1 or ROCK2 were recovered at the union of Venn diagrams of
481 DEGs in siCCM2 vs siNT and DEGs in siCCM2 vs siCCM2+ROCK1 or ROCK2 respectively.
482 Gene Set Enrichment Analyses were conducted using GSEA software from Broad Institute,
483 UC San Diego(70).

484 **Quantitative RT-PCR:**

485 Purified RNA (1 μg) were reverse transcribed using the iScript Reverse Transcription Supermix
486 (Biorad). Quantitative real-time PCR (Q-PCR) was performed with iTaq Universal SYBR
487 Green Supermix (Biorad) in a 20 μl reaction on a thermal cycler (C-1000 Touch; Bio-Rad
488 Laboratories). Product sizes were controlled by DNA gel electrophoresis and the melt curves
489 were evaluated using CFX Manager (Bio-Rad Laboratories). A total of three housekeeping
490 genes were selected for their stability in HUVECs or HBMVECs under our experimental
491 conditions, using the three analytical software programs, geNorm, Normfinder and Bestkeeper
492 (71,72). We used the relative expression software tool CFX Manager for relative quantification,
493 and normalization was achieved using a normalization factor from all reference genes(71). The
494 mean of three technical replicates was calculated per biological replicate.

495 **Immunofluorescence staining:**

496 HUVECs or HBMVECs were seeded at 5×10^4 cells or 2×10^5 cells in 24-well plates on
497 coverslips coated with 10 $\mu\text{g}/\text{ml}$ fibronectin (from human plasma, Sigma Aldrich) and
498 incubated overnight in complete EBM-2 medium. Cells were fixed with 4% PFA,
499 permeabilized with 0.2% Triton X-100, and incubated with anti-activated $\beta 1$ integrin clone
500 9EG7 ((BD Biosciences, 1/200), anti-Ki67 (AN9260 Millipore, 1/200), anti-HIRA (WC119,
501 Millipore, 1/200) and anti β -catenin clone 8E7 (Merck, 1/2000). After rinsing, coverslips were
502 incubated in Goat anti-Mouse or anti-Rabbit IgG (H+L) highly cross-adsorbed secondary
503 antibody, Alexa Fluor conjugated AF 488, AF 546, AF 647 (Invitrogen, 1/1000) and phalloidin
504 conjugates with Atto 647 (Sigma, 1/2000). The coverslips were mounted in Mowiol/DAPI
505 solution and imaged on an epifluorescent Axiomager microscope (Zeiss) at 63X magnification.

506 **SA-B-Galactosidase staining:**

507 HUVECs or HBMVECs were seeded 48 hours after the second siRNA transfection at a density
508 of 5×10^4 in 24-well plates coated with 10 $\mu\text{g}/\text{mL}$ fibronectin and incubated overnight in
509 complete EBM-2-medium. Senescence-Associated β -galactosidase activity was assessed using
510 a SA- β -galactosidase staining kit according to the manufacturer's instructions (Cell Signaling).
511 Positive cells were counted manually out of more than 100 cells total.

512 **BrdU assay:**

513 HUVECs were seeded 48 hours after the second siRNA transfection at a density of 2×10^5 cells
514 in 6-well plates coated with $100 \mu\text{g}/\text{mL}$ collagen 1 and incubated overnight in complete EBM-
515 2 medium. The BrdU assay was performed with the BD Accuri C6 flow cytometer using the
516 APC-BrdU flow kit according to the manufacturer's instructions (Cell Signaling).

517 **xCELLigence proliferation assay:**

518 Proliferation assay was performed using the xCELLigence Real-Time Cell Analysis (RTCA)
519 DP instrument in combination with E-plate 16 (ACEA Biosciences) coated with $100 \mu\text{L}$ of
520 $100 \mu\text{g}/\text{mL}$ collagen 1 (from rat tail) for 30 min at 37°C and washed 2 times with PBS 1X. 40
521 μL of complete EBM-2 medium was added and baseline without cells was made with RTCA
522 software. SiRNA transfected HUVECs or HBMVECs were seeded at 5-10% confluence 48h
523 after the second round of transfection in $100 \mu\text{L}$ of complete EBM-2-medium. Impedance
524 measurements were recorded every 5 min during 24h. Impedance was normalized at 4 hours
525 after seeding to eliminate the contribution of cell spreading and adhesion to the signal. Slope
526 measurement was performed between 4h and 24h.

527 **Matrigel invasion assay:**

528 This assay was performed using the xCELLigence RTCA DP instrument in combination with
529 CIM-Plate 16 (ACEA Biosciences). A layer of 3.3% of growth factor reduced Matrigel
530 (Corning) diluted with EBM basal media was poured on ice in the upper chamber and incubated
531 overnight at 37°C for polymerization. $40 \mu\text{L}$ basal EBM media was added to the upper chamber
532 and the baseline made after equilibration at 37°C . After setting the baseline, 3×10^4 WT
533 HUVECs were added to the upper chamber. The conditioned media from siRNA transfected
534 cells was recovered 48 h after the second round of transfection and centrifuged at 12000 rpm
535 for 5 min and $160 \mu\text{L}$ was added to the lower chamber of the CIM-plate. After one hour of
536 equilibration at 37°C , impedance was measured every 3 minutes for 24 hours.

537 **Chemo-attraction assay:**

538 This assay was performed using the xCELLigence Real-Time Cell Analysis (RTCA) DP
539 instrument in combination with CIM-Plate 16 (ACEA Biosciences). After setting the baseline,
540 3×10^5 IMAC were added in $100 \mu\text{L}$ of basal EBM in the upper chamber and 48h-conditioned
541 media from siRNA transfected HUVECs or HBMVECs added to the lower chamber of the
542 CIM-plate. After 30 min equilibration at 37°C , impedance was measured every 3 minutes for
543 24 hours.

544 **Gelation degradation assay:**

545 Coverslips in 24 well-plates were coated with gelatin-Alexa488 dye as previously described
546 (73). SiRNA transfected HUVECs or HBMVECs were seeded 48h after the second siRNA
547 transfection at 5×10^4 /well and incubated in OptiMEM medium overnight at 37°C in a 5% CO_2
548 incubator, fixed with 4% PFA, and washed with PBS twice. For a quantitative analysis of the
549 degradative skill of siRNA transfected cells, 5 images per condition were acquired over the
550 entire coverslip with an epifluorescent Axiomager microscope (Zeiss) at 40X magnification,
551 and were converted to binary images using B&W thresholding on ImageJ. The total area of the

552 black zones corresponding to the total area of degradation of the fluorescent gelatin was
553 measured.

554 **3D-PEG invasion assays:**

555 Poly-ethylene glycol (PEG) hydrogels were prepared on ice in EBM-2 complete media by
556 combining an MMP-sensitive peptide modified PEG precursor (8-arm 40kDa,(74)) at 1.5%
557 polymer concentration, 50 μ M Lys-RGD peptide (Pepmic), and 1 μ M sphingosine-1-phosphate
558 (S1P, Sigma-Aldrich). The hydrogels were enzymatically crosslinked using a reconstituted and
559 thrombin-activated Factor XIII (Fibrogammin, CSL Behring), prepared as previously described
560 (75)) at 10% of total hydrogel volume. A 20 μ L volume of the hydrogel suspension was pipetted
561 into a modified imaging chamber (Secure-Seal™ hybridization sealing systems, ThermoFisher
562 Scientific) attached to the bottom of a 24-well plate held vertically (63). The hydrogel was
563 allowed to polymerize for 30 minutes in this orientation at room temperature prior to cell
564 seeding. Depending on the study, a confluent cell monolayer composed of either 5×10^4 siRNA
565 transfected GFP-HUVECs or a 1:1 ratio mixture of siRNA transfected GFP-HUVECs and naïve
566 RFP-HUVECs was allowed to adhere to the PEG meniscus at 37°C, 5% CO₂ for 1 hour and
567 then placed back horizontally with 1mL of EBM-2 complete media. Fixation with 4% PFA in
568 DPBS was performed after 24 hours. Sprouts invading the PEG hydrogel were imaged using a
569 Leica SP8 inverted confocal microscope with an HC PL APO 10x, 0.4 numerical aperture dry
570 objective to obtain image stacks at 1024x1024 pixels with a 50 μ m Z-stack at 1-1.5 μ m Z-
571 spacing. A Z-projection of each image was used to manually quantify invasion distances using
572 the line measurement tool of ImageJ. More than 100 sprouts were analyzed per technical
573 replicate.

574

575 **Statistical tests:**

576 Results were assessed by the Tukey's multiple comparison tests post-ANOVA to compare with
577 control; a 0.5 alpha level was used for all comparisons. Prism software was used to conduct the
578 statistical analysis of all data. $P < 0.05$ was considered to be significant. * $P < 0.05$, ** $P < 0.05$,
579 *** $P < 0.005$. n represents biological replicates.

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593 **Author contributions**

594

595 D.R.V., A.S., H.V.O., E.F. conceived the project and designed experiments; D.R.V., A.S., S.M.,
596 E.P., E.F. performed experiments; F.C., D.R.V., E.F. analyzed the bioinformatical data; O.D.
597 contributed to scientific discussion; P.R. provided reagents; C.A.R., E.F. and H.V.O. provided
598 funding; E.F. wrote the manuscript which has been revised by all authors.

599

600 **Figure legends:**

601

602 **Table S1: Level of expression of DEGs with $\log_2FC > 1$ ($P < 0.05$, FDR corrected using**
603 **Benjamini Horchberg method) in the 6 different conditions of siRNA.**

604 **Table S2: Gene ontology analysis of DEGs in siCCM2 HUVECs with $\log_2FC > 1$ ($P < 0.05$,**
605 **FDR corrected using Benjamini Horchberg method).**

606 **Table S3: Gene sets of Senescence and SASP used for GSEA analyses.**

607 **Table S4: Lists of DEGs with $\log_2FC > 1$ ($P < 0.05$, FDR corrected using Benjamini**
608 **Horchberg method) in siCCM2 rescued by ROCKs.**

609 **Table S5: Gene Ontology Analysis on DEGs with $\text{Log}_2FC > 1$ rescued by ROCKs.**

610 **Table S6: Cytokines upregulated with $FC > 2$ in siCCM2 HUVECs and their biological**
611 **effects on immune and endothelial cells.**

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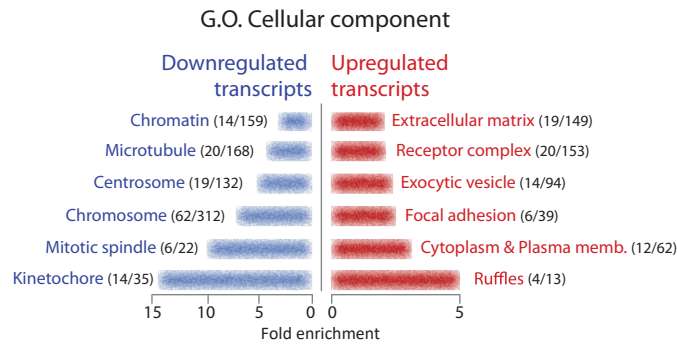
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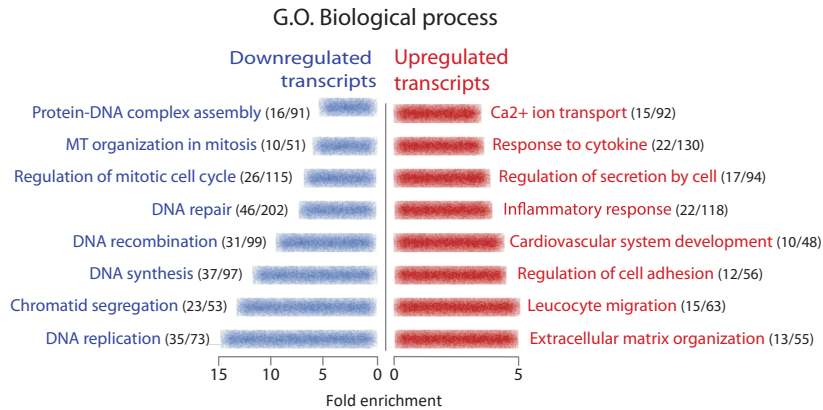
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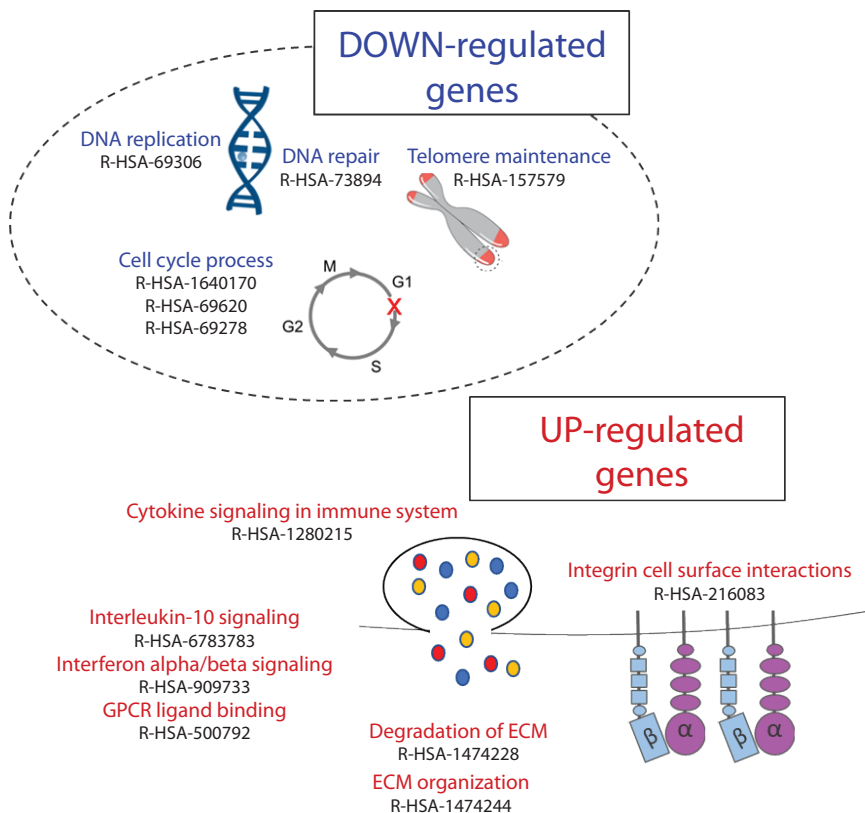
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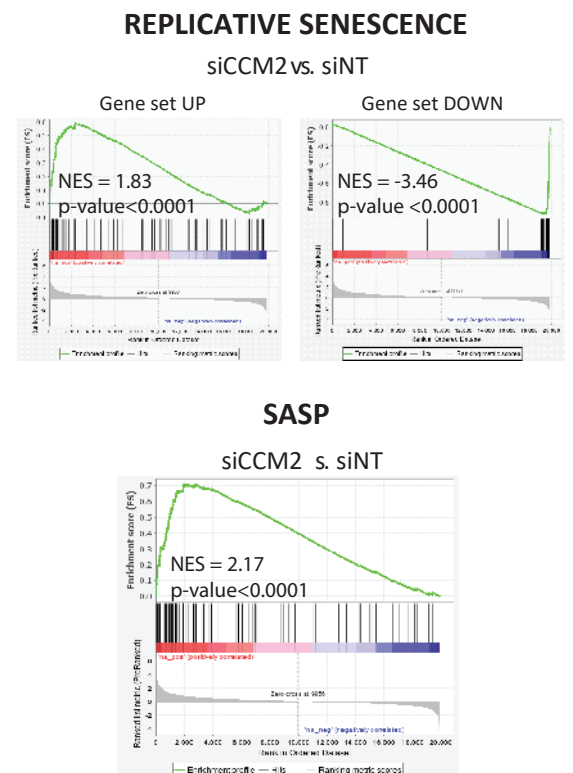


Figure 1: The loss of CCM2 turns on a SASP transcriptomic program.

(A) Gene Ontology enrichment analysis of cellular components in downregulated and upregulated genes in siCCM2 HUVEC compared to siNT HUVEC, bar graphs represent the fold enrichment. (B) Gene Ontology enrichment analysis of biological functions in downregulated and upregulated genes in siCCM2 HUVEC compared to siNT HUVEC, bar graphs represent the fold enrichment. (C) Schematic representation of the of enriched pathways in siCCM2 HUVEC with their reactome identification numbers. (D) GSEA profiles showing a significant normalized enrichment score (NES) of gene sets associated with replicative senescence (37) and SASP (39) in siCCM2 HUVEC transcriptome.

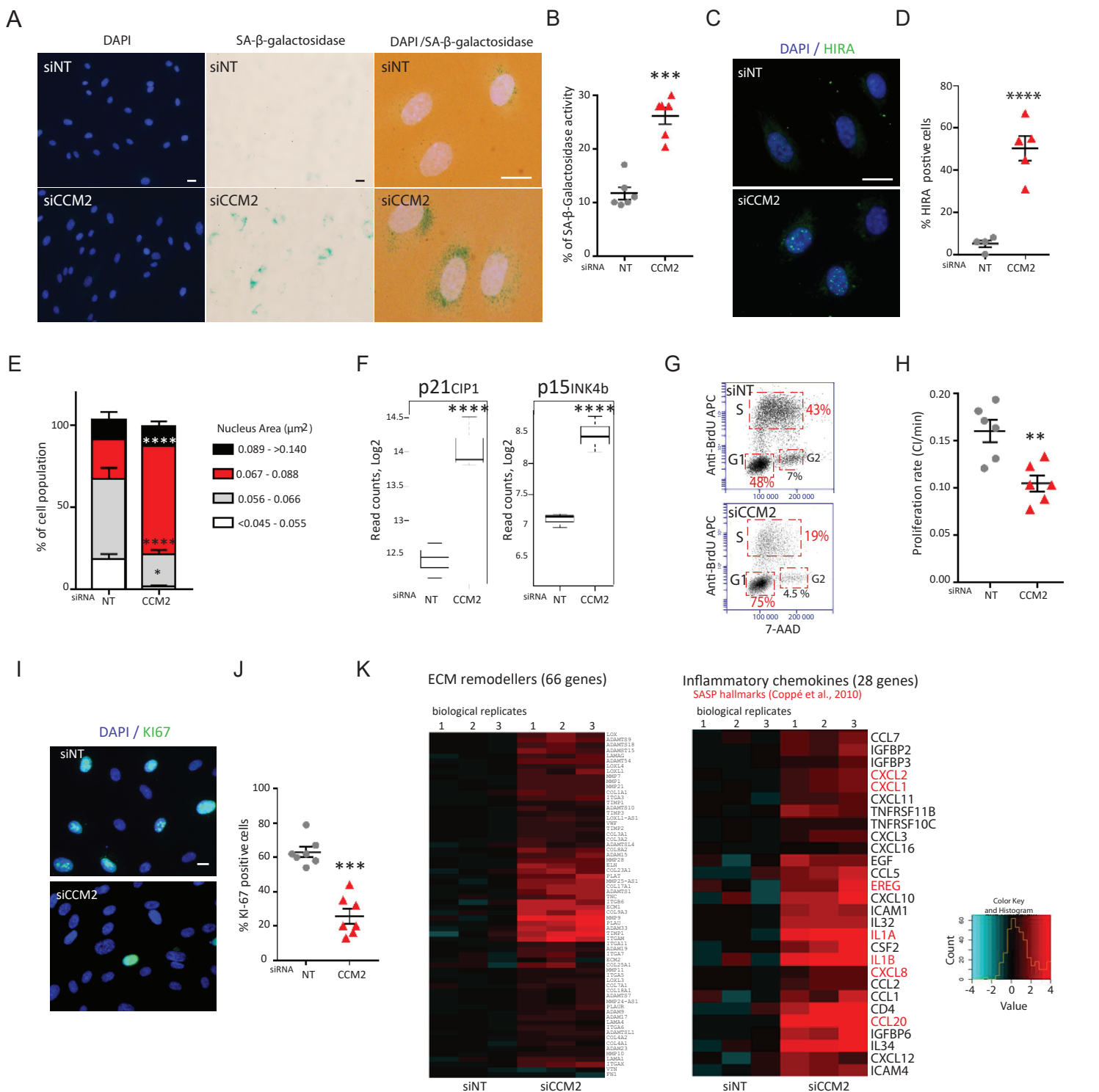


Figure 2: CCM2-depleted EC display hallmarks of SASP.

(A) Representative images of DAPI and SA-βgalactosidase staining of siNT and siCCM2 HUVEC. (Middle) Merge of DAPI and SA-β-galactosidase stainings at higher magnification. (B) Quantification of the % of positive cells for SA-β-galactosidase. Error bars are means \pm SEM from 6 independent experiments. (C) Representative images of merged DAPI and HIRA stainings of siNT and siCCM2 HUVEC. (D) Quantification of the % of HIRA positive cells. Error bars are means \pm SEM from 5 independent experiments. (E) Histogram of the cell population in function of their nucleus area. Error bars are means \pm SEM from 4 independent experiments. (F) Boxplots of the read counts for p21/CIP1 and p15/INK4b mRNA. Error bars are means \pm SEM from 3 independent experiments. (G) Representative plot showing BrdU incorporation as a function of total DNA content. The percentage of cells in each phase of the cell cycle is indicated. (H) Proliferation rate of siRNA transfected HUVEC measured by impedance using XCELLigence. Error bars are means \pm SEM from 4 independent experiments. (I) Representative images of the proliferation marker Ki-67 staining (green) merged with DAPI staining. (J) Quantification of the percentage of cells positive for Ki-67 staining. Error bars are means \pm SEM from 7 independent experiments. (K) Heatmap of expression of ECM remodelling proteins (left) (see Fig S4 for enlarged panel) and of SASP factors (right) over the 2 siRNA conditions, 3 biological replicates per condition. (*) P-value<0.05; (**) P-value<0.005; (***) P-value<0.0005; (****) P-value<0.00005. Scale bars are equal to 10 μm .

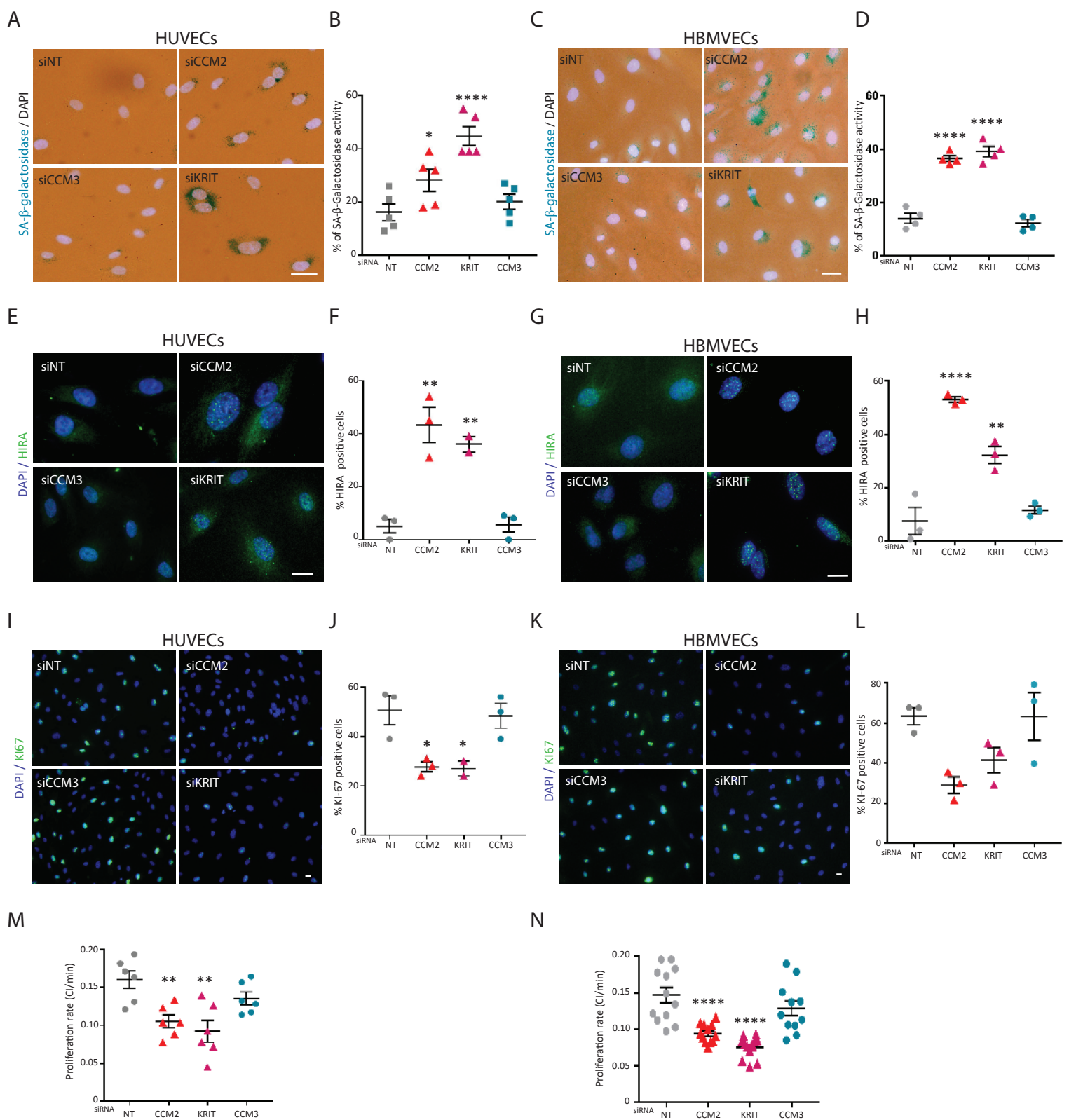
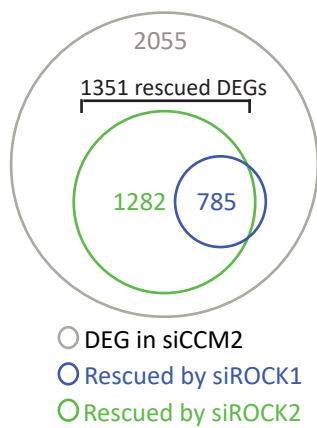


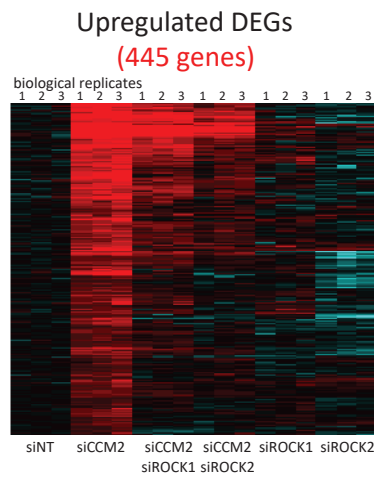
Figure 3: The loss of KRIT similarly to that of CCM2 leads to senescence in ECs whereas the loss of CCM3 does not.

Representative merged images of DAPI and SA-β-galactosidase stainings in HUVECs (A) and HBMVECs (C). Quantification of the % of positive cells for SA-β-galactosidase in HUVECs (B) and HBMVECs (D). Error bars are means ± SEM from 5 (HUVECs) or 3 (HBMVECs) independent experiments. Representative images of merged DAPI and HIRA stainings for HUVECs (E) and HBMVECs (G). Quantification of the % of HIRA positive cells in HUVECs (F) and HBMVECs (H). Error bars are means ± SEM from 3 (2 for KRIT) (HUVECs) and 3 (HBMVECs) independent experiments. Representative images of the proliferation marker Ki-67 staining (green) merged with DAPI staining in HUVECs (I) and HBMVECs (K). Quantification of the percentage of cells positive for Ki-67 staining for HUVECs (J) and HBMVECs (L). Error bars are means ± SEM from 3 (2 for KRIT) (HUVECs) and 3 (HBMVECs) independent experiments. Proliferation rate of siRNA transfected HUVECs (M) and HBMVECs (N) measured by impedance using XCELLigence. Errors bars are means ± SEM from 6 to 12 technical replicates over 3 independent experiments.

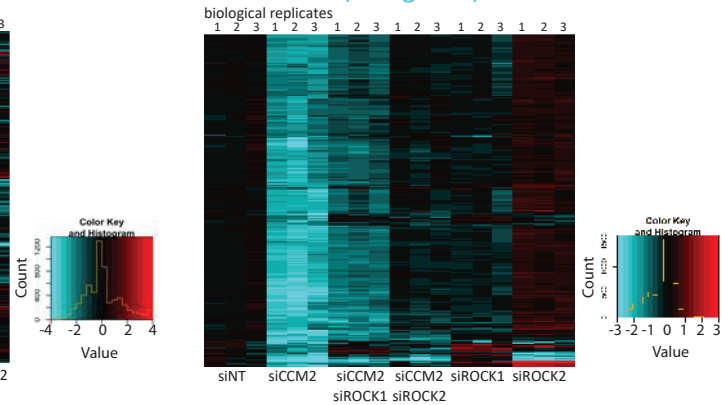
A



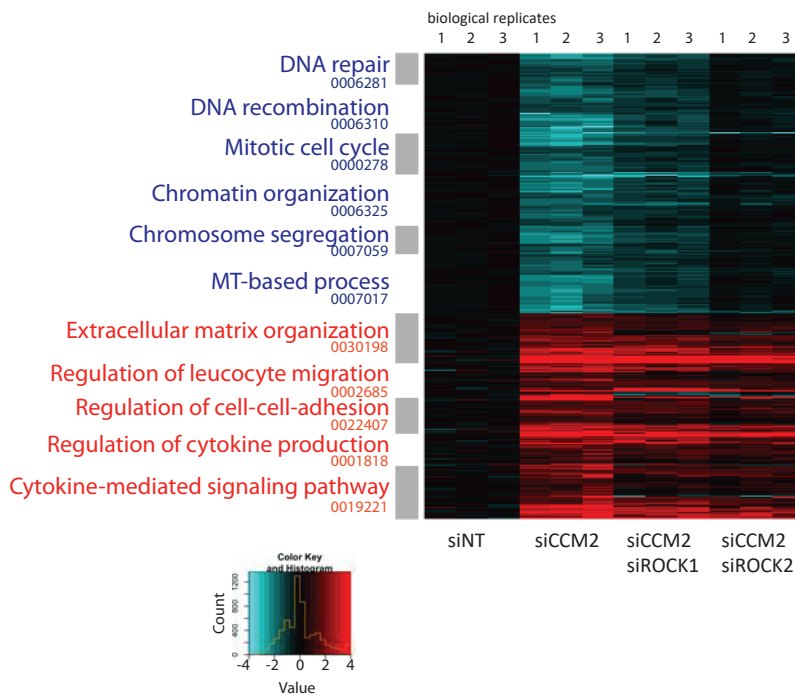
B



Downregulated DEGs (355 genes)



C



D

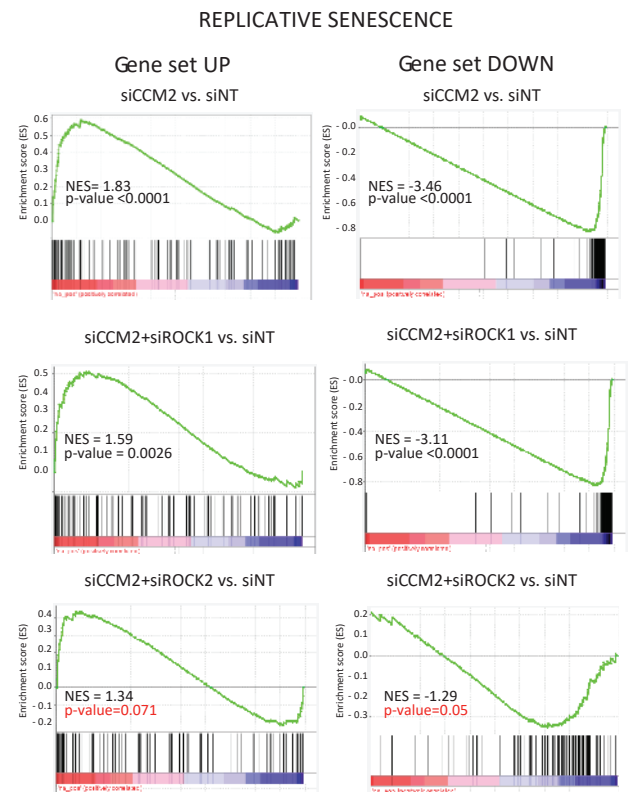


Figure 4: ROCK2 controls the SASP transcriptomic program of CCM2-depleted EC

(A) Venn diagrams showing overlap of DEGs with $FC \geq 2$; $P < 0.05$ in siCCM2 HUVEC (grey) with DEG rescued in siCCM2+siROCK1 (Blue) siCCM2+siROCK2 (Green). (B) Heatmap of the DEGs with $FC \geq 2$; $P < 0.05$ restored in common by ROCK1 and ROCK2. Upregulated (left) and downregulated (right) genes over the 6 siRNA conditions, 3 biological replicates per condition. (C) Clustered heatmap of GO enriched in up- and down-regulated genes in siCCM2 HUVEC. (D) GSEA enrichment plot showing the loss of significant enrichment in replicative senescence signature in siCCM2+ROCK2 but not in siCCM2+ROCK1 HUVEC transcriptome.

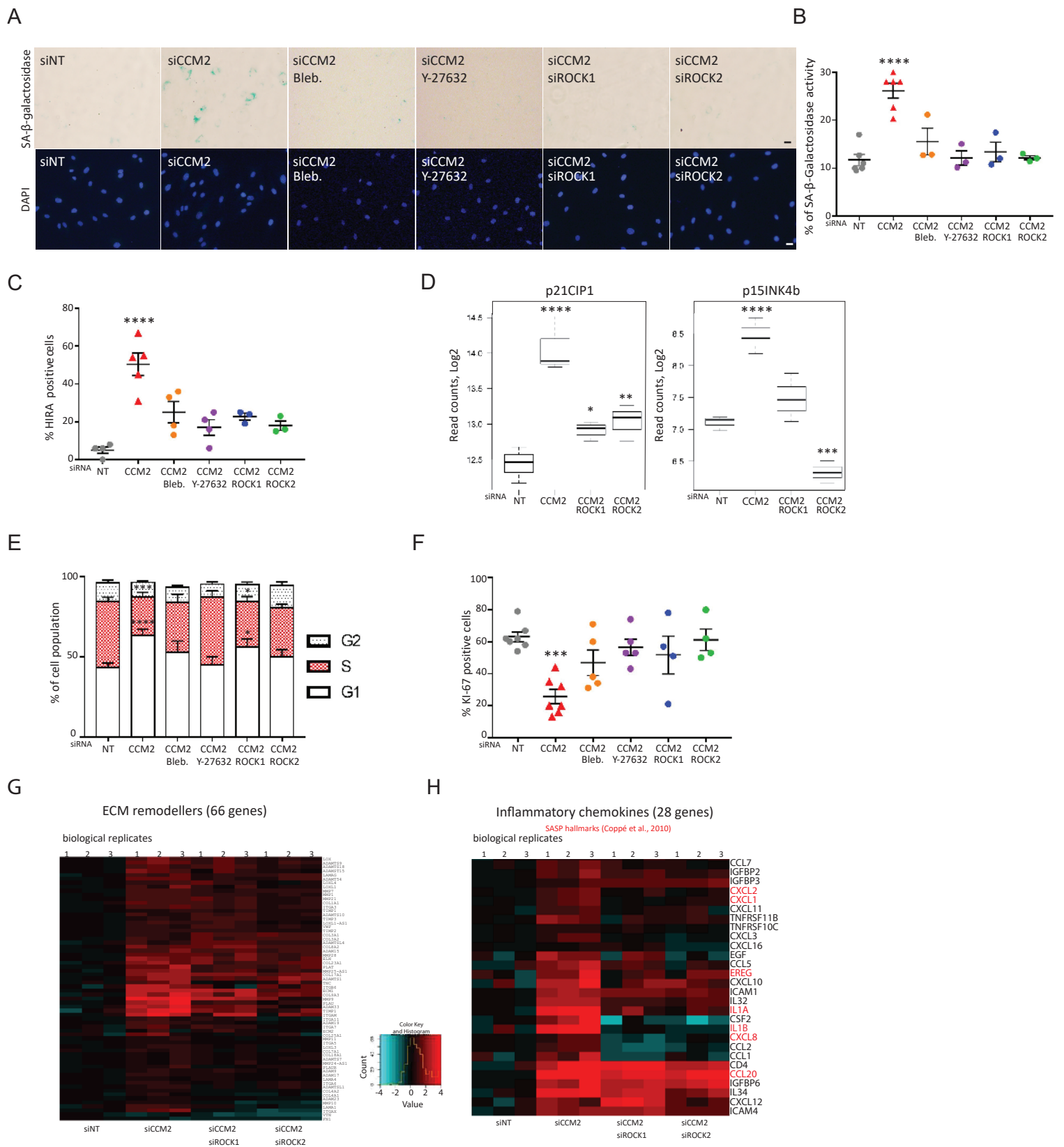
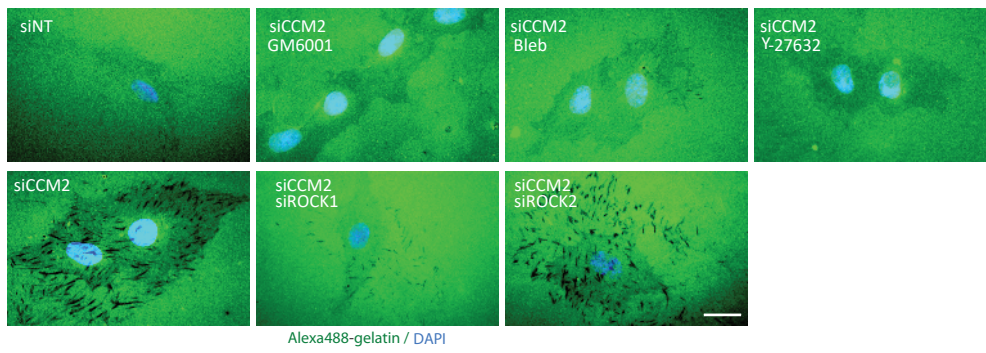


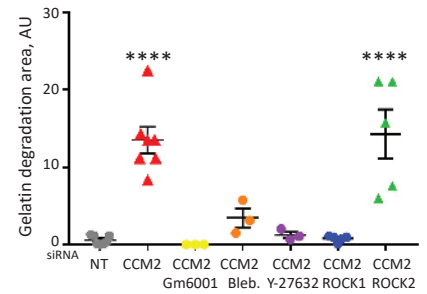
Figure 5: ROCKs dysfunctions induce premature senescence in CCM2-depleted ECs.

(A) Representative images of SA- β -galactosidase and DAPI stainings of siRNA-transfected HUVECs treated or not with blebbistatin or Y27632 at 10 μ M. (B) Quantification of the % of positive cells for SA- β -galactosidase. Error bars are means \pm SEM from 3 independent experiments. (C) Quantification of the % of HIRA positive cells in siRNA-transfected HUVECs treated or not with blebbistatin or Y27632. Error bars are means \pm SEM from 4 (drug treatments) and 3 (ROCKs silencing) independent experiments. (D) Boxplots of the read counts for p21/CIP1 and p15/INK4b after depletion of ROCK1 or ROCK2. Error bars are means \pm SEM from 3 independent experiments. (E) Quantification by BrdU assay of the percentage of cells in each phase of the cell cycle for siRNA-transfected HUVECs treated or not with blebbistatin or Y27632. Error bars are means \pm SEM from 5 (drug treatments) and 8 (ROCKs silencing) independent experiments. (F) Quantification of the percentage of cells positive for Ki-67 staining. Error bars are means \pm SEM from 5 (drug treatments) to 4 (ROCKs silencing) independent experiments. (G) Heatmap of expression of ECM remodelling proteins (left) and of SASP factors (right) over the 4 siRNA conditions, 3 biological replicates per condition. (*) P-value < 0.05; (**) P-value < 0.005; (***) P-value < 0.0005; (****) P-value < 0.00005. Scale bars are equal to 10 μ m. Data for siNT and siCCM2 HUVECs are the same as in figure 2.

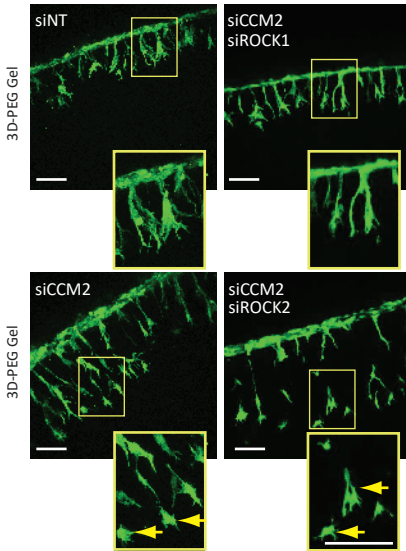
A



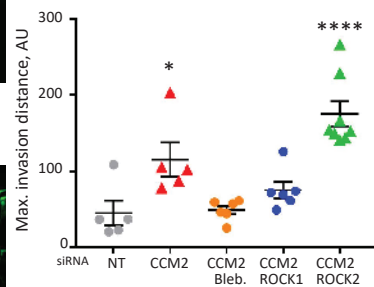
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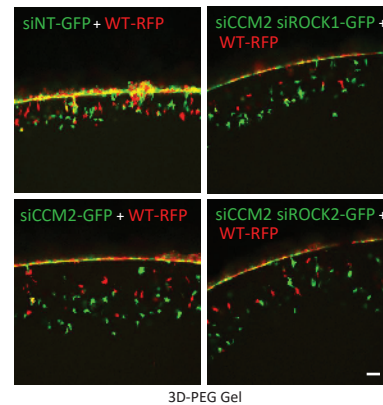
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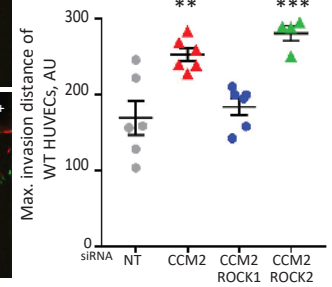
D



E



F



G

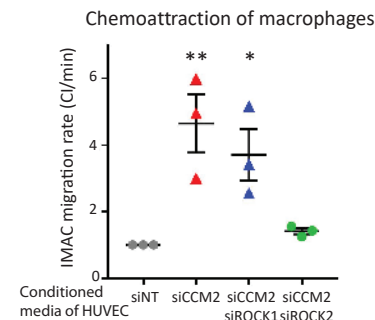
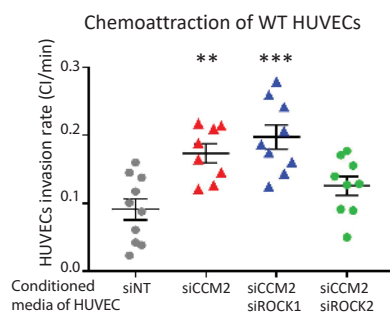









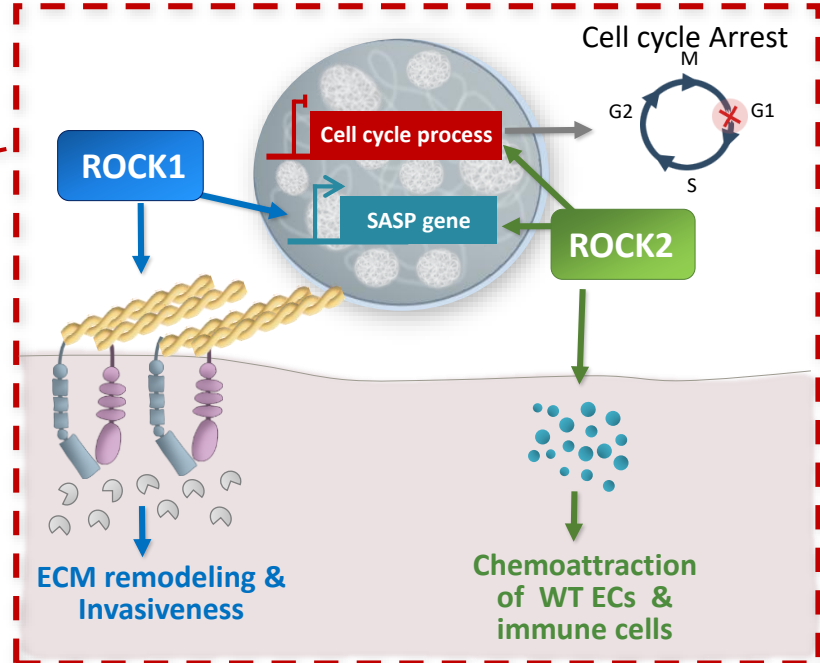
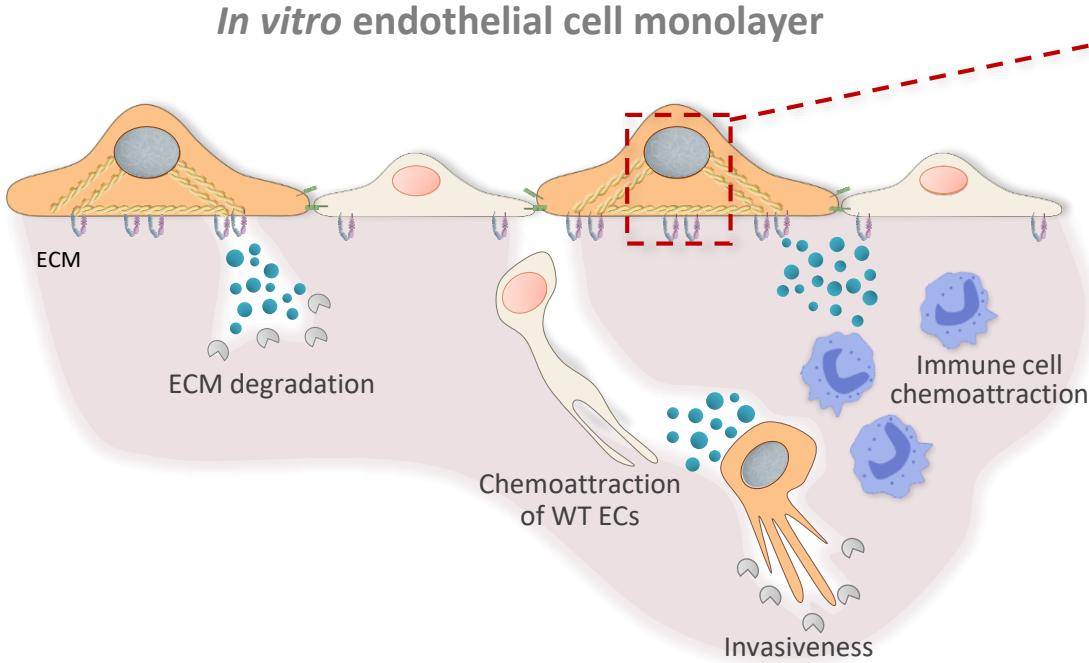
Figure 6: ROCK1 causes ECM degradation and invasion by CCM2-depleted HUVECs and neighbouring WT EC while ROCK2 causes chemo-attraction of WT ECs and macrophages.

(A) Representative images of the degradation of fluorescent gelatin by siRNA transfected HUVECs treated or not with GM6001, blebbistatin or Y-27632. Scale bars, 10 μ m. (B) Quantification of the area of gelatin degradation. Error bars are means \pm SEM from 3 (drug treatments) or 5 (silencing of ROCKs) independent experiments. (C) Representative images of siRNA transfected GFP-HUVECs after invasion of 3D-PEG gels. **Yellow squares are magnified zones of the images. Yellow arrows point at isolated invading cells.** Scale bars, 50 μ m. (D) Quantification of the maximum invasion distance of siRNA transfected HUVECs treated or not with blebbistatin. Error bars are means \pm SEM from 3 independent experiments (2 to 3 technical replicates per condition) for siRNA transfected HUVECs and 2 for blebbistatin treated HUVECs. (E) Representative images of siRNA transfected GFP-HUVECs and RFP WT-HUVECs after invasion of 3D-PEG gels Scale bar, 50 μ m. (F) Quantification of the maximum invasion distance of RFP WT-HUVECs. Error bars are means \pm SEM from 3 independent experiments (2 to 3 technical replicates per condition). (G) Quantification of the rate of invasion of WT HUVECs upon chemo-attraction by conditioned media of siRNA-transfected cells measured in a modified Boyden chamber in real time using xCELLigence. Error bars are means \pm SEM from 3 independent experiments (2-4 technical replicates per condition). (H) Quantification of the rate of transmigration of IMAC macrophages upon chemo-attraction by conditioned media of siRNA-transfected cells measured in a modified Boyden chamber in real time using xCELLigence. Error bars are means \pm SEM from 3 independent experiments.

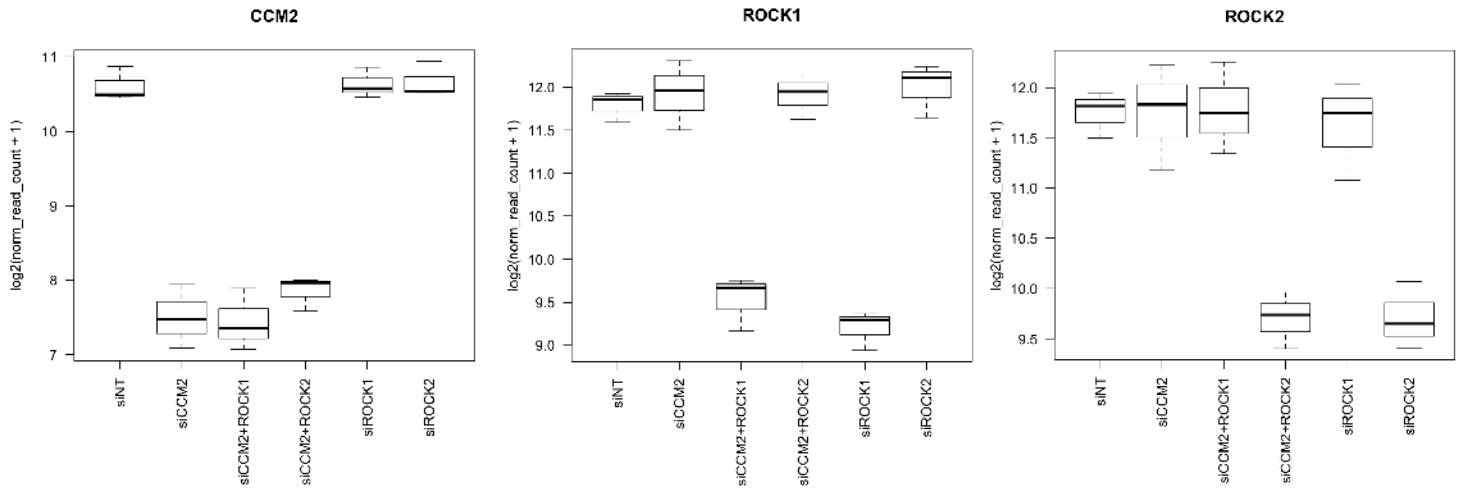
(*) P-value<0.05; (**) P-value<0.005; (***) P-value<0.0005; (****) P-value<0.00005.

ROCKs-dependent Senescence-Associated with Secretory Phenotype

-  WT Endothelial cell
-  CCM2 mutant Endothelial cell
-  Immune cell
-  VE-cadherin
-  Focal adhesion
-  Actomyosin stress fibers
-  SASP factors



A



B

C

FigS1: Validation of the silencing of the target genes by siRNA in HUVECs and HBMVECs

PREMATURE AGING
from Ly DH et al., science 2000

OLD AGING
from Ly DH et al., science 2000

SENESCENCE
from Casella et al, NAR 2019

Gene set UP

Gene set DOWN

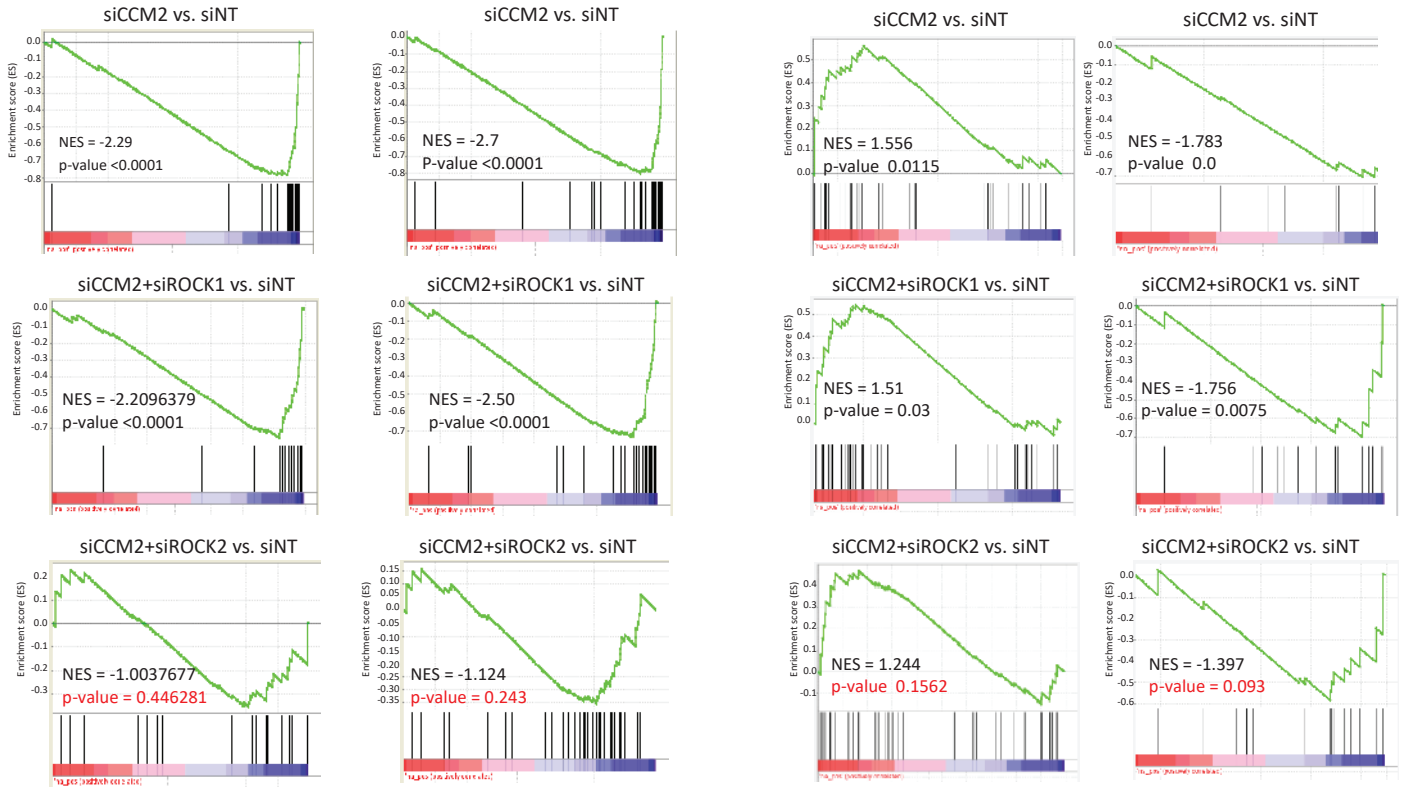
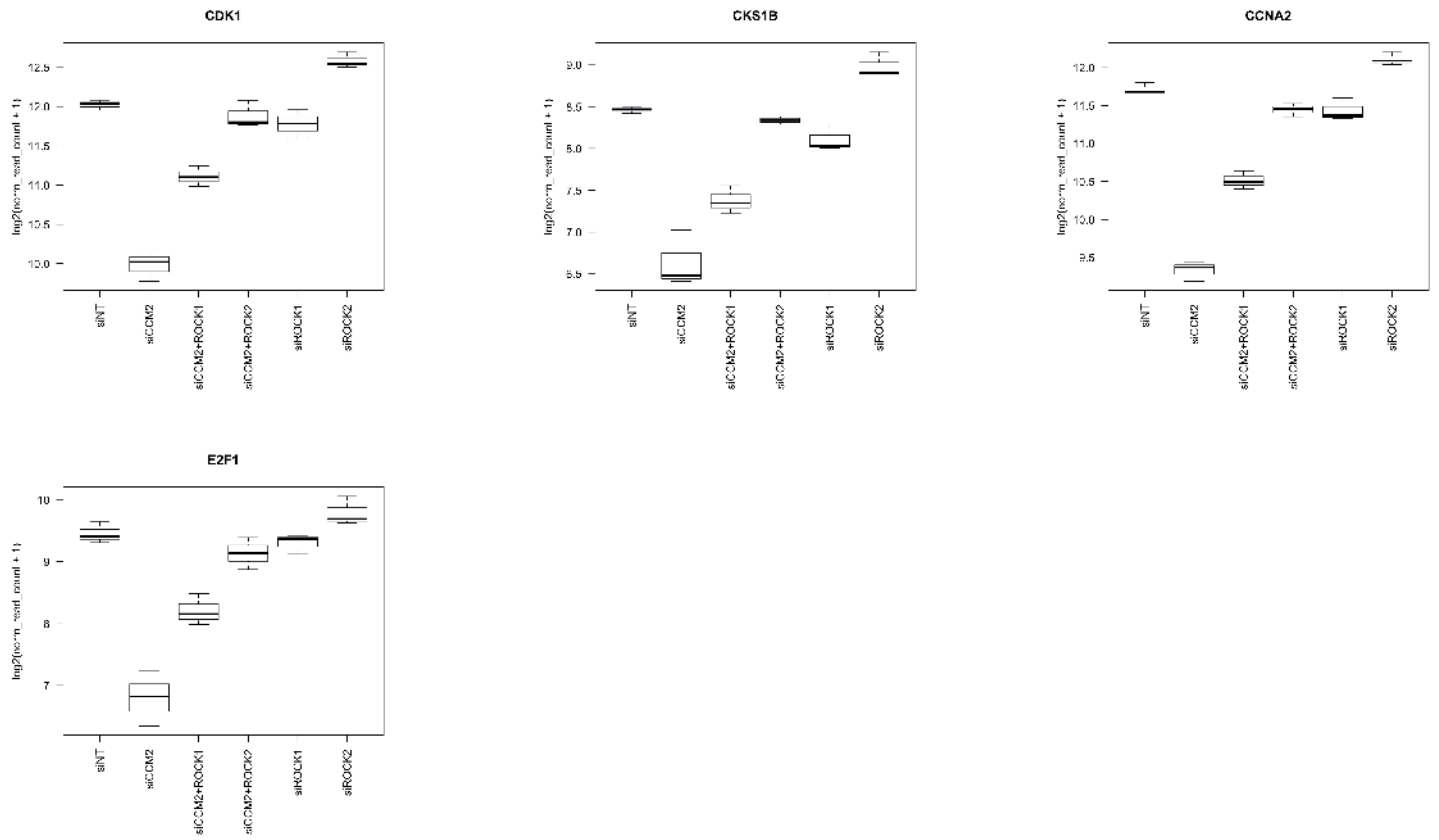


Fig S2: GSEA analyses using different gene sets of senescence.

Comparison of the enrichment in different senescence and SASP signatures(38,40) in siCCM2, siCCM2+ROCK1 and siCCM2+ROCK2.



FigS3: Boxplots of the expression level of cell cycle regulators in the 6 siRNA conditions, 3 biological replicates per condition, as measured by RNA seq.

ECM remodellers (66 genes)

biological replicates

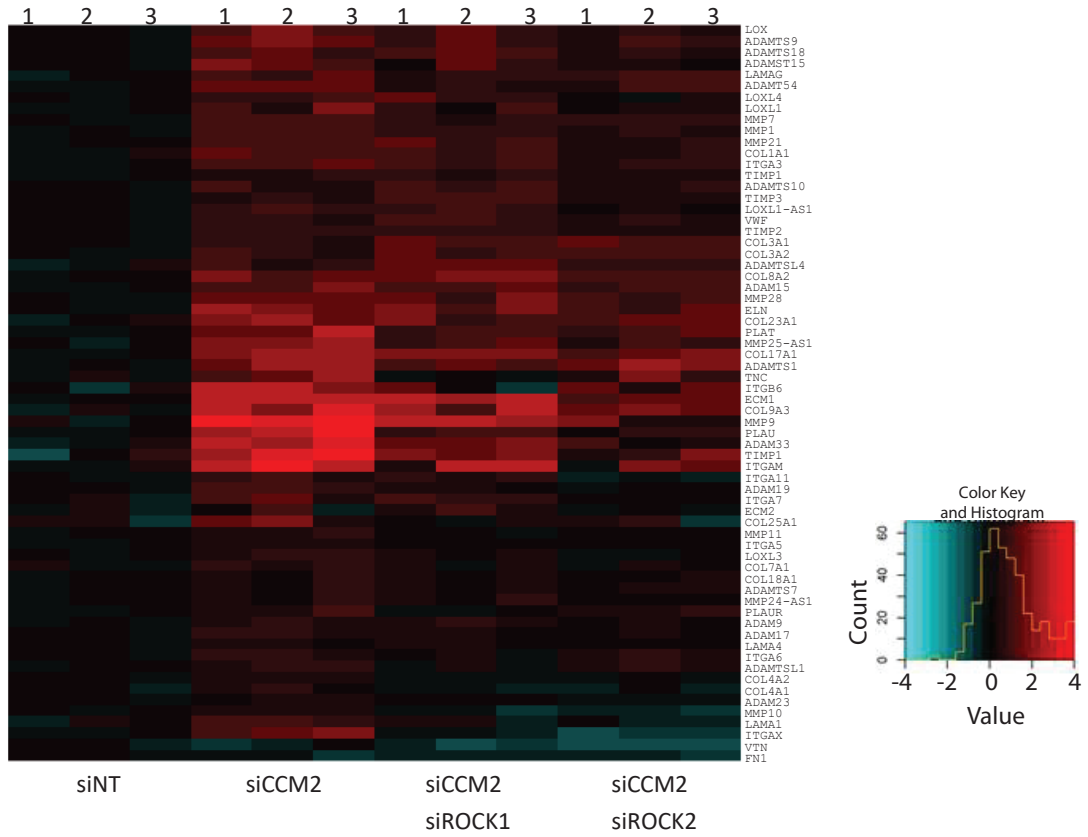
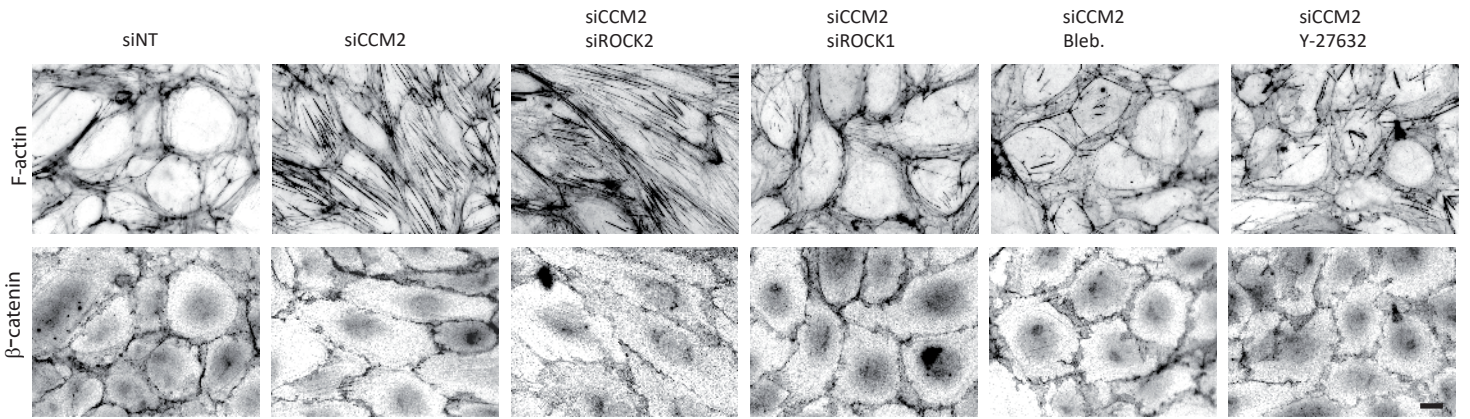


Fig S4 : Heatmap of expression of ECM remodelling proteins over the 4 siRNA conditions, 3 biological replicates per condition.

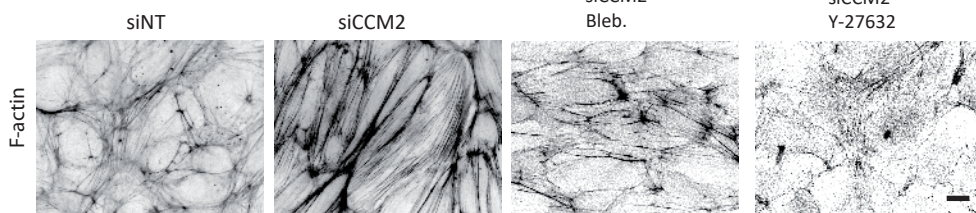
A

HBMVECs

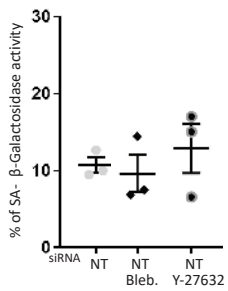


B

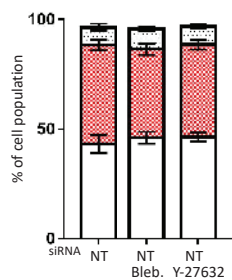
HUVECs



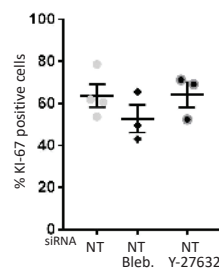
C



D



E



F

β1 integrin

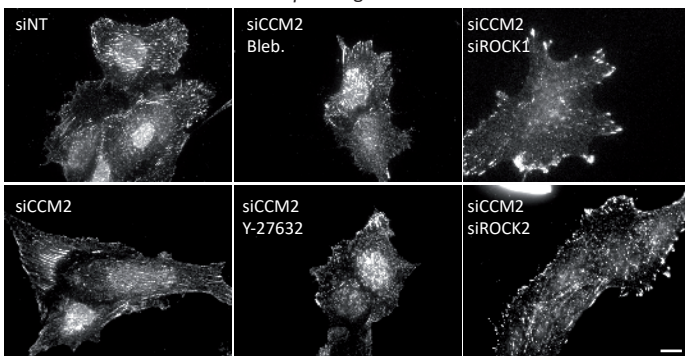


Fig S5: Effects of ROCKs silencing or drug treatments on the acto-adhesive phenotype of siCCM2 HBMVECs and HUVECs and on senescence marks in siNT HUVECs

(A) Representative immunofluorescence images of F-actin and β-catenin stainings in siRNA transfected HBMVECs. Scale bar 10 μm.

(B) Representative immunofluorescence images of the actin cytoskeleton of CCM2 transfected HUVECs treated or not with Blebbistatin or Y-27632, scale bar 10 μm. Effects these drugs on SA-β-galactosidase activity

(C) cell cycle progression (D) and Ki67 staining (E) of siNT HUVECs.

(F) Representative immunofluorescence images of β1 integrin staining in siRNA transfected HUVECs treated or not with Blebbistatin or Y-27632. Scale bar 10 μm

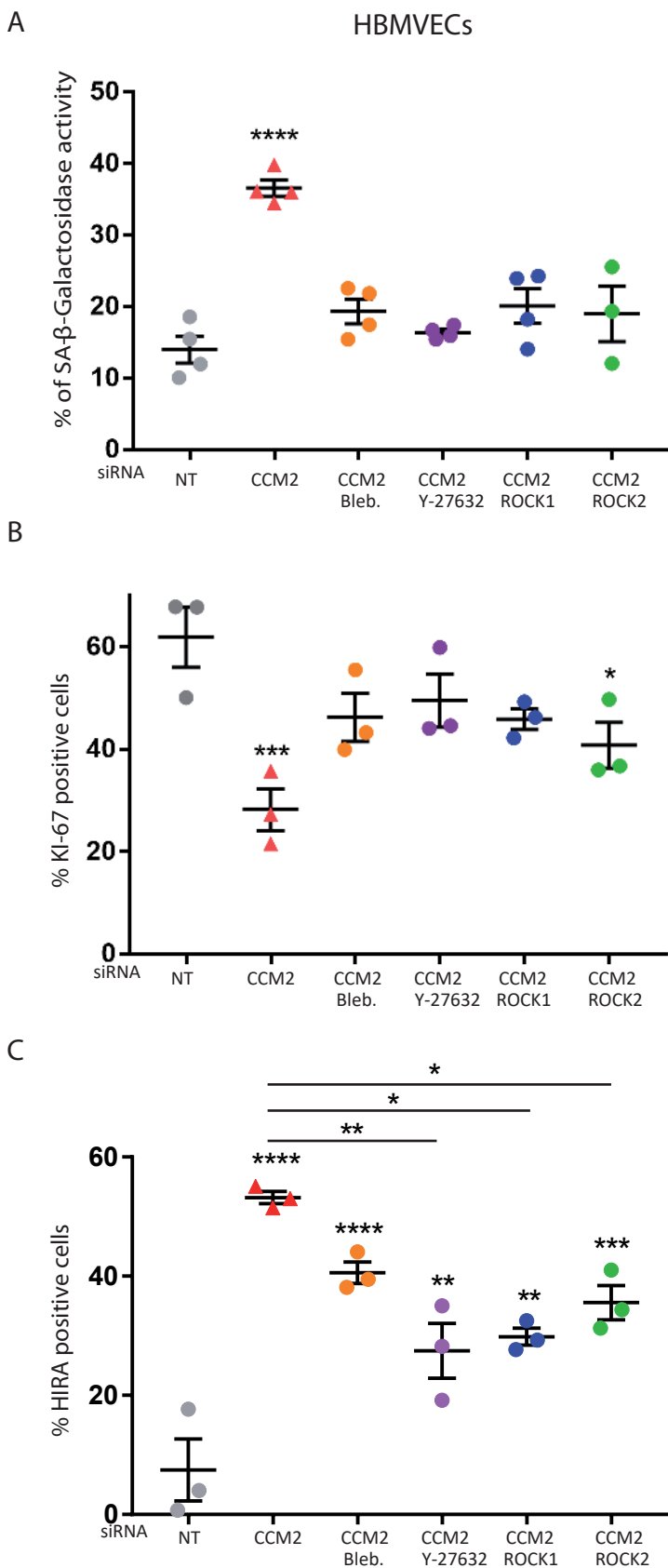


Figure S6: ROCKs dysfunctions induce senescence marks in CCM2-depleted HBMVECs.

(A) Quantification of the % of cells positive for SA-β-galactosidase in siRNA-transfected HBMVECs treated or not with blebbistatin or Y-27632. Error bars are means ± SEM from 3 independent experiments. (B) Quantification of the % of cells positive for Ki-67 staining in siRNA-transfected HBMVECs treated or not with blebbistatin or Y-27632. Error bars are means ± SEM from 3 independent experiments. (C) Quantification of the % of HIRA positive cells in siRNA-transfected HBMVECs treated or not with blebbistatin or Y-27632. Error bars are means ± SEM from 3 independent experiments. (*) P-value<0.05; (**) P-value<0.005; (***) P-value<0.0005; (****) P-value<0.00005. Data for siNT and siCCM2 HBMVECs are the same as in figure 3.

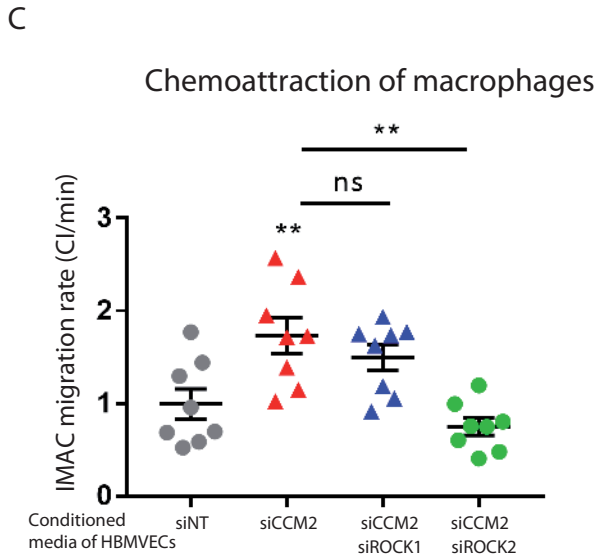
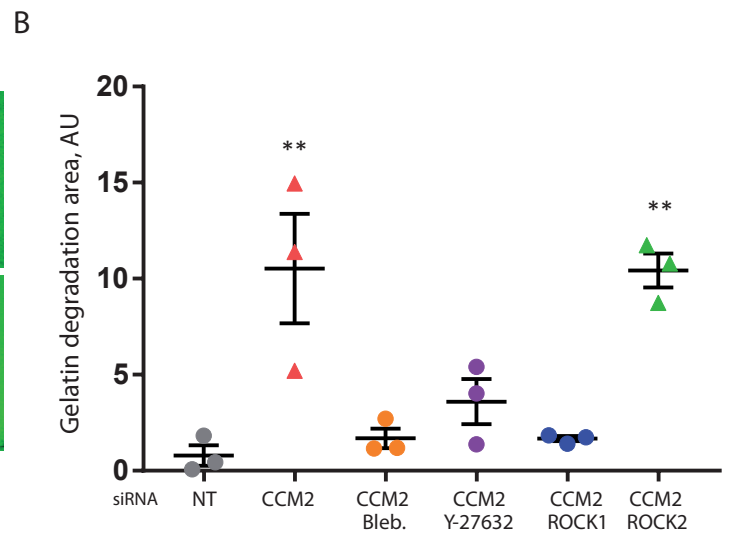
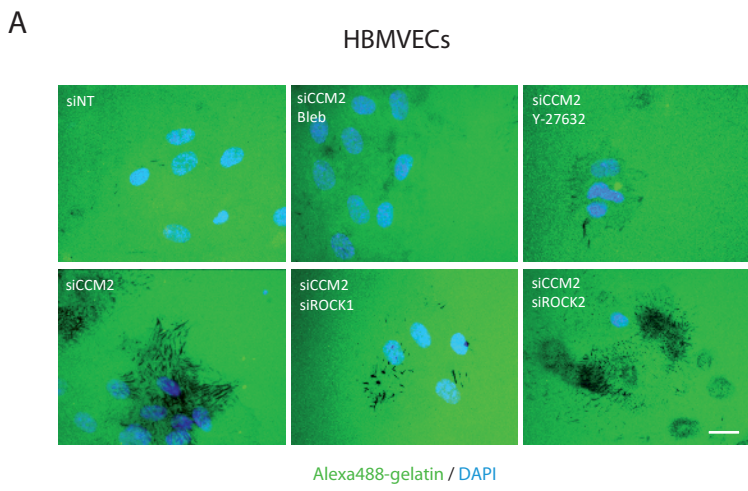
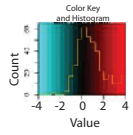
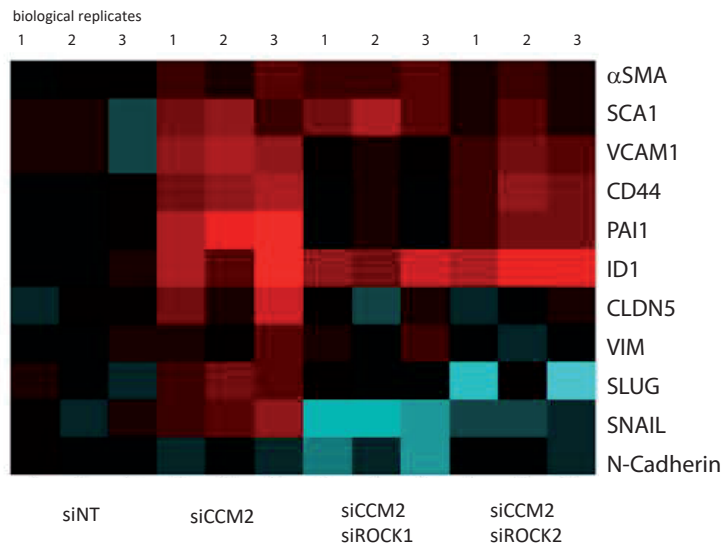
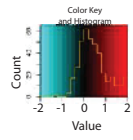


Figure S7 : As in HUVECs, ROCK1 causes ECM degradation by CCM2-depleted HBMVECs and ROCK2 causes chemo-attraction of macrophages.

(A) Representative images of the degradation of fluorescent gelatin by siRNA transfected HBMVECs treated or not with blebbistatin or Y-27632. Scale bars, 10 μ m. (B) Quantification of the area of gelatin degradation. Error bars are means \pm SEM from 3 drug treatments independent experiments. (C) Quantification of the rate of transmigration of IMAC macrophages measured in a modified Boyden chamber in real time using xCELLigence upon chemo-attraction by conditioned media of siRNA-transfected HBMVECs. Error bars are means \pm SEM from 3 independent experiments.

(*) P-value < 0.05; (**) P-value < 0.005.



FigS8: Heatmap of EndMT markers over the 4 biological conditions