

CCM2 deficient endothelial cells undergo a mechano-dependent reprogramming into senescence associated secretory phenotype used to recruit endothelial and immune cells.

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1 **Abstract:**

2 Cerebral Cavernous Malformations (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 diseases is unknown. Here, we reveal that CCM1- and CCM2-silenced endothelial
6 cells enter into senescence associated with secretory phenotype (SASP) that they use to invade
7 the extracellular matrix and attract surrounding wild-type endothelial and immune cells.
8 Further, we demonstrate that this SASP is driven by the mechanical and molecular disorders
9 provoked by ROCKs dysfunctions. By this, we identify CCM1/2 and ROCKs as parts of a
10 scaffold controlling senescence, bringing new insights into the emerging field of the control of
11 aging by cellular mechanics. This discovery reconciles the dysregulated traits of CCM1/2-
12 deficient endothelial cells into a unique mechano-dependent endothelial fate that links
13 perturbed mechanics to microenvironment remodelling and long-range activation of endothelial
14 and immune cells.

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18 **Introduction:**

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

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

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

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

58 Here, we reveal that the transcriptome of CCM2-silenced EC presents a signature of Senescence
59 Associated Secretory Phenotype (SASP). Cellular senescence contributes to a wide variety of
60 human age-related pathologies, including cancer, fibrosis, cardiovascular diseases, or
61 neurological disorders(33). Further, we demonstrate that CCM2-silenced EC indeed enter into
62 premature senescence and acquire degradative and invasive skills that stimulate angiogenesis
63 *in vitro*. CCM2-deficient EC gain paracrine functions through secreted factors that attract wild-
64 type EC and immune cells. Remarkably, we show that this SASP is a mechano-dependent
65 process triggered by dysfunctional ROCK1 and ROCK2 and by increased EC contractility. By
66 this, we identify CCM1/2 proteins and ROCKs as part of a mechanotransduction scaffold
67 controlling senescence. This unexpected endothelial fate transition triggered by the loss of
68 CCM2 unifies all the known dysregulated features of CCM2-deficient EC and establishes a new
69 molecular mechanism supporting the mosaicism of the CCM lesions and their inflammatory
70 state.

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73 **Results:**

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

75 KRIT and CCM2 proteins interact with each other to form a molecular scaffold(34). We
76 previously showed that, owing to the stabilizing effect of CCM2 on KRIT, both proteins are
77 lost when CCM2 is silenced(19). We therefore chose to silence CCM2 in order to deplete the
78 entire KRIT/CCM2 complex. To study the gene expression program of a pure population of EC
79 depleted for CCM2, we performed RNA sequencing on monolayers of human umbilical vein
80 endothelial cells (HUVEC) after two consecutive rounds of transfection with CCM2 targeting
81 siRNA (siCCM2) or with non-targeting siRNA (siNT). CCM2 silencing was of 86% (figS1A)
82 as reported in Lisowska et al., 2018(29). A total number of 2057 differentially expressed genes
83 (DEGs) (fold change [FC] ≥ 2 ; $P < 0.05$, FDR corrected using Benjamini Hochberg method
84 (35)) were identified in siCCM2-treated compared to siNT-treated HUVEC among which 1318
85 genes were upregulated while 739 were downregulated (Table S1A).

86

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

101

102 To comfort the hypothesis that a SASP transcriptomic program is turned on in siCCM2
103 HUVEC, we search for specific transcriptomic signatures of senescence and SASP in the
104 literature corresponding to gene sets enriched in senescent cells among which fibroblasts and
105 endothelial cells(37–40). These gene sets relate to up- and down-regulated genes (Table S2).
106 We then searched for an enrichment in these gene sets in the CCM2-depleted transcriptome
107 using 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 EC
110 undergo a SASP when CCM2 is lost.

111 **2) CCM2- and KRIT-depleted EC 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). HUVEC 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 S3A), CCM2-depleted EC
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). In addition, their nuclei displayed
119 senescence-associated heterochromatin foci (SAHF) as revealed by spots in DAPI and HIRA
120 staining (Fig 2B) and their area was increased (Fig 2C). As senescence leads to cell cycle arrest,
121 we looked at the expression level of the cell cycle inhibitors CDKNs. Among them, p21/CIP1
122 and p15/INK4b were 3-fold upregulated. On the contrary, cyclin dependent kinase 1, its
123 regulator CKS1 and cyclin 2A as well as the transcription factor E2F1, a driver of S phase entry,
124 were all dramatically downregulated (fig S1B). Using BrdU incorporation, we detected a 2-fold
125 reduction in the percentage of cells in S phase and an accumulation in G1 indicative of a defect
126 in the G1/S transition of the cell cycle (Fig 2E). This translated into a significantly lowered rate
127 of proliferation of the EC population as shown by impedance measurements (Fig 2F), and a
128 two-fold lowered percentage of cells positive for the proliferative marker Ki67 (Fig 2G).
129 Overall, the combination of all these traits confirms that the loss of CCM2 indeed induced
130 premature senescence in HUVEC.

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

138 Having shown that the loss of CCM2 leads to SASP, we wondered whether this would be a
139 common feature with the loss of KRIT and CCM3. KRIT-depleted HUVEC displayed the same
140 senescent phenotype as CCM2-depleted HUVEC as shown by a significant increase in cells
141 expressing SA- β gal activity (Fig 3A) and SAHF (Fig 3B), a significant decrease in Ki67-
142 positive cells (Fig 3C) and with a lowered rate of proliferation (Fig 3D). Interestingly, CCM3-
143 depleted EC did not display marks of senescence in good agreement with its distinct role in EC
144 biology and onset of the disease(44). Indeed, CCM3-depleted HUVEC behaved as control cells
145 in these assays. They did not show increased SA- β gal activity (Fig 3A), nor SAHF (Fig 3B).
146 They had a normal level of Ki67 positive cells (Fig 3C) and they did not proliferate differently
147 from control cells (Fig 3D). Therefore, consistently with their strong association and regulation
148 of common signaling pathways(19,44), KRIT and CCM2 loss similarly lead to premature
149 cellular senescence.

150 **3) ROCK2 controls the SASP transcriptomic program of CCM2-depleted EC**

151 ROCK-dependent perturbations in the mechanotransduction of EC have a major role in the
152 genesis and progression of CCM lesion. Inhibition of ROCK is sufficient to block the formation
153 and the maturation of CCM lesions (30–32). We previously showed that the CCM1/2 complex
154 is a scaffold recruiting ROCK2 at VE-cadherin complexes thereby limiting ROCK1 kinase

155 activity to maintain the tensional homeostasis between cell-cell and cell-ECM adhesions and to
156 preserve the integrity of the endothelial monolayer(29). However, it is yet unknown whether
157 ROCKs are also involved in the control of gene expression downstream of CCM2 and in
158 particular in the regulation of this SASP transcriptomic program. Hence, we analysed the
159 contribution of ROCK1 and ROCK2 by performing RNA sequencing on monolayers of CCM2-
160 silenced HUVEC that were additionally silenced for ROCK1 or ROCK2 (Fig S1A) in the same
161 set of experiments as that shown in figure 1. We have previously shown that the additional
162 depletion of ROCK1 but not ROCK2 restores the morphological defects of the CCM2-deficient
163 EC and their permeability barrier(29). Depletion of ROCK1 or ROCK2 alone were performed
164 as controls. Strikingly, 40% of the DEGs with FC>2 (54% of all DEGs) had their expression
165 significantly returned toward the control level by the additional silencing of ROCKs (Fig 4A,
166 Table S1B). The silencing of ROCK2 had a stronger restoring effect than that of ROCK1 on
167 the number of restored genes (Fig 4A) and their level of expression (Fig 4B, Fig S2A).
168 Importantly, silencing of ROCK2 alone had overall an opposite effect to that of CCM2 on gene
169 expression (Fig 4B), suggesting that ROCK2 acquires a gain of transcriptional function when
170 CCM2 is lost.

171 We then studied the restoring effect of ROCK1 or ROCK2 silencing on the biological functions
172 perturbed by CCM2 depletion. Figure 4C shows a clustered expression heatmap of DEGs
173 belonging to the Gene Ontology (G.O) biological processes presented in figure 1B. The
174 expression of DEGs related to the down-regulated nuclear functions was fully restored by
175 ROCK2 depletion while ROCK1 depletion had only a partial effect. In addition, up-regulated
176 peri-membrane functions were rescued by the silencing of ROCK2 to a higher extent than that
177 of ROCK1 (Fig 4C). Going further, we focused on the effect of ROCKs on the signatures of
178 senescence or SASP found in siCCM2 transcriptome. While these signatures of senescence
179 were still present upon ROCK1 depletion, they were not anymore significantly enriched in
180 HUVEC doubly silenced for CCM2 and ROCK2 (Fig 4D, Fig S2B) highlighting the crucial
181 role of dysregulated ROCK2 in the onset of the SASP transcriptomic program.

182 Overall, our transcriptomic data reveal that, beyond their role on the tensional homeostasis of
183 the endothelial monolayer, ROCK2 and to a lesser extent ROCK1 control the expression of an
184 important fraction of the genes regulated by CCM2. These genes are involved in a
185 transcriptomic program supporting the onset of SASP when CCM2 is lost.

186 **4) ROCKs dysfunctions induce premature senescence in CCM2-depleted EC.**

187 Having shown that ROCKs control the expression of genes involved in SASP, we next asked
188 whether dysfunctional ROCKs played a causal role in the entry into SASP of CCM2-depleted
189 EC. Additional silencing of ROCK1 or ROCK2 was similarly efficient in preventing the
190 appearance of most of the features tested i.e. SA- β -gal activity, HIRA-positive SAHF and
191 restored normal level of Ki67+ cells (Fig 5A, B, E). However, ROCK2 silencing was more
192 efficient in preventing the accumulation in G1 (Fig 5D), consistently with its higher efficiency
193 in lowering the expression of p21/CIP1 and p15/INK4b (Fig 5C) and in restoring the expression
194 of down-regulated cyclins and cyclin dependent kinases (Fig S1B). Moreover, the silencing of

195 ROCK2 and to a lesser extent that of ROCK1 lowered the expression of SASP factors, i.e. ECM
196 remodelers or inflammatory chemokines (Fig 5F).

197 Our next goal was then to know whether the mechanical defects provoked by dysregulated
198 ROCKs play a direct role in the premature senescence of CCM2-depleted HUVEC. To answer
199 this question, we treated siCCM2 HUVEC with blebbistatin, an inhibitor of myosin II that
200 blocks cell contractility or with Y27632, an inhibitor of ROCK1 and 2 kinase activities. Both
201 treatments inhibited the production of transversal actin stress fibers by siCCM2 HUVEC and
202 restored a more cortical actin rim alike the one observed in control HUVEC (Fig S3A).
203 Interestingly, these treatments inhibited all the senescent traits studied above, i.e. SA- β -gal
204 activity, HIRA-positive SAHF and accumulation in G1 phase of the cell cycle and restored
205 normal level of Ki67+ cells (fig 5A, B, D, E) supporting the fact that increased contractility is
206 involved in the premature senescence of CCM2-depleted EC. Noticeably, blebbistatin and
207 Y27632 had no effect on siNT HUVEC in these assays (Fig S3B, C, and D).

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

210

211 **5) ROCK1 causes ECM degradation and supports invasiveness of CCM2-depleted EC** 212 **and neighbouring WT EC.**

213 Cancer cells undergoing SASP can promote tumour development through a juxtacrine effect on
214 their microenvironment by secreting matrix metalloproteinases (MMPs) and ECM remodeling
215 enzymes that facilitate tumour cell invasiveness and metastasis(42). Similar to cancer
216 progression, the formation of CCM lesions could result from a SASP-dependent invasion of the
217 brain tissue by EC. Consistently, MMPs have been found around CCM lesions in human(14)
218 and in mouse(13), or zebrafish models(22) and their upregulation plays a role in CCM defects
219 (12,20). To know whether upregulated expression of SASP factors confers invasive skills to
220 siCCM2 HUVEC, we tested the ability of these cells to degrade ECM and invade a 3D matrix.
221 To visualize the degradation of the ECM, siRNA transfected HUVEC were cultured overnight
222 on fluorescent gelatin. siNT HUVEC barely degraded the gelatin as expected for differentiated
223 EC (Fig 6A). Conversely and consistently with their new SASP expression program, siCCM2
224 HUVEC degraded the gelatin through scratch zones appearing dark in the fluorescently labelled
225 layer (Fig 6A). The linear shapes of these scratch zones suggested that they were produced
226 under focal adhesions. They were dependent on the activity of MMPs as demonstrated by their
227 complete disappearance upon MMP inhibitor GM-6001 treatment (Fig 6A). Together with
228 blocking the mechanosensitive assembly of focal adhesions (Fig S4), Y27632 and blebbistatin
229 blocked the degradation of gelatin (Fig 6A). We previously showed that additional silencing of
230 ROCK1, but not of ROCK2, limits excessive focal adhesion formation in siCCM2
231 HUVEC(29). Accordingly, only the additional depletion of ROCK1 but not ROCK2 inhibited
232 ECM degradation by siCCM2 HUVEC (Fig 6A). Overall, these data show that ROCK1-
233 dependent increase in cell contractility and in cell-ECM adhesive sites together with the
234 overexpression of MMPs are responsible for the acquisition of ECM degradative skills by
235 siCCM2 HUVEC.

236 We next tested whether their new degradative capacities gave invasive skills to siCCM2
237 HUVEC. To measure 3D invasiveness, GFP-expressing siRNA treated HUVEC were plated on
238 3D-degradable polyethylene glycol gels. Invasive sprouts were imaged after 18 hours and the
239 maximum invasion distance was quantified. siCCM2 HUVEC invaded the 3D gel twice as deep
240 as siNT HUVEC and they mostly invaded as isolated cells with filopodia at their front compared
241 to the cohesive invasion mode of siNT HUVEC (Fig 6B). Silencing of ROCK1 or blebbistatin
242 treatment reduced the invasiveness of siCCM2 HUVEC to the level of siNT HUVEC whereas
243 silencing of ROCK2 enhanced it (Fig 6B) consistently with the increased traction forces upon
244 additional depletion of ROCK2(29).

245 CCM lesions are mosaics of mutant and WT EC as shown in human and murine lesions(9–11).
246 Moreover, it has recently been shown that CCM3 KO EC can attract WT EC *in vivo* in the brain
247 vasculature and *in vitro* on mixed monolayers(11). Therefore, we asked whether senescent
248 siCCM2 HUVEC could also stimulate the sprouting of WT HUVEC when mixed together on
249 the surface of 3D-PEG gels. Strikingly, we observed that RFP-expressing WT HUVEC invaded
250 the gels twice as deep when they were mixed with GFP-expressing siCCM2 HUVEC as when
251 mixed with GFP-expressing siNT HUVEC (Fig 6C). Interestingly, this increased invasion was
252 significantly reduced when WT HUVEC were mixed with siCCM2+siROCK1 HUVEC but
253 remained unchanged when they were mixed with siCCM2+siROCK2 HUVEC (Fig 6C).

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

256

257 **6) ROCK2 causes the expression of paracrine factors by CCM2-depleted EC that chemo-** 258 **attract WT EC and immune cells.**

259 Apart from remodelling their surrounding ECM, senescent cells secrete paracrine factors that
260 have been shown to promote invasiveness of neighbouring cells and engage deleterious
261 inflammatory responses (42). Therefore, we tested whether secreted factors would have a long
262 distance paracrine effect allowing the recruitment of WT EC. We measured the capacity of a
263 media conditioned by siCCM2 HUVEC to chemo-attract serum-starved WT HUVEC through
264 a layer of Matrigel in a modified Boyden chamber measuring the impedance of cells.
265 Remarkably, siCCM2-conditioned media significantly increased the speed of invasion of WT
266 HUVEC through the Matrigel showing that chemo-attractive factors secreted by siCCM2
267 HUVEC could also attract EC (Fig 6D). Interestingly, this effect was inhibited by ROCK2 but
268 not ROCK1 additional silencing (Fig 6D). Therefore, siCCM2 HUVEC produce paracrine
269 factors that attract WT EC in a ROCK2-dependent manner.

270 Since a chronic inflammation is observed at the site of human and murine CCM lesions through
271 the recruitment of activated lymphocytes and monocytes(45), we tested whether the loss of
272 CCM2 leads to the secretion of chemo-attractive cues for immune cells. Thus, we measured the
273 capacity of media conditioned by siCCM2 HUVEC to chemo-attract IMAC, an immortalized
274 macrophage cell line in a modified Boyden chamber as above. Almost no transmigration of
275 macrophages was observed in the case of siNT-conditioned media (Fig 6E). Strikingly,
276 siCCM2-conditioned media provoked a rapid transmigration of the macrophages suggesting

277 that siCCM2 HUVEC had secreted chemo-attractive factors that siNT HUVEC had not (Fig
278 6E). Importantly, this chemo-attraction was inhibited when ROCK2 but not ROCK1 was
279 additionally silenced (Fig 6E). Therefore, siCCM2 HUVEC secrete paracrine factors that can
280 attract immune cells such as macrophages. Moreover, these chemo-attraction skills depend on
281 ROCK2.

282 Overall, these results demonstrate for the first time to our knowledge that, when CCM2 is lost,
283 HUVEC undergo a SASP that is driven by the mechanical and molecular disorders provoked
284 by ROCKs dysfunctions. A major consequence of this SASP is a profound mechano-dependent
285 remodelling of the microenvironment leading to the recruitment of wild-type EC and immune
286 cells to generate mosaic CCM lesions.

287 Discussion

288 Many cellular pathways are dysregulated in CCM-deficient EC and it has been difficult to
289 propose a mechanistic model that could take into account all these aspects. Transition in cellular
290 fate(18) upon morphological and mechanical changes during the loss of cell-cell
291 junctions(19,29,46) are associated with overproduction of ROS(16), decreased autophagy(17),
292 secretion of metalloproteases(12–14) or cytokines(15) and increased integrin(19,20), p38(21),
293 MEKK3/KLF2(20,22,23) and TLR4(24) signaling. The first breakthrough of this current study
294 is to show, for the first time to our knowledge that CCM2-deficient EC engage towards a
295 Senescence Associated Secretory Phenotype (SASP) (Fig 7). Multiple senescent traits were
296 validated such as transcriptomic SASP signature, lysosomal SA- β -galactosidase activity,
297 upregulation of CDK inhibitors, cell cycle blockage in G1, presence of SAHF along with the
298 secretion of chemo-attractant factors. Moreover, we show that the functional consequence of
299 this SASP is an acquired ability of CCM2-deficient EC to invade the ECM and recruit wild-
300 type EC and immune cells.

301
302 SASP is characterized by cell growth arrest, widespread changes in chromatin organization and
303 gene expression(36). These changes also include the secretion of numerous pro-inflammatory
304 cytokines, chemokines, growth factors and proteases(43). Acute senescence is beneficial in
305 development, tissue regeneration or cancer through the clearance of senescent cells by the
306 immune system(47). Chronic senescence triggers chronic inflammation that can on the contrary
307 favour age-related diseases including cancer, fibrosis, cardiovascular diseases, type 2 diabetes,
308 osteoarthritis or neurological disorders(33). Indeed, the long-term secreted pro-inflammatory
309 factors promote cell proliferation, microenvironment remodelling, angiogenesis and
310 inflammation in a paracrine manner. Remarkably, decreased autophagy(48), ROS
311 overproduction(49), P38 MAPK(50), KLF2/4(51,52) and TLR4(53) signaling pathways have
312 all been involved in the induction of SASP either through regulation of CDK inhibitors or
313 through the NF κ B or C/EBP β -driven expression of cytokines. Therefore, we propose that each
314 of the dysregulated features of CCM-deficient EC represents a different facet of the same
315 cellular state. Future research should help reconstructing the complex chronology of the
316 different events in the framework of this SASP. This should identify key therapeutic targets for
317 either single or combinatorial drug treatments to block at their root the defective molecular
318 pathways involved in the CCM2 disease. Senolytic drugs and drugs targeting the SASP *per se*
319 are currently under pre-clinical trials for cancer therapy or other age-related diseases(33). They
320 could be tested to prevent the formation and expansion of CCM lesions. A recent study strongly
321 supports that premature aging of the neuro-vascular system could be the cause of CCM disease
322 by showing that aging and CCM brains share common dysregulated features including impaired
323 endothelial barrier function, inflammation, 320 DEGs, plasma molecules and imaging
324 biomarkers (54).

325
326 This SASP not only reconciles all the CCM defects but also brings a molecular explanation for
327 the mosaicism of the CCM lesion (Fig 7). Secreted chemokines and cytokines attract wild-type
328 EC in paracrine and juxtacrine manners and trigger an inflammatory response, another major
329 feature of CCM lesions(11). Not only are WT EC attracted by CCM-deficient EC but they are

330 triggered to undergo EndMT and express stem cell markers(11). Interestingly, NF κ B signalling
331 has been shown to activate WNT/ β catenin signalling to induce the EMT of cells into cancer
332 stem cells(55) and to induce cancer cell metastasis in response to IL1 β (56). Therefore, one can
333 assume that a similar mechanism might operate in CCM lesion where senescent cells could
334 induce the dedifferentiation of WT EC through EndMT.

335

336 Whereas it has also been observed that Ccm3-null EC attract wild-type EC at the site of the
337 lesion in Ccm3 mouse model, clonal expansion of Ccm3-null EC precedes the expansion of the
338 lesion(10,11). Moreover, differently from KRIT and CCM2, the loss of CCM3 actually
339 preserves primary EC from replicative senescence *in vitro*(57) which is consistent with our
340 findings. Therefore, opposite mechanisms may lead to the formation of CCM lesions upon the
341 loss of CCM3 and CCM2 or KRIT. A rapid clonal proliferation of CCM3-deficient EC may
342 trigger the formation of aggressive lesions at early age whereas an entry in SASP of KRIT- or
343 CCM2-deficient EC may lead to the progressive maturation of lesions and to the later onset of
344 the CCM1/2 disease. Accordingly, the absence of increased Ki67 or phospho-histone 3 staining
345 in the EC lining the lesions in the acute Ccm2^{-/-}(59) and in the Ccm1^{+/-}; Msh2^{-/-} (7) mouse
346 models or in ccm1^{-/-}mutant zebrafish embryo(60) do not argue in favour of an early clonal
347 endothelial proliferation upon CCM1 or CCM2 loss. Studies are ongoing in several laboratories
348 to better characterize the different cellular states that co-exist in mutant and WT EC populations
349 over time within the CCM lesions (58). However, the complexity of the situation renders these
350 *in vivo* investigations very challenging and do not tackle the relationship between
351 mechanotransduction and CCM progression. This current study on pure or mixed populations
352 of CCM2-depleted and WT EC has led to significant insights towards a better understanding of
353 the complex *in vivo* situation. It should constitute the foundation for new specific *in vivo* studies.
354 Using the confetti reporter system in Ccm1/2 mouse models compatible with SA-
355 β galactosidase staining would help studying the initiation and expansion of the lesions in
356 comparison with the Ccm3 model.

357 The second major breakthrough of this study is that CCM proteins are involved in a
358 mechanotransduction pathway controlling the onset of the SASP. The fact that cellular
359 mechanical defects can provoke senescence is an emerging area of investigations. Not only
360 does our study make a clear demonstration of it, but it also identifies CCM proteins and ROCKs
361 as part of a crucial scaffold controlling senescence. We previously showed that upon the loss
362 of CCM1/2, a vicious cycle sets up between aberrant ECM remodelling and increased
363 intracellular contractility which breaks the permeability barrier of the endothelium(19).
364 Moreover, we demonstrated that placing WT EC on ECM remodelled by mutant EC provoke
365 CCM-like morphological defects in these cells(19). Going deeper into mechanistic
366 investigations, we showed that upon the loss of CCM2, ROCK2 is delocalized from VE-
367 cadherin junctions while ROCK1 gets over activated, enhancing the polymerization of β 1
368 integrin-anchored stress fibers(29). Here we uncover for the first time that, beyond their role in
369 the disruption of the endothelial architecture, dysregulated ROCKs are instrumental in the
370 transition of CCM2-depleted EC towards SASP (Fig 7). We propose that this SASP allows the
371 amplification of local mechanical perturbations into a systemic response that propagates to

372 other EC and immune cells, a phenomenon that could also exist for other mechano-dependent
373 pathologies such as cancer or fibrosis.

374

375 ROCK2 controls the expression of a very significant fraction of the genes dysregulated upon
376 the loss of CCM2 among which genes involved in senescence, cell cycle and paracrine SASP
377 factors. The opposite expression profiles in absence of CCM2 or ROCK2 suggest that ROCK2
378 gains a broad transcriptional regulatory function when its scaffold CCM2 is lost. In fact,
379 ROCK2 has already been involved in cellular senescence(61). In this study, the loss of the two
380 ROCK isoforms led to senescence of MEF and was due to the downregulation of CDK1, CKS1
381 and cyclin2A. Intriguingly, we observe a similar downregulation of these genes in HUVEC
382 when CCM2 is lost (Fig S2B), but contrary to this other study, their expression is rescued by
383 the additional silencing of ROCK2. This suggests that the presence or absence of CCM2 has a
384 strong influence on the transcriptional activity of ROCK2. Future mechanistic studies will be
385 necessary to understand how ROCKs interplay with the CCM complex to regulate gene
386 expression.

387

388 We previously showed that ROCK1 over activation allows CCM2-depleted HUVEC to exert
389 stronger traction forces on the ECM (19,29). Here we show that ROCK1 enables the
390 mechanosensitive degradation and invasion of the ECM by the senescent CCM2-depleted EC
391 and by neighbouring WT EC. Since EC pull on their microenvironment to invade it(62), it is
392 likely that these stronger forces are responsible for enhanced invasion. Further investigations
393 will help better understand the molecular events at play. Like ROCK2, ROCK1 affects gene
394 expression downstream of CCM2 though to a lesser extent. Moreover, inhibition of the myosin
395 motor or silencing of ROCK1 is enough to block senescence. These data suggest that cross talks
396 exist between the contractility of the acto-myosin cytoskeleton and the transcription of genes
397 involved in SASP. Several mechanosensitive transcription factors are known to be regulated by
398 acto-myosin polymerization or contractility(63). Moreover, ROCK2 can shuttle into the
399 nucleus where it phosphorylates the chromatin remodeller p300HAT(64). Future investigations
400 should unravel how ROCK1 and ROCK2 cooperate to regulate chromatin organization and
401 gene expression through the control of mechanosensitive transcription machineries.

402

403 Overall, this study demonstrates that CCM2-deficient EC undergo a SASP that is driven by the
404 mechanical and molecular disorders provoked by ROCKs dysfunctions. Remarkably, this
405 discovery unifies all the known dysregulated traits of CCM1/2-deficient EC into a unique
406 cellular state. A major consequence of this mechano-dependent SASP is the remodelling of a
407 microenvironment that sustains chronic recruitment of wild-type EC and immune cells to the
408 site of the lesion. Thus, this work unravels the molecular basis of the mosaicism of CCM1/2
409 lesions and their inflammatory status. It opens new avenues for mechanistic *in vivo* studies on
410 the dynamics and penetrance of the CCM1/2 disease as well as for exploring new therapies.

411

412 **Material and Methods**

413 **Cell culture and transfections:**

414 Pooled HUVEC were obtained from Lonza. Upon reception, HUVEC at P0 were expanded
415 over 2 passages in collagen 1 (from rat tail, BD) coated flasks in complete EGM-2 medium
416 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ - 3%
417 O₂ humidified chamber. HUVEC at passage 3 were transfected twice at 24 h-intervals with 30
418 nM siRNA and Lipofectamine RNAi max (Life Technologies, ref. 13778-150) according to the
419 manufacturer's instructions in 37°C in a regular 5% CO₂ in a humidified chamber. For double
420 transfections, 30 nM of each siRNA duplexes (Dharmacon smartpool ON-TARGET plus
421 Thermo Scientific) was used; Non-targeting siRNA #1, CCM2; ref. L-014728-01), KRIT; ref.
422 L-003825-00, CCM3; ref. L-004436-00, ROCK1; ref. L-003536-00 and ROCK2; ref. L-
423 004610-00. When required, blebbistatin, Y27632 and GM6001 were used at 10 µM final.

424 **RNA sequencing and differential analysis:**

425 One million of siRNA transfected HUVEC were seeded at confluency the day after the second
426 round of transfection in wells of 6-well plates coated with collagen 1 (from rat tail) and cultured
427 for 48h in complete media at 37°C, 5% CO₂. Total RNA were extracted from HUVEC using
428 the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions.
429 cDNA libraries were prepared with the TruSeq Stranded mRNA Sample Preparation (Illumina)
430 and sequenced on a HiSeq 2500 Illumina platform using single-end 50 basepair reads at the
431 MGX facility (Montpellier). Fastq files were aligned using STAR (2.5.2b) on UCSC hg19
432 genome. The contents of the annotation directories were downloaded from UCSC on: July 17,
433 2015. Bam files were counted using htseq-count (0.11.2.) with option -t exon -f bam -r pos -s
434 reverse -m intersection-strict -nonunique none. Differential analyses were performed with
435 SARTools (1.4.1) (65) using DESeq2 (1.12.3) (66) and default options. P-values were adjusted
436 with Benjamini-Hochberg procedure(35) set for 5% of FDR. Heatmaps using a correlation
437 matrix and boxplots were obtained with custom R script (3.3.0). The raw sequencing data used
438 in this study are available in the National Center for Biotechnology Information's Gene
439 Expression Omnibus (GEO) database and are accessible through GEO series accession number
440 (pending).

441
442 **Data Availability:** The raw data (FastQ files) and processed data (count files) are deposited in
443 the Gene Expression Omnibus database under ID code GEO: GSE165406.

444 **Bioinformatics analyses:**

445 Gene ontology analyses on DEG in siCCM2 condition (fold change [FC] ≥ 2; $P < 0.05$ with
446 FDR corrected using Benjamini-Hochberg method (35)) were made with PANTHER version
447 15.0 Released 2020-02-14 using slim cellular components and biological processes. Enriched
448 pathways analyses were conducted with Reactome. DEGs ($P < 0.05$, 5% FDR) rescued by
449 additional silencing of ROCK1 or ROCK2 were recovered at the union of Venn diagrams of
450 DEGs in siCCM2 vs siNT and DEGs in siCCM2 vs siCCM2+ROCK1 or ROCK2 respectively.

451 Gene Set Enrichment Analyses were conducted using GSEA software from Broad Institute,
452 UC San Diego(67).

453 **Quantitative RT-PCR:**

454 Purified RNA (1 µg) were reverse transcribed using the iScript Reverse Transcription Supermix
455 (Biorad). Quantitative real-time PCR (Q-PCR) was performed with iTaq Universal SYBR
456 Green Supermix (Biorad) in a 20 µl reaction on a thermal cycler (C-1000 Touch; Bio-Rad
457 Laboratories). Product sizes were controlled by DNA gel electrophoresis and the melt curves
458 were evaluated using CFX Manager (Bio-Rad Laboratories). A total of three housekeeping
459 genes were selected for their stability in our HUVEC cell line under our experimental
460 conditions, using the three analytical software programs, geNorm, Normfinder and Bestkeeper
461 (68,69). We used the relative expression software tool CFX Manager for relative quantification,
462 and normalization was achieved using a normalization factor from all reference genes(68). The
463 mean of three technical replicates was calculated per biological replicate.

464 **Immunofluorescence staining:**

465 HUVEC were seeded at 5×10^4 cells or 2×10^5 cells in 24-well plates on coverslips coated with
466 10 µg/ml fibronectin (from human plasma, Sigma Aldrich) and incubated overnight in complete
467 EBM-2 medium. Cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, and
468 incubated with anti-activated $\beta 1$ integrin clone 9EG7 ((BD Biosciences, 1/200), anti-Ki67
469 (AN9260 Millipore, 1/200), anti-HIRA (WC119, Millipore, 1/200) antibodies. After rinsing,
470 coverslips were incubated in Goat anti-Mouse or anti-Rabbit IgG (H+L) highly cross-adsorbed
471 secondary antibody, Alexa Fluor conjugated AF 488, AF 546, AF 647 (Invitrogen, 1/1000) and
472 phalloidin conjugates with Atto 647 (Sigma, 1/2000). The coverslips were mounted in
473 Mowiol/DAPI solution and imaged on an epifluorescent Axiomager microscope (Zeiss) at 63X
474 magnification.

475 **SA-B-Galactosidase staining:**

476 HUVEC were seeded 48 hours after the second siRNA transfection at a density of 5×10^4 in 24-
477 well plates coated with 10µg/mL fibronectin and incubated overnight in complete EBM-2-
478 medium. Senescence-Associated β -galactosidase activity was assessed using a SA- β -
479 galactosidase staining kit according to the manufacturer's instructions (Cell Signaling). Positive
480 cells were counted manually out of more than 100 cells total.

481 **BrdU assay:**

482 HUVEC were seeded 48 hours after the second siRNA transfection at a density of 2×10^5 cells
483 in 6-well plates coated with 100µg/mL collagen 1 and incubated overnight in complete EBM-
484 2 medium. The BrdU assay was performed with the BD Accuri C6 flow cytometer using the
485 APC-BrdU flow kit according to the manufacturer's instructions (Cell Signaling).

486 **xCELLigence proliferation assay:**

487 Proliferation assay was performed using the xCELLigence Real-Time Cell Analysis (RTCA)
488 DP instrument in combination with E-plate 16 (ACEA Biosciences) coated with 100 μ L of
489 100 μ g/mL collagen 1 (from rat tail) for 30 min at 37°C and washed 2 times with PBS 1X. 40
490 μ L of complete EBM-2 medium was added and baseline without cells was made with RTCA
491 software. 5x10³ siRNA transfected HUVEC were seeded 48h after the second round of
492 transfection in 100 μ L of complete EBM-2-medium. Impedance measurements were recorded
493 every 5 min during 24h. Impedance was normalized at 4 hours after seeding to eliminate the
494 contribution of cell spreading and adhesion to the signal. Slope measurement was performed
495 between 4h and 24h.

496 **Matrigel invasion assay:**

497 This assay was performed using the xCELLigence RTCA DP instrument in combination with
498 CIM-Plate 16 (ACEA Biosciences). A layer of 3.3% of growth factor reduced Matrigel
499 (Corning) diluted with EBM basal media was poured on ice in the upper chamber and incubated
500 overnight at 37°C for polymerization. 40 μ l basal EBM media was added to the upper chamber
501 and the baseline made after equilibration at 37°C. After setting the baseline, 3x10⁴ WT HUVEC
502 were added to the upper chamber. The conditioned media from siRNA transfected cells was
503 recovered 48 h after the second round of transfection and centrifuged at 12000 rpm for 5 min
504 and 160 μ L was added to the lower chamber of the CIM-plate. After one hour of equilibration
505 at 37°C, impedance was measured every 3 minutes for 24 hours.

506 **Chemo-attraction assay:**

507 This assay was performed using the xCELLigence Real-Time Cell Analysis (RTCA) DP
508 instrument in combination with CIM-Plate 16 (ACEA Biosciences). After setting the baseline,
509 3x10⁴ IMAC were added in 100 μ L of basal EBM in the upper chamber and 48h-conditioned
510 media from siRNA transfected cells added to the lower chamber of the CIM-plate. After 30 min
511 equilibration at 37°C, impedance was measured every 3 minutes for 24 hours.

512 **Gelation degradation assay:**

513 Coverslips in 24 well-plates were coated with gelatin-Alexa488 dye as previously described
514 (70). SiRNA transfected HUVEC were seeded 48h after the second siRNA transfection at
515 5x10⁴/well. HUVEC were incubated in OptiMEM medium overnight at 37°C in a 5% CO₂
516 incubator, fixed with 4% PFA, and washed with PBS thrice. For a quantitative analysis of the
517 degradative skill of siRNA transfected cells, 5 images per condition were randomly acquired
518 with an epifluorescent Axiomager microscope (Zeiss) at 40X magnification, and were
519 converted to binary images using B&W thresholding on ImageJ. The total area of the black
520 zones corresponding to the total area of degradation of the fluorescent gelatin was measured.

521 **3D-PEG invasion assays:**

522 Poly-ethylene glycol (PEG) hydrogels were prepared on ice in EBM-2 complete media by
523 combining an MMP-sensitive peptide modified PEG precursor (8-arm 40kDa,(71)) at 1.5%
524 polymer concentration, 50 μ M Lys-RGD peptide (Pepmic), and 1 μ M sphingosine-1-phosphate
525 (S1P, Sigma-Aldrich). The hydrogels were enzymatically crosslinked using a reconstituted and

526 thrombin-activated Factor XIII (Fibrogammin, CSL Behring), prepared as previously described
527 (72)) at 10% of total hydrogel volume. A 20 μ L volume of the hydrogel suspension was pipetted
528 into a modified imaging chamber (Secure-Seal™ hybridization sealing systems, ThermoFisher
529 Scientific) attached to the bottom of a 24-well plate held vertically (62). The hydrogel was
530 allowed to polymerize for 30 minutes in this orientation at room temperature prior to cell
531 seeding. Depending on the study, a confluent cell monolayer composed of either 5×10^4 siRNA
532 transfected GFP-HUVEC or a 1:1 ratio mixture of siRNA transfected GFP-HUVEC and naïve
533 RFP-HUVEC was allowed to adhere to the PEG meniscus at 37°C, 5% CO₂ for 1 hour and then
534 placed back horizontally with 1mL of EBM-2 complete media. Fixation with 4% PFA in DPBS
535 was performed after 24 hours. Sprouts invading the PEG hydrogel were imaged using a Leica
536 SP8 inverted confocal microscope with an HC PL APO 10x, 0.4 numerical aperture dry
537 objective to obtain image stacks at 1024x1024 pixels with a 50 μ m Z-stack at 1-1.5 μ m Z-
538 spacing. A Z-projection of each image was used to manually quantify invasion distances using
539 the line measurement tool of ImageJ. More than 100 sprouts were analyzed per technical
540 replicate.

541

542 **Statistical tests:**

543 Results were assessed by either performing a paired t-test for comparing 2 conditions or for
544 more than 2 conditions by the Tukey's multiple comparison tests post-ANOVA to compare
545 with control; a 0.5 alpha level was used for all comparisons. Prism software was used to conduct
546 the statistical analysis of all data. $P < 0.05$ was considered to be significant. * $P < 0.05$, ** $P < 0.05$,
547 *** $P < 0.005$. n represents biological replicates.

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560 **Author contributions**

561

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

566

567 **Figure legends:**

568

569 **Table S1: Level of expression of the dysregulated genes in the 6 different conditions of**
570 **siRNA** (A) DEG with $FC \geq 2$ ($P < 0.05$, FDR corrected using Benjamini Hochberg method) in
571 siCCM2 HUVEC. Rescued DEG with $FC \geq 2$ upon additional silencing of ROCK1 (B) or
572 ROCK2 (C).

573 **Table S2: Gene sets of Senescence and SASP used for GSEA analyses.**

574

575

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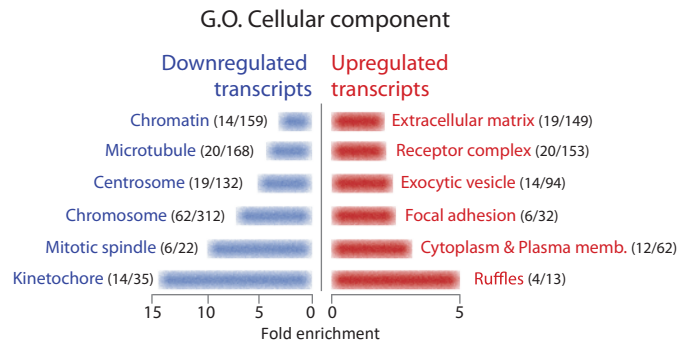
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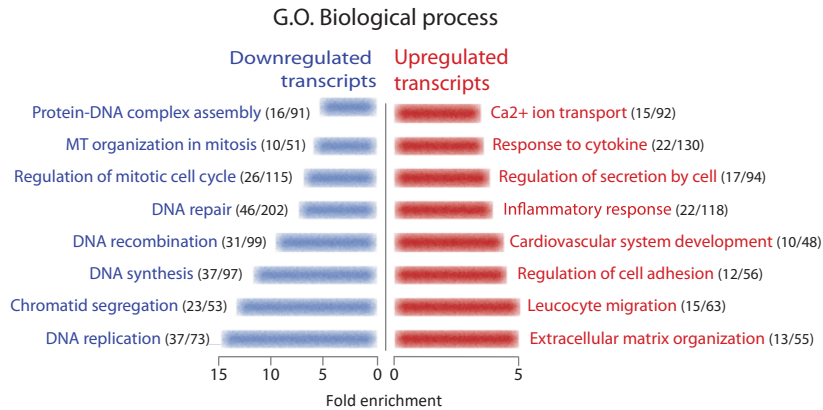
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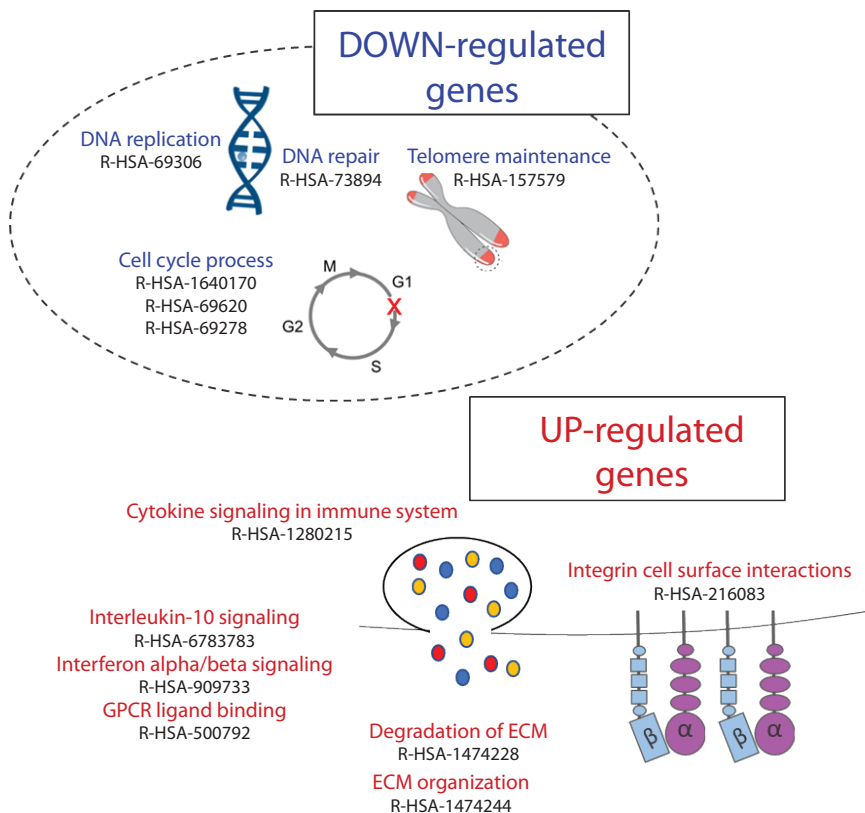
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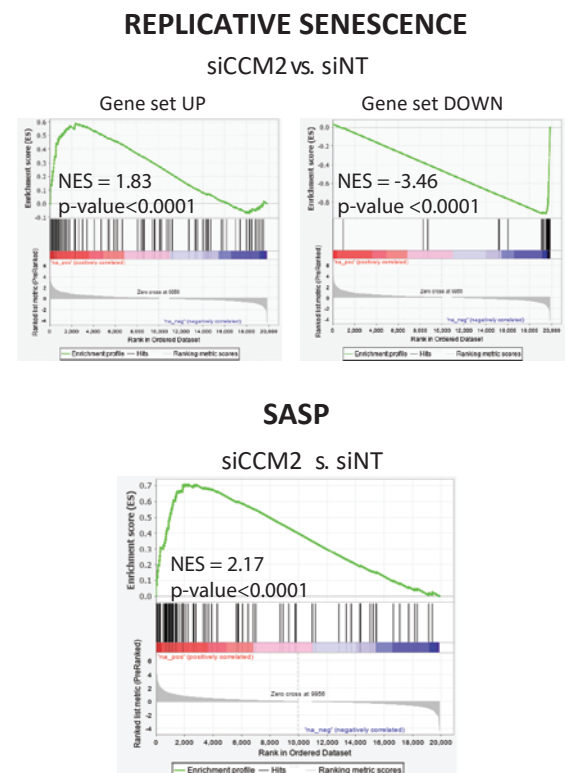


Figure 1: The loss of CCM2 turns on a SASP transcriptomic program.

(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 Reactome analysis of enriched pathways in siCCM2 HUVEC. (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.

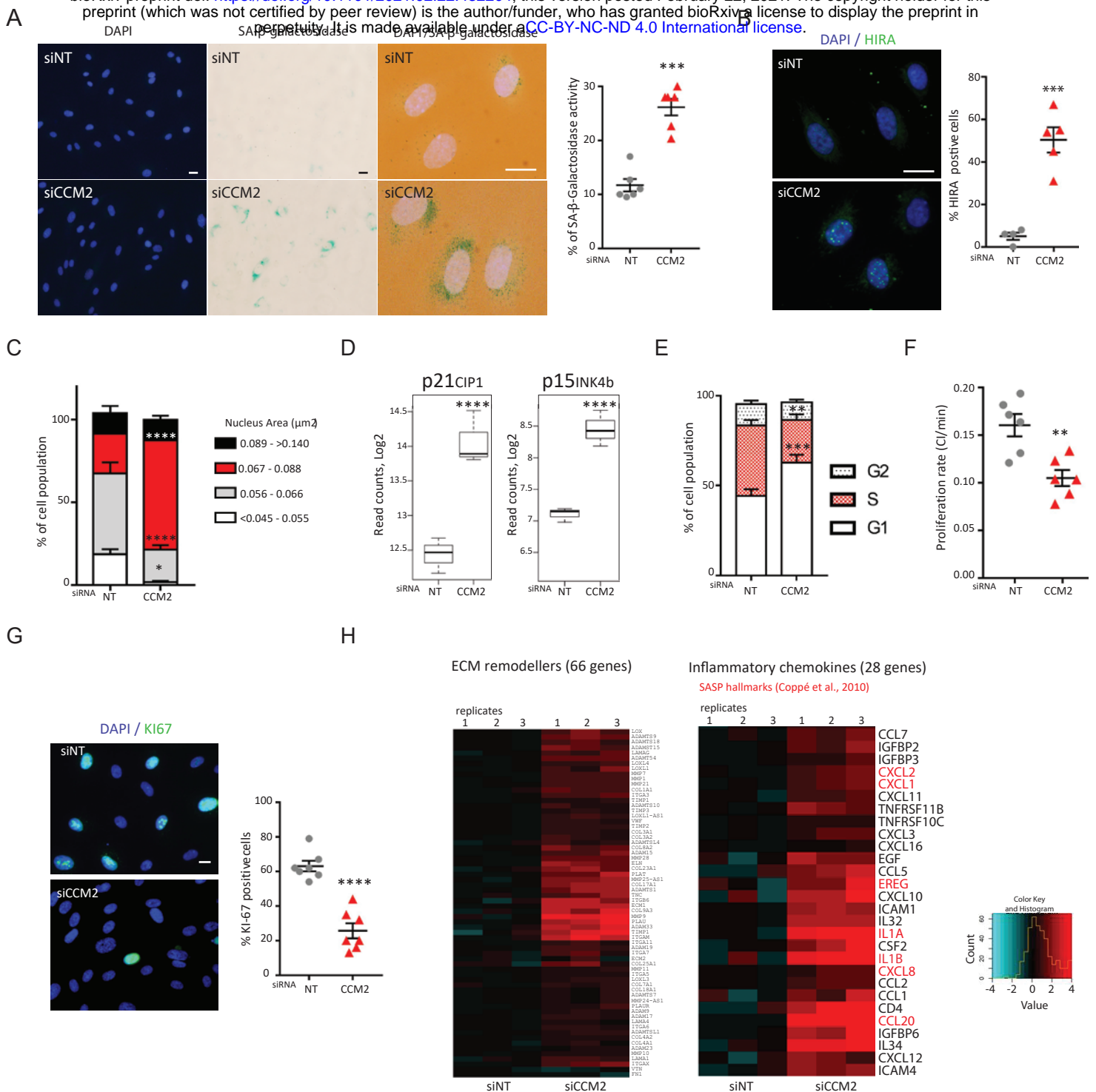
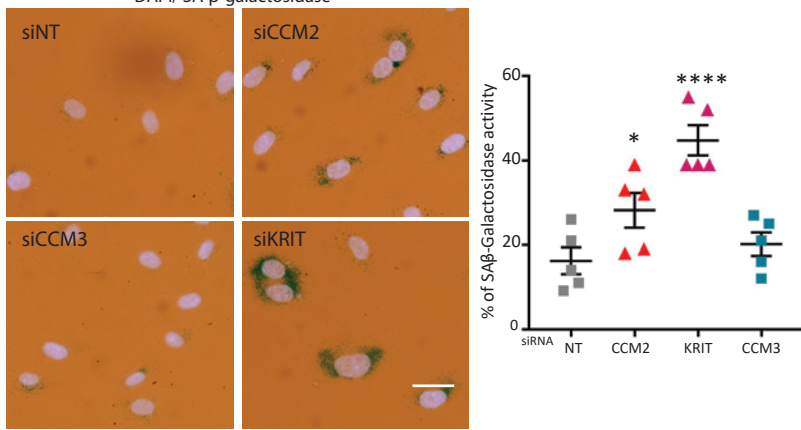


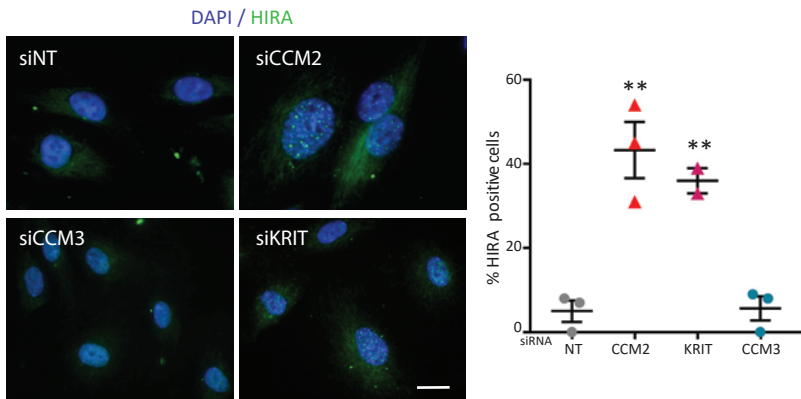
Figure 2: CCM2-depleted EC display hallmarks of SASP.

(A)(left) 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. (Right) Quantification of the % of positive cells for SA-β-galactosidase. Error bars are means ± SEM from 6 independent experiments. (B)(left) Representative images of merged DAPI and HIRA stainings of siNT and siCCM2 HUVEC. (Right) Quantification of the % of HIRA positive cells. Error bars are means ± SEM from 5 independent experiments. (C) Histogram of the cell population in function of their nucleus area. Error bars are means ± SEM from 4 independent experiments. (D) Boxplots of the read counts for p21/CIP1 and p15/INK4b mRNA. Error bars are means ± SEM from 3 independent experiments. (E) Quantification by BrdU assay of the percentage of cells in each phase of the cell cycle. Error bars are means ± SEM from 8 independent experiments. (F) Proliferation rate of siRNA transfected HUVEC measured by impedance using XCELLigence. Error bars are means ± SEM from 4 independent experiments. (G)(left) Representative images of the proliferation marker Ki-67 staining (green) merged with DAPI staining. (Right) Quantification of the percentage of cells positive for Ki-67 staining. Error bars are means ± SEM from 7 independent experiments. (H) Heatmap of expression of ECM remodelling proteins (left) and of SASP factors (right) over the 2 siRNA conditions, 3 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.

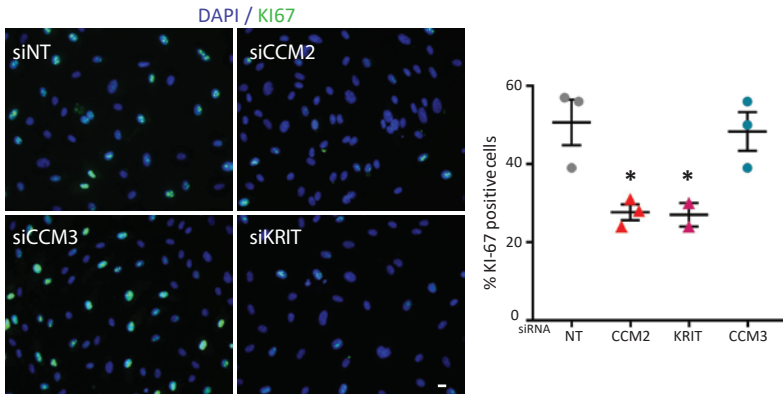
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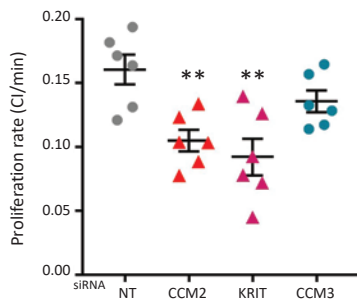
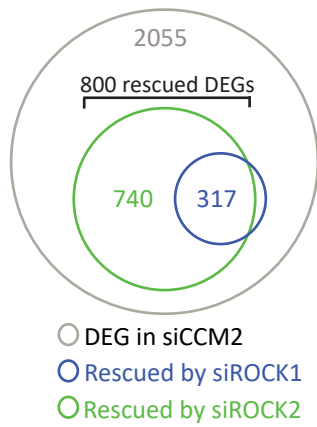


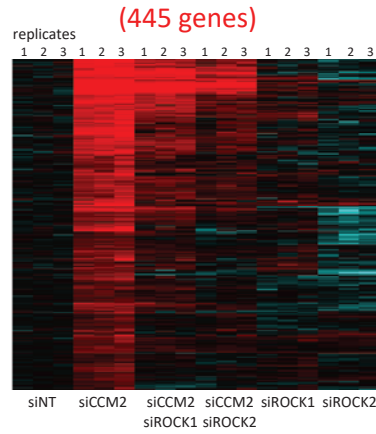
Figure 3: The loss of KRIT similarly to that of CCM2 leads to senescence in EC whereas the loss of CCM3 does not.

(A) (left) Representative merged images of DAPI and SA- β -galactosidase stainings. (Right) Quantification of the % of positive cells for SA- β -galactosidase. Error bars are means \pm SEM from 5 independent experiments. (B) Representative images of merged DAPI and HIRA stainings. (Right) Quantification of the % of HIRA positive cells. Error bars are means \pm SEM from 3 (2 for KRIT) independent experiments. (C) Representative images of the proliferation marker Ki-67 staining (green) merged with DAPI staining. (Right) Quantification of the percentage of cells positive for Ki-67 staining. Error bars are means \pm SEM from 3 (2 for KRIT) independent experiments. (D) Proliferation rate of siRNA transfected HUVEC measured by impedance using XCELLigence. Errors bars are means \pm SEM from 3 independent experiments.

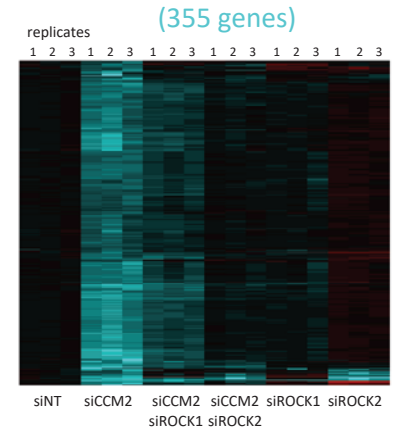
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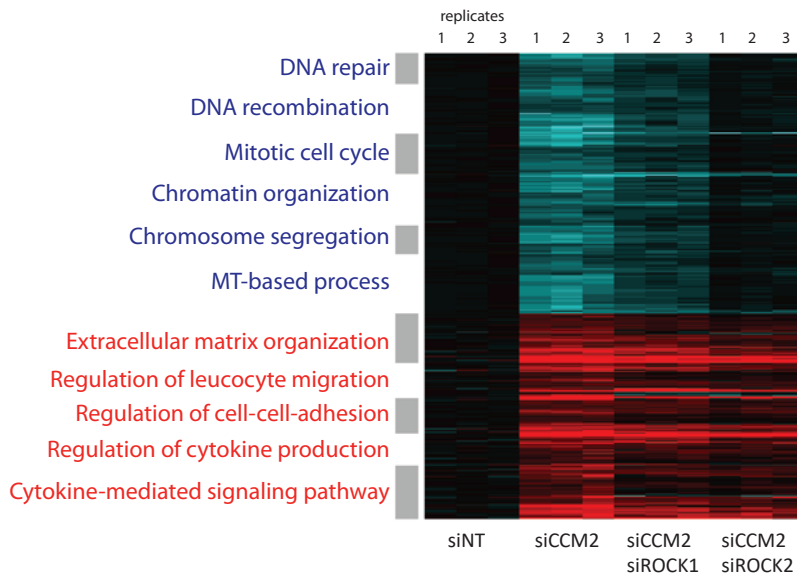
Upregulated DEGs



Downregulated DEGs



C



D

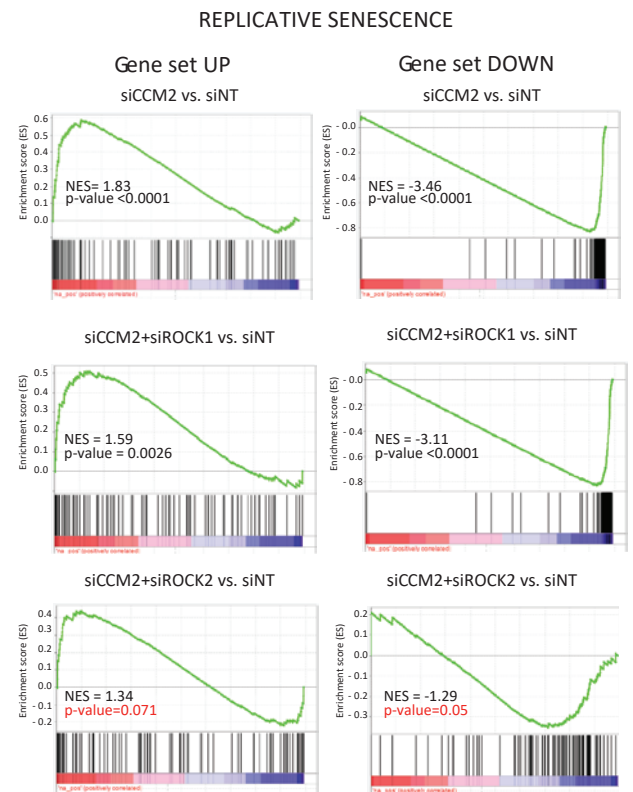
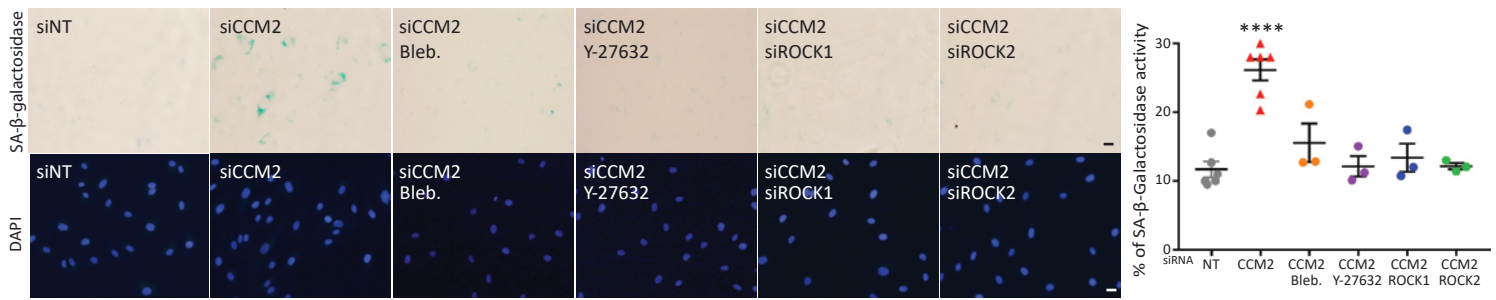


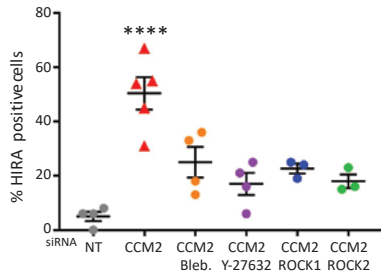
Figure 4: ROCK2 controls the SASP transcriptomic program of CCM2-depleted EC

(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 DEGs with $FC \geq 2$; $P < 0.05$ in siCCM2 HUVEC rescued by ROCKs. Upregulated (left) and downregulated (right) genes over the 6 siRNA conditions, 3 replicates per condition. (C) Clustered heatmap of GO enriched in up- (left) and down- (right) 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.

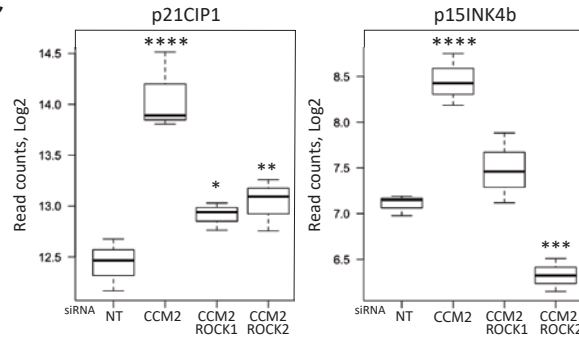
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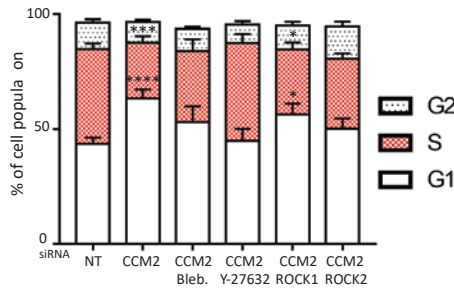
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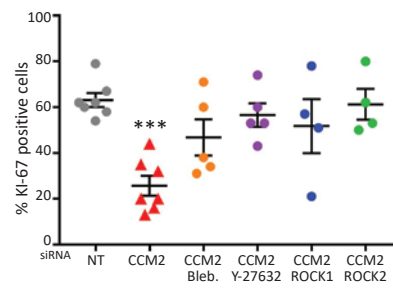
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D



E



F

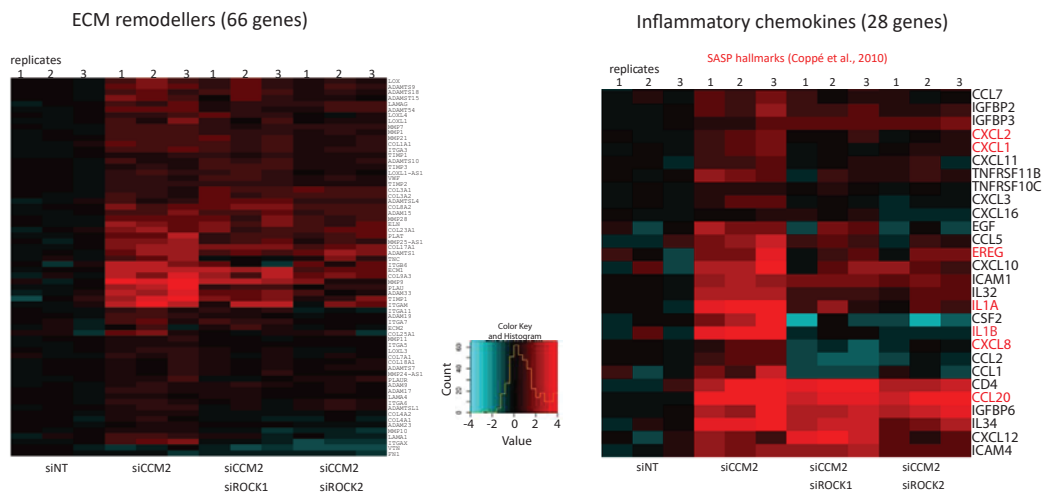
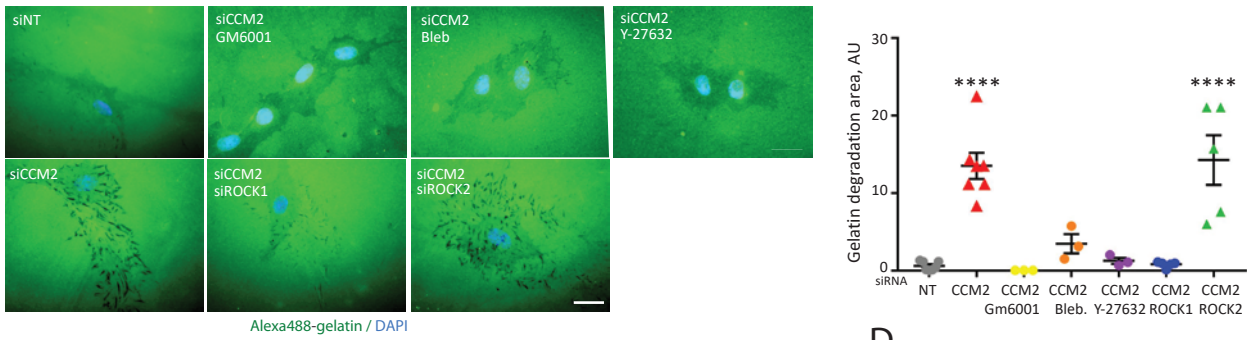


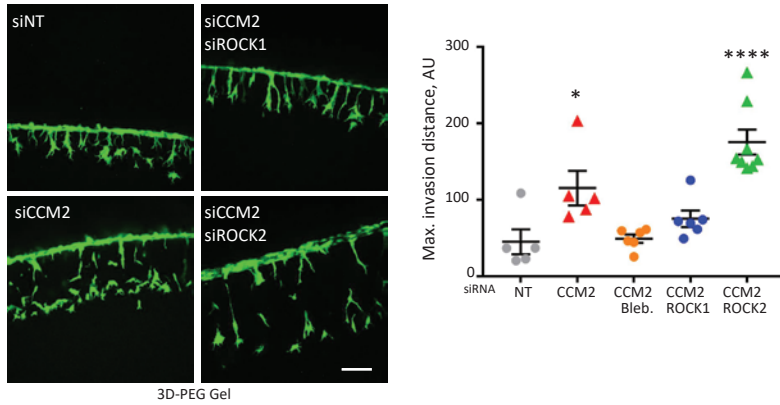
Figure 5: ROCKs dysfunctions induce premature senescence in CCM2-depleted EC.

(A)(left) Representative images of SA- β -galactosidase and DAPI stainings of siRNA-transfected HUVEC or treated with blebbistatin or Y27632 at 10 μ M. (Right) Quantification of the % of positive cells for SA- β -galactosidase. Error bars are means \pm SEM from 3 independent experiments. (B) Quantification of the % of HIRA positive cells in siRNA-transfected HUVEC or treated with blebbistatin or Y27632. Error bars are means \pm SEM from 4 (drug treatments) and 3 (ROCKs silencing) independent experiments. (C) Boxplots of the read counts for p21/CIP1 and p15/INK4b mRNA after depletion of ROCK1 or ROCK2. Error bars are means \pm SEM from 3 independent experiments. (D) Quantification by BrdU assay of the percentage of cells in each phase of the cell cycle for siRNA-transfected HUVEC or treated with blebbistatin or Y27632. Error bars are means \pm SEM from 5 (drug treatments) and 8 (ROCKs silencing) independent experiments. (E) 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. (F) Heatmap of expression of ECM remodelling proteins (left) and of SASP factors (right) over the 4 siRNA conditions, 3 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 HUVEC are the same as in figure 2.

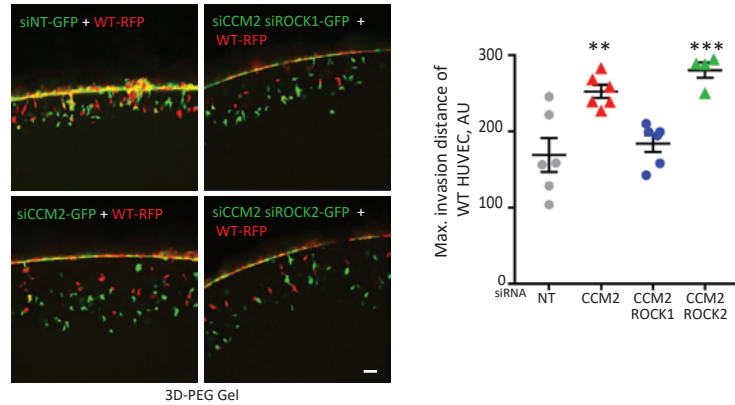
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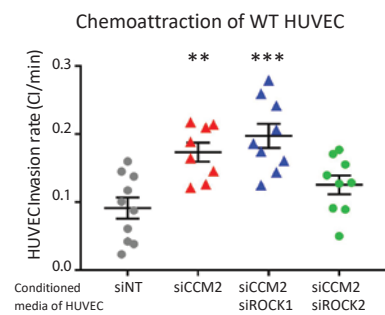
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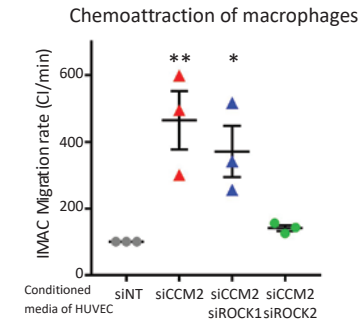


Figure 6: ROCK1 causes ECM degradation and invasion by CCM2-depleted HUVEC and neighbouring WT EC while ROCK2 causes chemo-attraction of WT EC and macrophages.

(A)(left) Representative images of the degradation of fluorescent gelatin by siRNA transfected HUVEC or treated with GM6001, blebbistatin or Y27632. Scale bars, 10 μ m. (right) Quantification of the area of gelatin degradation. Error bars are means \pm SEM from 3 (drug treatments) or 5 (silencing of ROCKs) independent experiments. (B)(left) Representative images of siRNA transfected GFP-HUVEC after invasion of 3D-PEG gels. Scale bar, 50 μ m. (right) Quantification of the maximum invasion distance of siRNA transfected HUVEC or treated with blebbistatin. Error bars are means \pm SEM from 3 independent experiments (2 to 3 technical replicates per condition) for siRNA transfected HUVEC and 2 for blebbistatin treated HUVEC.

(C)(left) Representative images of siRNA transfected GFP-HUVEC and RFP WT-HUVEC after invasion of 3D-PEG gels Scale bar, 50 μ m. (right) Quantification of the maximum invasion distance of RFP WT-HUVEC. Error bars are means \pm SEM from 3 independent experiments (2 to 3 technical replicates per condition).

(D) 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 cells. Error bars are means \pm SEM from 3 independent experiments. (E) Quantification of the rate of invasion of WT HUVEC measured in a modified Boyden chamber in real time using xCELLigence upon chemo-attraction by conditioned media of siRNA-transfected cells. Error bars are means \pm SEM from 3 independent experiments (2-4 technical replicates per condition).

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

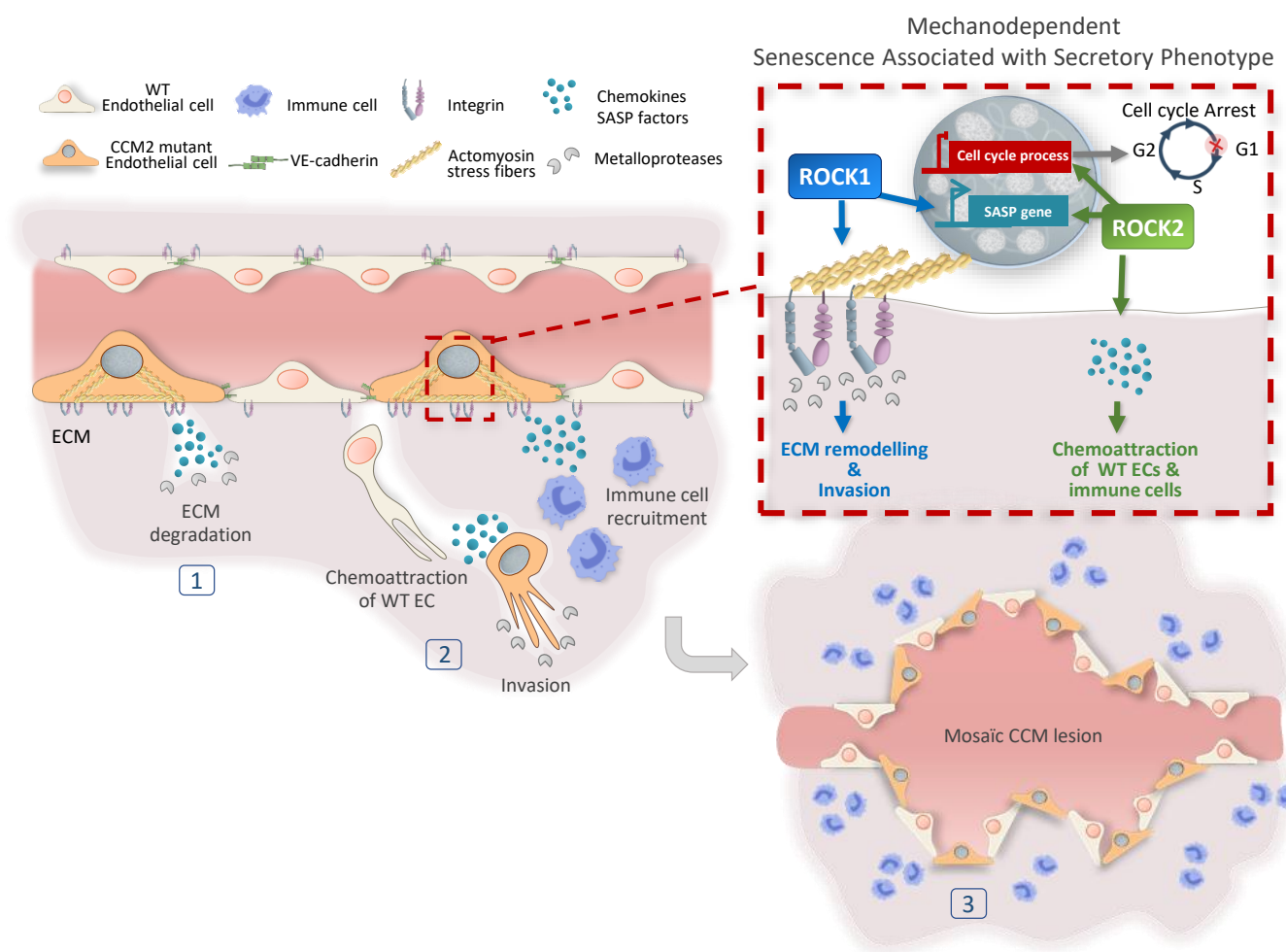
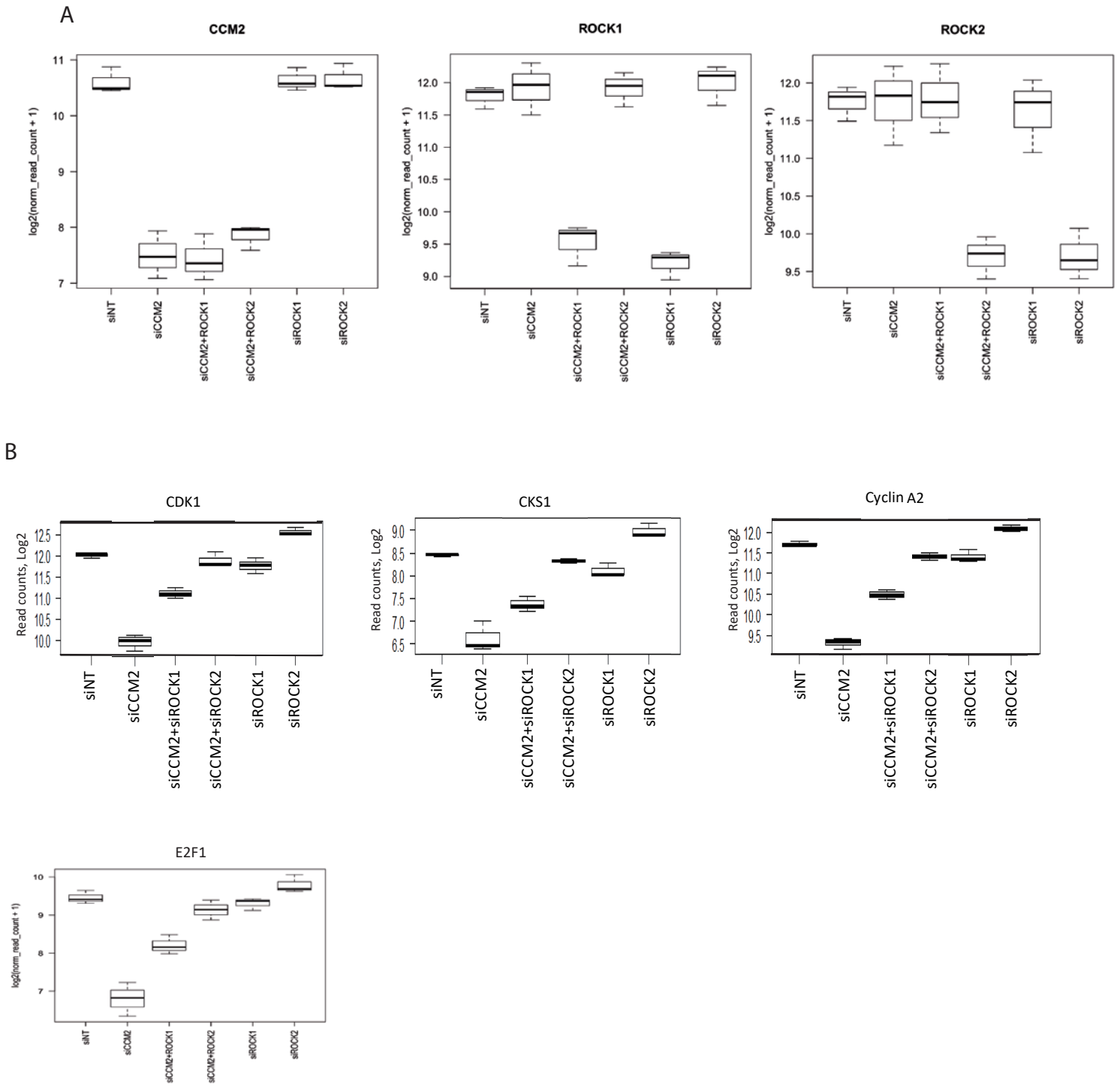
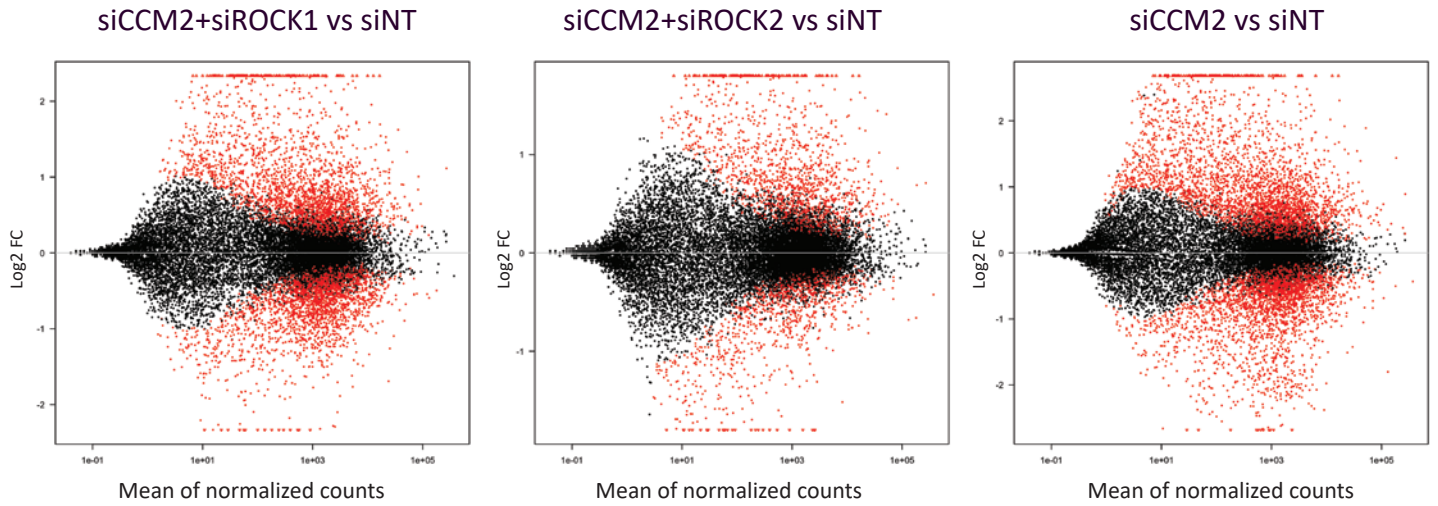


Figure 7: Proposed mechanism for CCM2 lesion mosaicism: a mechano-dependent SASP with complementary roles for ROCK1 and ROCK2. In low shear stress capillaries, the loss of CCM2 protein upon the second-hit mutation induces a ROCK-dependent premature senescence of the mutant endothelial cell associated with secretory phenotype (SASP). (1) This senescent cell acquires ECM degradative skills by secreting metalloproteases. (2) It invades the surrounding tissue, providing tracks for neighbouring WT EC and produces SASP factors that chemo-attract WT EC and immune cells. (3) These processes allow the formation and expansion of a mosaic CCM lesion. Dysregulated ROCKs have complementary key roles. Whereas, they both control the expression of genes involved in the SASP, with ROCK2 having a more prominent regulatory role, ROCK1 is specifically required for ECM invasion by mutant and WT EC. ROCK2 is specifically required for paracrine chemo-attraction of WT EC and immune cells.



FigS1: Boxplots of the expression level of CCM2, ROCK1, ROCK2 (A) and cell cycle regulators (B) in the 6 siRNA conditions as measured by RNA seq.

A



B

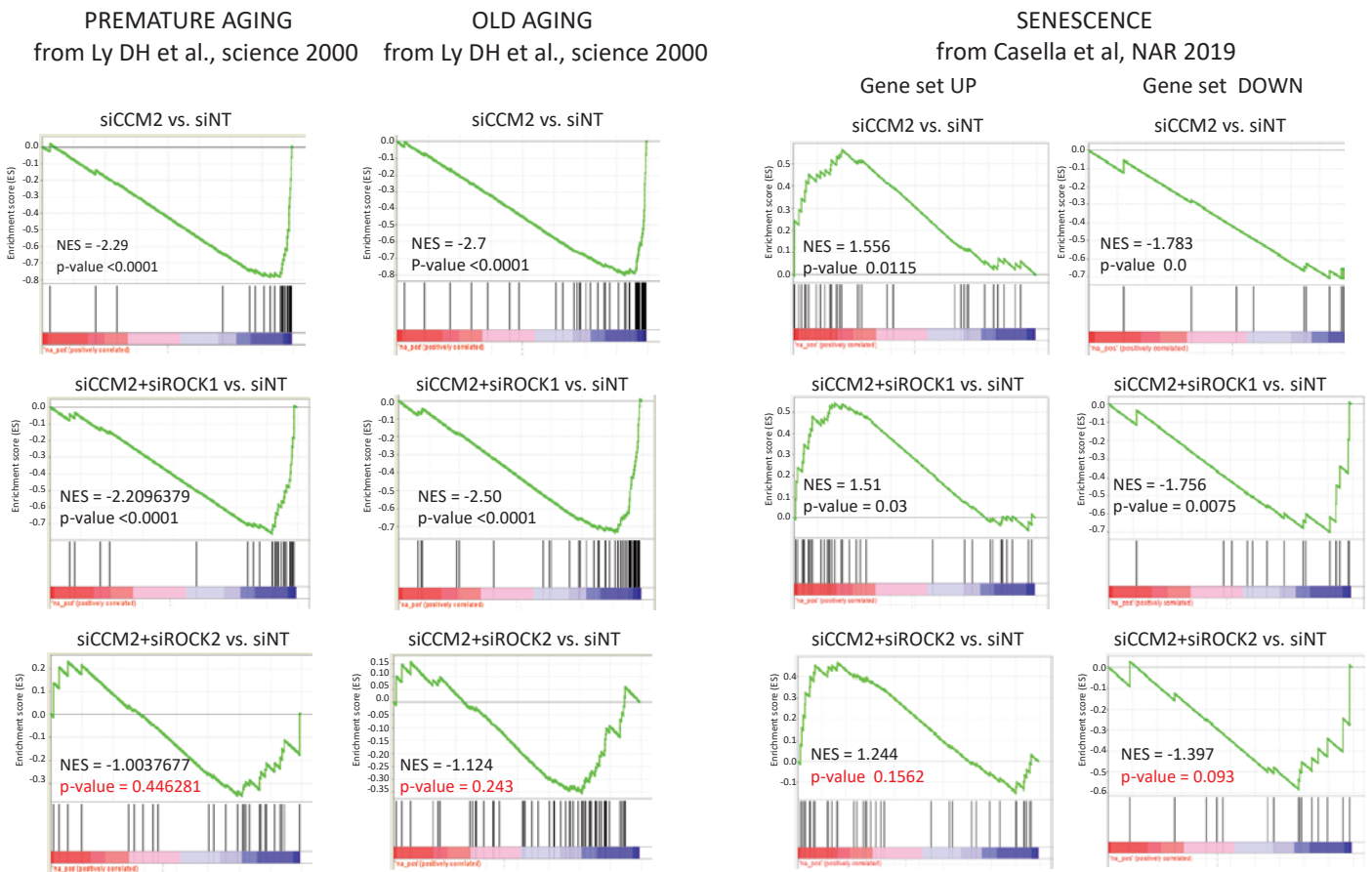


Fig S2: GSEA analyses using different gene sets of senescence.

(A) MA-plots showing the the better restoring effect of ROCK2 over ROCK1 on the DEG of CCM2-depleted HUVEC.
 (B) Comparison of the enrichment in different senescence and SASP signatures(38,40) in siCCM2, siCCM2+ROCK1 and siCCM2+ROCK2.

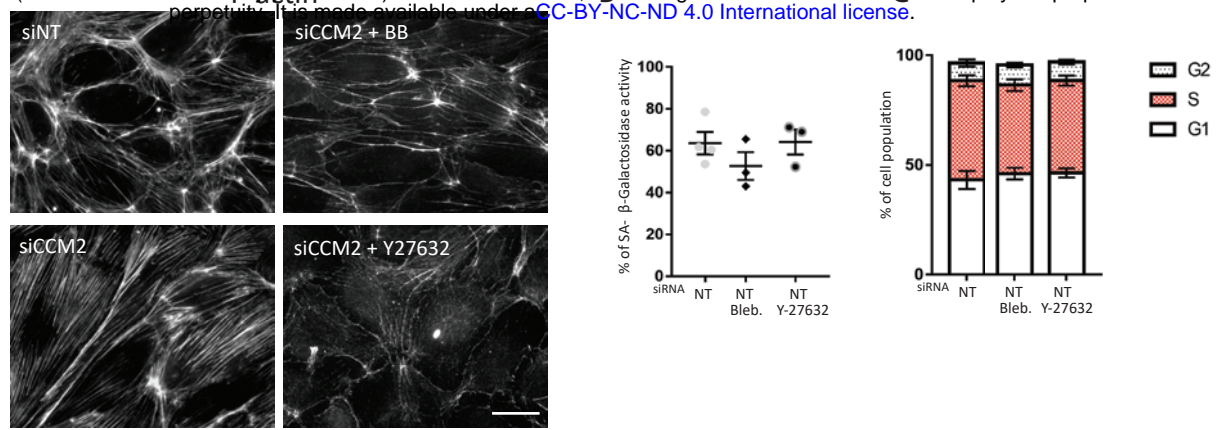


Fig S3: Effect of drugs on the actin cytoskeleton of siCCM2-HUVEC and on marks of senescence of siNT HUVEC.

(A) Representative immunofluorescence images of the actin cytoskeleton of CCM2 transfected HUVEC or HUVEC treated with drugs, scale bar 10 μ m. Effects of drug treatments on SA- β -galactosidase activity (B) cell cycle progression (C) and Ki67 staining (D) of siNT HUVEC.

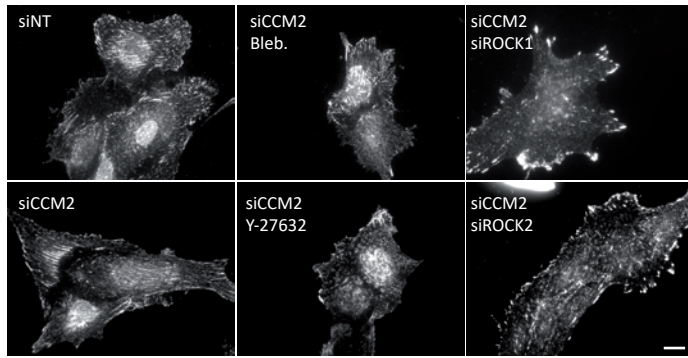


Fig S4: β1 integrin staining of siRNA transfected or drug treated HUVEC spread on gelatin.
Scale bar, 10 μm.