

1 **Title**

2 **Cytoplasmic innate immune sensing by the caspase-4 non-canonical**
3 **inflammasome promotes cellular senescence.**

4
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18 **Running title: control of cellular senescence by caspase-4**

19
20 **Key words**

21 Cellular senescence, SASP, caspase-4, non-canonical inflammasomes, gasdermin-D, oncogene-
22 induced senescence, LPS, innate immune signaling.

23
24 **Summary**

25 Cytoplasmic recognition of microbially derived lipopolysaccharides (LPS) in human cells is elicited by
26 the inflammatory cysteine aspartic proteases caspase-4 and caspase-5, which activate non-canonical
27 inflammasomes inducing a form of inflammatory programmed cell death termed pyroptosis. Here we
28 show that LPS mediated activation of the non-canonical inflammasome also induces cellular
29 senescence and the activation of tumour suppressor stress responses in human diploid fibroblasts.

30 Interestingly, this LPS-induced senescence is dependent on caspase-4, the pyroptotic effector protein
31 gasdermin-D and the tumour suppressor protein p53. Also, experiments with a catalytically deficient
32 mutant suggest that caspase-4 proteolytic activity is not necessary for its role in senescence.
33 Furthermore, we found that the caspase-4 non-canonical inflammasome is induced and assembled
34 during Ras-mediated oncogene-induced senescence (OIS). Moreover, targeting caspase-4 in OIS
35 showed that the non-canonical inflammasome is critical for SASP activation and contributes to
36 reinforcing the cell cycle arrest in OIS. Finally, we observed that caspase-4 induction occurs *in vivo* in
37 models of tumour suppression and ageing. Altogether, we are unveiling that cellular senescence is
38 induced by cytoplasmic microbial LPS recognition by the caspase-4 non-canonical inflammasome and
39 that this pathway is conserved in the senescence program induced by oncogenic stress.

40

41 **Introduction**

42 Cellular senescence is a cell state characterized by a proliferative cellular arrest, a secretory phenotype,
43 macromolecular damage and altered metabolism that can be triggered by several different stress
44 mechanisms (Gorgoulis et al., 2019). Senescent cells produce and secrete a myriad of soluble and
45 insoluble factors, including cytokines, chemokines, proteases and growth factors, collectively known as
46 the senescence-associated secretory phenotype (SASP) (Acosta et al., 2008; Coppe et al., 2008;
47 Kuilman et al., 2008) and interleukin-1 (IL-1) signalling is one of its critical signalling pathways (Acosta
48 et al., 2013; Orjalo et al., 2009). The role of the SASP in cancer is complex and mechanistically ill-
49 defined in ageing-associated diseases, two of the critical pathophysiological contexts where
50 senescence is functionally relevant (Faget et al., 2019; McHugh and Gil, 2018) . More recent evidence
51 proposes that different triggers might induce distinctive SASP subsets with concrete functions (Herranz
52 and Gil, 2018). Nonetheless, the SASP has started to incite interest as a potential therapeutic target in
53 disease (Paez-Ribes et al., 2019; Soto-Gamez and Demaria, 2017) . Therefore, a better understanding
54 of the molecular machinery regulating the SASP is needed.

55 Pattern recognition receptors (PRRs) of the innate immune system are molecular sensors that are
56 activated by microbial-derived pathogen-associated molecular patterns (PAMPs) or by damage-
57 associated molecular patterns (DAMPs or alarmins) generated endogenously in cells under certain
58 conditions of stress and damage (Takeuchi and Akira, 2010). Emerging data indicate a close
59 relationship between these PRRs and cellular senescence. For example, the SASP is initiated following

60 nucleic acid sensing by the cGAS-STING pathway and serum amyloid signalling through the PRR toll-
61 like receptor-2 (TLR2) during oncogene-induced senescence is critical for the SASP and the cell cycle
62 arrest (Dou et al., 2017; Gluck et al., 2017; Hari et al., 2019). Moreover, we have previously shown that
63 inflammasomes are crucial for the SASP (Acosta et al., 2013). Inflammasomes are multiprotein
64 platforms that induce the proteolytic activity of the inflammatory cysteine-aspartic protease caspase-1
65 which activates by proteolytic cleavage the proinflammatory cytokines IL-1 β and interleukin-18 (IL-18).
66 The canonical inflammasomes are assembled by PRRs of the nod-like receptor family, pyrin or by the
67 cytoplasmic DNA sensor AIM2 (Lamkanfi and Dixit, 2014; Man and Kanneganti, 2016). Alternatively,
68 the related inflammatory caspase-4 and caspase-5 (caspase-11 in mice) function as independent PRRs
69 for cytoplasmic microbial lipopolysaccharide (LPS) activating a non-canonical inflammasome. Critically,
70 activated non-canonical inflammasomes cleave the effector protein gasdermin-D, which induce a form
71 of inflammatory programmed cell death termed pyroptosis (Kayagaki et al., 2015; Shi et al., 2015; Shi
72 et al., 2014). Because the mechanism of SASP regulation by inflammasomes remain ill-defined, we
73 decided to define the role of these inflammatory caspases in senescence. We show here that caspase-
74 4 activation by cytoplasmic LPS triggers a senescence phenotype. Nonetheless, caspase-4 induction
75 and non-canonical inflammasome assembly were observed in RAS^{G12V}-oncogene-induced senescence
76 (OIS). Moreover, we show here that the caspase-4 non-canonical inflammasome contributes critically
77 to the establishment of the SASP and the reinforcement of the cell cycle arrest program during OIS, in
78 a mechanism that is independent on its catalytic activity over its downstream pyroptotic target
79 gasdermin-D. In all, we describe a new and critical function for cytoplasmic sensing by the caspase-4
80 non-canonical inflammasome in cellular senescence.

81

82 **Results**

83 ***Cytoplasmic LPS recognition by caspase-4 induces a senescent response in human diploid*** 84 ***fibroblasts.***

85 While the canonical inflammasome can be activated with the microbial-derived molecule muramyl-
86 dipeptide (MDP), the caspase-4 non-canonical inflammasome detects cytoplasmic bacterial LPS
87 inducing gasdermin-D cleavage and pyroptosis. Gasdermin-D is the best characterized functional
88 substrate of non-canonical inflammasomes, eliciting IL-1 β secretion and pyroptotic cell death (He et al.,
89 2015; Kayagaki et al., 2015; Shi et al., 2015). To compare the effect of activating the canonical and

90 non-canonical inflammasome in senescence, we activated the caspase-1 or the caspase-4
91 inflammasomes by transfection of MDP or LPS in human IMR90 fibroblasts respectively. Similarly to
92 other human cells expressing caspase-4 (Shi et al., 2014), IMR90 cells were sensitive to intracellular
93 LPS in a dose-dependent manner (Figure S1A-C). In contrast, MDP transfection did not induce cell
94 death (Figure S1A). LPS addition without further electroporation did not result in cell death, confirming
95 the requirement of an intracellular location for LPS to trigger pyroptosis (Figure S1C). Knockdown of
96 the pyroptosis effectors caspase-4 and gasdermin-D, but not caspase-1 impaired cell death mediated
97 by LPS transfection, confirming that the cells were dying by pyroptosis (Figure S1D-F). Noticeably, cell
98 death was detectable within the first hours after LPS transfection (Figure S1G). However, the fraction
99 of cells surviving cell death after these hours remained stable and viable (Figure S1H), and this
100 subpopulation was further examined.

101 Interestingly, this subset of cells displayed features of senescent cells such as decreased cell
102 proliferation, increased levels of the cyclin-dependent kinase inhibitors p16^{INK4a} and p21^{CIP1} and
103 increased senescence-associated- β -galactosidase (SA- β -galactosidase) activity (Figure S1I-J). In
104 contrast, MDP transfection did not induce a senescent response (Figure S1J), indicating a specific role
105 for the non-canonical inflammasome in PAMP-induced cellular senescence. Furthermore, the
106 acquisition of a senescent phenotype was also accompanied by increased levels of caspase-4 (Figure
107 S1J).

108 To examine the contribution of inflammatory caspases to the acquisition of a senescent phenotype
109 following LPS transfection, we downregulated the expression of *CASP1* or *CASP4* with shRNA before
110 transfection (Figure 1A). In contrast to caspase-1, caspase-4 was required for the acquisition of
111 senescent features such as increased SA- β -galactosidase activity, decreased cell proliferation and
112 induction of p21^{CIP1} and p16^{INK4a} in the subpopulation of cells surviving cell death (Figure 1B-D). Next,
113 human *CASP1* and *CASP4* were ectopically expressed in IMR90 fibroblasts (Figure S2A).
114 Overexpression of *CASP1* or *CASP4* alone resulted in a mild senescence induction with reduced cell
115 proliferation and increased SA- β -galactosidase activity (Figure S2B-C). However, *CASP4*
116 overexpression exacerbated the acquisition of senescent features in the presence of intracellular LPS
117 (Figure 1E-F, S2D), indicating that caspase-4 expression is critical for non-canonical inflammasome
118 induced senescence. Overall, these results suggest that the acquisition of a senescent phenotype
119 following intracellular LPS exposure is mediated through caspase-4 in a dose-dependent manner.

120 We decided to investigate the role of the caspase-4 substrate gasdermin-D and the critical senescence
121 regulator p53 in LPS-induced senescence and pyroptosis by targeting their expression with shRNAs.
122 In contrast to *GSDMD*, *TP53* knockdown failed to impair LPS mediated cell death, indicating a negligent
123 role for p53 regulating caspase-4 dependent pyroptosis (Figure S1F). However, *TP53* and *GSDMD*
124 knockdown significantly reduced LPS dependent cell growth arrest, and p53 and p21^{CIP1} induction
125 (Figure 1G-H, S2E). Interestingly, *GSDMD* knockdown also had a strong effect on p16^{INK4a} and
126 caspase-4 induction during LPS-induced senescence (Figure 1I, S2F). Altogether, these results
127 indicate that cytoplasmic LPS sensing by the non-canonical inflammasome induced a senescence
128 response that is dependent on caspase-4, gasdermin-D and p53 expression.

129

130 ***The caspase-4 mediated LPS-induced senescent response is independent of IL-1 β priming.***

131 Because overproduction of activated IL-1 β can have detrimental effects (Afonina et al., 2015), the
132 inflammasome activation is tightly regulated by a two-step mechanism. In some cases, a first signal,
133 also called priming, is required to boost transcriptional levels of *IL1B*. The priming signal is then followed
134 by a second signal which induces the assembly of the inflammasome (Lamkanfi and Dixit, 2014).
135 Intriguingly, senescence induction by the sole overexpression of *CASP4* or *CASP1* in IMR90 fibroblasts
136 did not induce transcriptional activation of *IL1B* (Figure 2A). In contrast, *IL1B* transcriptional levels were
137 increased upon LPS transfection in a caspase-4 dependent fashion (Figure 2B). We have previously
138 shown that TLR2 has a role in controlling *IL1B* expression and the SASP in cellular senescence (Hari
139 et al., 2019). Thus, we decided to investigate if TLR2 mediated inflammasome priming could synergize
140 with LPS-mediated caspase-4 induced senescence. As expected, the addition of the synthetic
141 lipopeptides Pam2CSK4 and Pam3CSK4 (TLR2/6 and TLR1/2 agonists, respectively) but not LPS
142 (TLR4 agonist) nor MDP to IMR90 cells highly induced *IL1B* mRNA levels (Figure S2G). Then, we
143 observed that priming the inflammasome with the TLR2 agonist Pam2CKS4 significantly synergizes
144 with LPS transfection to produce a robust IL-1 β induction, and this induction was further enhanced by
145 *CASP4* ectopic overexpression (Figure 2C). However, we did not observe a dramatic increase in cell
146 cycle arrest or SA- β -galactosidase activity in LPS-induced senescence by addition of Pam2CKS4
147 (Figure 2D-E). Similar results were observed when TLR2 overexpressing cells were primed with the
148 endogenous senescence-associated TLR2 alarmin A-SAA (Hari et al., 2019) or Pam2CKS4 with LPS
149 transfection, which synergized in producing robust *IL1B* and SASP induction but without affecting

150 dramatically LPS-induced cell cycle arrest or SA- β -Galactosidase activity (Figure S2H-L). However, the
151 observed increase in *IL1B* mRNA levels in LPS transfected cells in all conditions is minimal if compared
152 to the logarithmic increase observed in OIS (Figure 2C). These results suggest that additional signals
153 to caspase-4 stimulation such as a sustained priming signalling are necessary for a robust *IL1B* and
154 SASP induction during LPS-mediated cellular senescence. Moreover, these data suggest that the LPS-
155 induced caspase-4 senescent response is independent of *IL1B* and the SASP.

156

157 ***The caspase-4 proteolytic activity is not necessary for LPS-induced senescence.***

158 The active site of human caspase-4 has been well characterized and is associated to the residue C258
159 (Faucheu et al., 1995), and point mutations of this amino acid renders the protein catalytically inactive
160 (Shi et al., 2014; Sollberger et al., 2012). To further study the contribution of the protease activity of
161 caspase-4 protease to senescence, *CASP4* wild-type and the catalytically death mutant C258A
162 (*CASP4*^{C258A}) were overexpressed in IMR90 cells, and the phenotypical outcomes were assessed.
163 Overexpression of wild-type *CASP4* or *CASP4*^{C258A} reduced cellular proliferation and increased SA- β -
164 galactosidase activity to a similar extent (Figure 3A-B). Next, *CASP4* wild-type and *CASP4*^{C258A} were
165 stably overexpressed in IMR90 fibroblasts before LPS transfection (Figure 3C). *CASP4*^{C258A} but not
166 *CASP4* wild-type expressing IMR90 cells were resistant to cell death after LPS transfection (Figure 3D,
167 S3A), indicating a dominant negative role for the caspase-4 defective form in pyroptosis. However, both
168 *CASP4* wild-type and *CASP4*^{C258A} overexpressing cells remained equally sensitive to the acquisition of
169 senescent features after LPS challenge (Figure 3E-F, S3B). These results suggest that, in contrast to
170 caspase-4 mediated pyroptosis, the role of caspase-4 in LPS-induced senescence is independent of
171 its catalytic activity.

172

173 ***The caspase-4 non-canonical inflammasome is induced and assembled during oncogene-***
174 ***induced senescence.***

175 Next, the role of inflammatory caspases in oncogene-induced senescence (OIS) was investigated. To
176 induce OIS, *HRAS*^{G12V} (hereafter *RAS*^{G12V}) was constitutively overexpressed in IMR90 human
177 fibroblasts. *RAS*^{G12V} overexpression reduced cellular proliferation and increased SA- β -galactosidase
178 activity (Figure 4A). Coinciding with the upregulation of the cell cycle inhibitors p21^{CIP1}, p16^{INK4a} and
179 p15^{INK4b}, caspase-4 expression was increased both at the mRNA and protein levels upon *RAS*^{G12V}

180 overexpression (Figure S4A-B, 4B-C). Next, we took advantage of an inducible system extensively
181 employed by us and others to exert tight control over the onset of senescence (Boumendil et al., 2019).
182 In this system, a mutant form of the estrogen receptor (ER) ligand-binding domain is fused to the protein
183 of interest (RAS); consequently, ER:RAS cells undergo OIS after addition of 4-hydroxytamoxifen
184 (4OHT) (Figure 4D). As expected, IMR90 ER:RAS cells underwent cell proliferation arrest and showed
185 increased SA- β -galactosidase activity compared to non-treated IMR90 ER:RAS upon 4OHT addition
186 (Figure 4D). A time-course experiment using this system revealed that *CASP4* mRNA levels increase
187 in parallel to the exponential increase in *IL1B* mRNA expression in cells undergoing OIS (Figure 4E-F),
188 and in caspase-4 protein (Figure 4G). Also, we observed caspase-4 induction in paracrine senescence,
189 and senescence induced by DNA damage with etoposide (Figure S4C-D). Oligomerization of caspase-
190 4 protein is essential for its activity (Shi et al., 2014). These caspase-4 oligomers can be revealed in
191 lysates crosslinked with disuccinimidyl suberate (DSS) as high weight migratory bands in a western blot
192 (Choi et al., 2019). Endogenous caspase-4 oligomerization was detected in IMR90 ER:RAS senescent
193 cells but not in control cells from 3 days after 4OHT treatment (Figure 4H, S4E). Moreover, caspase-4
194 proteolytic activity was also increased in IMR90 ER:RAS cells 4 and 8 days after the addition of 4OHT
195 (Figure 4I). Altogether these results demonstrate that caspase-4 expression is induced and the non-
196 canonical inflammasome is assembled in OIS.

197

198 ***The caspase-4 non-canonical inflammasome is required for inflammatory signalling in OIS.***

199 Next, a functional role for caspase-4 in OIS was investigated in our IMR90 ER:RAS system. Global
200 changes in mRNA expression upon *CASP4* depletion were analyzed. IMR90 ER:STOP and ER:RAS
201 cells were transfected with control and *CASP4*-targeting small-interfering RNA (siRNA), samples were
202 collected 5 and 8 days after 4OHT addition and subjected to transcriptomic analysis (Figure 5A).
203 Knockdown control of the experiment was performed by mRNA expression analysis for *CASP4*,
204 showing a substantial reduction of its expression upon siRNA targeting at both time points (Figure S5A).
205 Furthermore, *CASP4* was the top downregulated gene in *CASP4* siRNA-targeted compared to non-
206 target siRNA control RAS^{G12V} cells both 5 and 8 days after the addition of 4OHT (Figure S5B).
207 Similarities between replicates and differences between conditions were confirmed by principal
208 component analysis visualization and heatmap sample clustering (Figure S5C-D). Differentially
209 expressed gene analysis identified 557 and 478 genes significantly differentially expressed (FDR 10%)

210 upon *CASP4* knockdown, of which 340 and 240 were induced in a *CASP4* dependent fashion in
211 *RAS^{G12V}*-OIS cells 5 and 8 days after 4OHT addition respectively (Figure 5A). Gene set enrichment
212 analysis (GSEA) of 50 hallmark gene sets of the transcriptomic data showed a *CASP4* dependent
213 regulation in *RAS^{G12V}*-OIS cells of gene sets related to inflammatory processes, including TNF- α
214 signalling and interferon responses, both 5 and 8 days after the addition of 4OHT (Figure 5B).
215 Enrichment plots of the gene signature hallmark “INFLAMMATORY RESPONSE” showed a positive
216 correlation of this gene set expression with *CASP4* (Figure S5E). Plotting a heatmap of the fold change
217 values of control IMR90 ER:STOP and *CASP4* knockdown vs control IMR90 ER:RAS cells of all genes
218 included in the “INFLAMMATORY RESPONSE” gene set revealed a pattern by which the increased
219 expression of inflammatory-related genes in senescence is abrogated if *CASP4* is targeted, including
220 SASP factors (Figure 5C). Changes in the expression of *IL1A* and *IL1B* were validated by RT-qPCR
221 (Figure 5D-E). The serum amyloid A (SAA) proteins SAA1 and SAA2 belong to a family of
222 apolipoproteins known to activate innate and adaptive immune cells and have recently been identified
223 as SASP factors (Hari et al., 2019). Of note, the expression of SAA1 and SAA2 was also decreased
224 when *CASP4* was targeted in OIS (Figure 5F-G). Targeting *CASP4* also reduced the amount of
225 intracellular IL-1 α , IL-1 β , IL-6 and IL-8 protein to a similar extent than *CASP1* targeting (Figure S5F).
226 Moreover, the levels of intracellular mature IL-1 β were also significantly and similarly reduced when
227 either *CASP1* or *CASP4* were targeted (Figure 5H). Furthermore, the concentration of secreted IL-1 β
228 was significantly reduced in conditioned media of RAS induced cell cultures when *CASP4* was targeted
229 (Figure 5I). Overall, these results suggest that caspase-4 upstream of caspase-1 are required for a full
230 SASP activation in OIS.

231 The SASP can induce senescence in adjacent growing cells through paracrine signaling, which is
232 dependent on IL-1 signaling (Acosta et al., 2013). Because *CASP1* and *CASP4*-targeting reduced the
233 production of several SASP factors and, in particular, limited the secretion of IL-1 β , we next examined
234 whether inflammatory caspases are implicated in SASP induction during paracrine senescence (Figure
235 S5G). Conditioned media from IMR90-ER:RAS senescent cells added to growing IMR90 fibroblasts
236 produced the induction of *IL1A*, *IL1B*, *IL8* and *IL6*, which was impaired when *CASP4* was targeted
237 (Figure S5G). In contrast, *CASP1* targeting did not affect the induction of the paracrine SASP (Figure
238 S5G). Overall, these data suggest that the caspase-4 non-canonical inflammasome controls SASP
239 activation during paracrine senescence.

240 We then investigated the role of gasdermin-D in OIS. While mRNA levels remained unaffected (Figure
241 S6A), gasdermin-D was found to be cleaved during OIS (Figure S6B). However, in contrast to *CASP1*
242 or *CASP4*-targeting, *GSDMD* knockdown did not impair *IL1A*, *IL1B*, *IL8* or *IL6* mRNA induction (Figure
243 S6C-D). Moreover, whereas targeting either *CASP1* or *CASP4* resulted in a significantly lower
244 concentration of IL-1 β in conditioned media from OIS cells, *GSDMD* knockdown did not alter IL-1 β
245 secretion (Figure S6E), suggesting that IL-1 β secretion is dependent on caspase-1 and caspase-4 but
246 independent on gasdermin-D in OIS. Altogether, these results indicate that the caspase-4 non-
247 canonical inflammasome is a critical regulator of the SASP in OIS.

248

249 ***The caspase-4 non-canonical inflammasome contributes to the cell cycle arrest in OIS.***

250 To investigate the role of the non-canonical inflammasome in regulating the cell cycle arrest program
251 in OIS, we targeted *CASP4* using RNAi (Figure S6F). *CASP4*-targeting significantly rescued early
252 proliferation arrest during OIS (Figure 6A) and increased the total cell content in a long-term cell growth
253 assay (Figure 6B). Moreover, targeting *CASP4* during OIS modestly but significantly decreased SA- β -
254 galactosidase activity (Figure S6G-H). A GSEA of 50 hallmark gene sets showed a negative regulation
255 of *CASP4* in *RAS*^{G12V}-OIS of the gene signatures “G2M CHECKPOINT” and “E2F TARGETS”, and the
256 expression of the *CDKN2A* (p16^{INK4a}-p14^{ARF}) and *CDKN2B* (p15^{INK4b}) locus, but not p21^{CIP1} (Figure 5B,
257 6C, S6H). Indeed, targeting *CASP4* reduced p16^{INK4a} expression and rescued the phosphorylation of
258 pRb (Figure 6D, S6I) and resulted in a transcriptional increase in the levels of E2F target genes (Figure
259 6E), suggesting a role for caspase-4 in cell cycle regulation by controlling p16^{INK4a} and p15^{INK4b}
260 expression in OIS. Of note, activation of caspase-4 by intracellular LPS resulted in hypophosphorylated
261 pRb and increased levels of E2F target genes (Figure S6J-K). Finally, similarly to SASP regulation,
262 *GSDMD* targeting did not alter the cell cycle arrest in OIS (Figure S6L), suggesting that gasdermin-D
263 has no significant role in OIS.

264 Altogether, these results suggest that caspase-4 contributes to the proliferation arrest during
265 senescence, impacting ultimately on the phosphorylation state of pRb resulting in transcriptional
266 repression of E2F target genes.

267

268

269

270 ***Caspase-11 is induced during cellular senescence in vivo.***

271 We have shown that caspase-4 expression levels are critical in cellular senescence. To investigate
272 non-canonical inflammasome expression in senescence *in vivo*, we used three well-characterized
273 mouse models of senescence. We first analysed the caspase-4 murine homologous caspase-11
274 expression in a model of OIS in which conditional expression of *Kras*^{G12D} by *Pdx-CRE* induces
275 Pancreatic Intraepithelial Neoplasia (PanIN) in the pancreas of mice (Morton et al., 2010) (Figure 7A).
276 We observed that low grade PanINs stained positive for caspase-11 when compared to surrounding
277 pancreatic acinar cells, higher grade PanINs, and in the ducts and acinar cells in wild-type mice (Figure
278 7A). Importantly, quantification of Ki-67 staining in PanINs showed that the expression of caspase-11
279 was restricted to early senescent lesions with low proliferative index (Figure 7B), indicating that
280 caspase-11 expression correlates with senescence in low-grade PanIN lesions.

281 We then investigated the expression of caspase-11 in two additional models of senescence *in vivo*. We
282 detected increased expression of caspase-11 and the markers of senescence lipofuscin in lung airways
283 in a model of inflammatory mediated activation of senescence by constitutive activation of NF-κB by
284 knockout of its regulator *nfkb1*^{-/-} (*p50*^{-/-}) (Jurk et al., 2014) (Figure 7C). Moreover, we observed an
285 increase in the number of cells positively expressing caspase-11 in alveolar cells of the lung during
286 organismal ageing (Figure 7D). In summary, these results support the model that non-canonical
287 inflammasomes contribute to senescence *in vivo*.

288

289 **Discussion**

290 Here, we show that cytoplasmic LPS recognition by the caspase-4 non-canonical inflammasome
291 induces a senescence response, in which sublethal levels of LPS activate the p16^{INK4a}-pRb and p53-
292 p21^{CIP1} tumour suppressor pathways. Interestingly, while LPS-mediated pyroptosis requires caspase-4
293 and the effector protein gasdermin-D but not p53, the senescence response requires the participation
294 of p53. These results suggest a mechanism in which p53 controls the cellular stress responses to
295 microbial infection downstream of caspase-4 until a certain threshold level in which gasdermin-D-
296 dependent pyroptosis eliminates highly damaged cells, introducing a new context-dependent role for
297 p53 in innate immune sensing (Kastenhuber and Lowe, 2017). Further research will be necessary to
298 determine the functional role of senescence in response to infection.

299 Our results describe a new mechanism of senescence induction by cytoplasmic microbial sensing,
300 adding to the essential triggers such as the DNA Damage Response, telomere attrition, oncogenic
301 activation, mitochondrial damage or ribosome biogenesis inactivation (Gorgoulis et al., 2019; Pantazi
302 et al., 2019). We also show that both the expression and the assembly of the caspase-4 non-canonical
303 inflammasome are triggered upon oncogene activation. Furthermore, we observe that caspase-4 is
304 critical for SASP induction and contributes to the cell cycle arrest in OIS. Thus, these results suggest
305 that the role of caspase-4 in senescence is conserved in a sterile context and that there is crosstalk
306 between anti-microbial immune responses and tumour suppression. Notably, the assembly of the
307 caspase-4 inflammasome is an early event in OIS, peaking after day three to four after Ras activation
308 (Figure 4). Recent results suggest that OIS is a highly dynamic process, with distinct signalling waves
309 contributing to the establishment of the senescent phenotype. Interestingly, the specific time point
310 where caspase-4 is assembled coincides with the moment of transition to a proinflammatory, NF- κ B -
311 dependent SASP in OIS (Hoare et al., 2016) (Martinez-Zamudio et al., 2020). It is plausible that the
312 activation of the non-canonical inflammasome could play a critical role in this transition.

313 Unexpectedly, our results indicate that LPS-mediated caspase-4 induced senescence is not
314 accompanied by robust activation of IL-1 β and the SASP. Interestingly, a significant SASP induction is
315 only achieved when LPS stimulation and priming of the inflammasome with TLR2 ligands happen
316 simultaneously, suggesting that the control of cellular senescence by caspase-4 is independent of the
317 SASP. However, our data shows that caspase-4 heavily influences the SASP induction during OIS,
318 where sustained production of A-SAA signaling through TLR2 is critical to the SASP (Hari et al., 2019).
319 Thus, caspase-4 appears to be central regulating cell fate decisions occurring upon microbial-
320 cytoplasmic recognition or sterile cellular stresses controlling the induction of cellular senescence, the
321 SASP, or pyroptosis. Further research will be required to identify the nature of the signal responsible
322 for caspase-4 activation during OIS.

323 Mechanistically, our data suggest that the role of caspase-4 in senescence is independent from its
324 catalytic activity. Caspase-4 is a pattern recognition receptor that binds LPS directly (Shi et al., 2014);
325 therefore, it is plausible that upon ligand recognition, caspase-4 functions as a scaffold platform for the
326 activation of downstream senescence pathways in a proteolytic independent fashion. Our data suggests
327 that pyroptosis is triggered only at a certain threshold of caspase-4 induction, suggesting a dose-
328 dependent functional split between the caspase-4 pro-senescent and pyroptotic functions.

329 Inflammasomes have shown diverse pro- and anti-tumorigenic functions in cancer (Karki and
330 Kanneganti, 2019). Here, we show that caspase-4 expression is induced following tumour initiation in
331 a genetically engineered mouse model of pancreatic cancer and during ageing, suggesting a
332 suppressive role for this pathway in cancer initiation. In recent years several strategies have been
333 implemented to eliminate senescent cells or to modulate the activation of the SASP in anti-ageing and
334 cancer therapies. (Baker et al., 2016; Baker et al., 2011; Dorr et al., 2013). Furthermore, the
335 pharmacological targeting and removal of senescent cells has been shown to improve homeostasis
336 following tissue damage and ageing (Baar et al., 2017; Chang et al., 2016). Here we propose that
337 manipulation of non-canonical inflammasomes could provide a new rationale for senotherapies and the
338 implementation of pyroptosis for senolysis in cancer and ageing.

339

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349

350 **Author contributions**

351 Conceptualisation, I.F.D and J.C.A.; Formal Analysis, I.F.D, P.H, N.T., J.B., J.F.P. and J.C.A;
352 Investigation, I.F.D, P.H., F.R.M., N.T., A.Q., J.B., and J.C.A.; Resources, J.C.A and V.G.B.; Data
353 Curation, I.F.D. and J.C.A; Writing-Original Draft I.F.D and J.C.A.; Writing-Review & Editing, F.R.M.;
354 Visualisation, I.F.D., N.T., J.B., J.F.P. and J.C.A; Supervision, J.C.A.; Funding Acquisition, J.C.A.

355

356 **Declaration of interests**

357 The authors declare that they have no competing interest.

358

359 **Figures Legends**

360

361 **Figure 1**

362 **LPS-mediated caspase-4 activation induces a senescent phenotype in human primary**
363 **fibroblasts**

364 (A) Scheme of the experiment shown in B-D and Supplementary Figure D-E. IMR90 cells were infected
365 with an empty pRS vector (vector) or a pRS vector targeting either *CASP1* (shC1) or *CASP4* (shC4)
366 prior to transfection with 0.1 μg LPS / 5×10^5 cells. The acquisition of senescent features after LPS
367 transfection was assessed by IF, RT-qPCR and SA- β -Gal activity.

368 (B) SA- β -Gal activity was determined 4 days after transfection. Representative images for SA- β -Gal
369 activity are shown.

370 (C) BrdU incorporation and p16^{INK4a}, p21^{CIP1} and caspase-4 levels of surviving cells were measured by
371 IF 48 h after LPS transfection.

372 (D) *CDKN1A* (p21^{CIP1}) and *CDKN2A* (p16^{INK4a}) mRNA relative expression was quantified by RT-qPCR
373 48 h after transfection.

374 (E) *CASP4* was stably overexpressed prior to LPS transfection. Cells were transfected with increasing
375 concentrations of LPS (0.1 or 1 μg LPS / 5×10^5 cells) and BrdU incorporation was measured 48 h
376 after transfection.

377 (F) Cells were treated as in (E) and SA- β -Gal activity was determined 4 days after transfection.
378 Representative images for SA- β -Gal activity are shown.

379 (G to I) IMR90 cells were stably infected with an empty pRS vector (vector) or a pRS vector targeting
380 either *CASP4* (shC4), *GSDMD* (shGSDMD) or *TP53* (shP53) prior to transfection with 0.1 μg LPS / $5 \times$
381 10^5 cells. BrdU incorporation (G) and the levels of p21^{CIP1} (H) and p16^{INK4a} (I) were measured by IF 48
382 h after LPS transfection.

383 Statistical significance in B, D-I was calculated using one-way analysis of variance (ANOVA). Statistical
384 significance in C was calculated using two-tailed Student's *t*-test. *****P* < 0.0001, ****P* < 0.001, ***P* <
385 0.01, and **P* < 0.05. ns, not significant. All error bars represent mean \pm s.e.m; A represents 4 and B, D-
386 I represents 3 independent experiments.

387

388

389 **Figure 2**

390 **LPS-mediated caspase-4 induced senescence is independent on inflammasome priming**

391 (A) *IL1B* mRNA relative expression levels were quantified by RT-qPCR in IMR90 infected with *CASP4*,
392 *CASP1*, *RAS*^{G12V} expression vectors or empty vector (vector) control.

393 (B) Cells were treated as shown in Figure 1A. *IL1B* mRNA relative expression was quantified by RT-
394 qPCR 48 h after LPS transfection.

395 (C-E) IMR90 cells were infected with *CASP4* or *RAS*^{G12V} expression vectors or empty vector (vector)
396 control. After 3 h treatment with Pam2CSK4, cells were transfected with LPS (0.1 µg LPS / 5 x 10⁵
397 cells). *IL1B* mRNA relative expression (C) and BrdU incorporation (D) were measured by IF and RT-
398 qPCR respectively 48 h after LPS transfection. (E) SA-β-Gal activity was determined 4 days after LPS
399 transfection. Representative images are shown.

400 For A and B, sstatistical significance was calculated using one-way analysis of variance (ANOVA). ****P
401 < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. All error bars represent mean ± s.e.m of 3
402 independent experiments.

403 For C-E, sstatistical significance was calculated using one-way analysis of variance (ANOVA). ****P <
404 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05. Error bars represent the average ± range of 2
405 representative experiments.

406

407

408 **Figure 3**

409 **Caspase-4 mediated regulation of senescence is independent of its catalytic function**

410 (A-B) IMR90 cells were infected with wild-type (WT) *CASP4*, catalytically inactive (C258A) *CASP4* or
411 the empty vector (vector). Overexpression of *RAS*^{G12V} was used as a positive control for the induction
412 of senescence. (A) Caspase-4 levels, BrdU incorporation and SA-β-galactosidase activity were
413 measured 4 days after equal number of cells were seeded. (B) Relative cell content (left) was quantified
414 15 days after equal number of cells were seeded; representative images (right) of crystal violet stained
415 cells are shown.

416 (C) Scheme of the experiment shown in D-F and Supplementary Figure A-B. IMR90 cells were infected
417 with wild-type (WT) *CASP4*, catalytically inactive (C258A) *CASP4* or the empty vector (vector) prior to
418 transfection with 0.1 µg LPS / 5 x 10⁵ cells.

419 (D) Cell viability was measured 24 h after transfection.

420 (E) BrdU incorporation was measured 48 h after transfection.

421 (F) SA- β -Gal activity was determined 4 days after transfection.

422 Statistical significance in A and B was calculated using one-way analysis of variance (ANOVA).

423 Statistical significance in D-F was calculated using two-tailed Student's *t*-test. *****P* < 0.0001, ****P* <

424 0.001, ***P* < 0.01, and **P* < 0.05. ns, not significant. All error bars represent mean \pm s.e.m of 3

425 independent experiments.

426

427 **Figure 4**

428 **The caspase-4 non-canonical inflammasome is activated in Oncogene-induced senescence**

429 (A) IMR90 cells were infected with *RAS*^{G12V} expression vector to induce OIS or empty vector (vector).

430 BrdU incorporation and SA- β -Gal activity were measured 4 days after equal number of cells were

431 seeded (left). Representative images (right) for SA- β -Gal activity are shown.

432 (B) *CASP4* mRNA relative expression was quantified in IMR90 cells undergoing *RAS*^{G12V} after infection

433 with *RAS*^{G12V} expression vector-

434 (C) IMR90 cells were infected with *RAS*^{G12V} expression vector or empty vector (vector). BrdU

435 incorporation and caspase-4 levels were measured by IF in *RAS*^{G12V}-OIS and control cells 4 days after

436 equal number of cells were seeded.

437 (D) IMR90 cells were infected with a control (ER:STOP) or an ER:RAS vector. Upon addition of 4OHT,

438 ER:RAS cells undergo OIS.

439 (E) Time-course experiment of *CASP4* mRNA relative expression in IMR90 ER:STOP and ER:RAS

440 cells treated with 4OHT for 0, 2, 4, 6 and 8 days.

441 (F) Time-course experiment of *IL1B* mRNA relative expression in IMR90 ER:STOP and ER:RAS cells

442 treated with 4OHT for 0, 2, 4, 6 and 8 days.

443 (G) IMR90 ER:STOP and ER:RAS were treated or not with 4OHT during 8 days. Caspase-4, IL-1 β and

444 IL-8 levels were analyzed by immunoblotting.

445 (H) IMR90 ER:STOP and ER:RAS cells were treated with 4OHT for five days, then cells were collected

446 and subjected to disuccinimidyl suberate (DSS) crosslinking. After SDS-PAGE separation, both DSS-

447 crosslinked samples and inputs were probed for caspase-4 by immunoblotting.

448 (I) IMR90 ER:STOP and ER:RAS cells were treated with 4OHT and LEVD-AFC cleavage was
449 measured in low serum (0.5% FBS) cultured cells 0, 4 and 8 days after 4OHT addition.

450 All statistical significance was calculated using using two-tailed Student's *t*-test. ****P* < 0.001, ***P* <
451 0.01, and **P* < 0.05 and ns, not significant. All error bars represent mean ± s.e.m of 3 independent
452 experiments.

453

454 **Figure 5**

455 **Caspase-4 activation controls the proinflammatory SASP**

456 (A) Schematic diagram of the experimental approach. ER:STOP and ER:RAS IMR90 cells were
457 targeted with either control (non-targeting pool, NTP) or *CASP4*-targeting siRNA. All cells were treated
458 with 4OHT from day 0. RNA was extracted at day 5 and 8 after the addition of 4OHT and subjected to
459 transcriptomic analysis. Differentially expressed gene (DEG) analysis was performed and the number
460 of significant upregulated and downregulated genes 5 and 8 after the addition of 4OHT upon *CASP4*-
461 targeting in ER:RAS cells is shown.

462 (B) Normalized Enriched Scores (NES) of a set of 50 curated hallmark gene signatures were calculated
463 based on the DEG analysis performed between control and *CASP4*-knockdown ER:RAS samples after
464 5 and 8 days of 4OHT treatment. Gene sets with a false discovery rate (FDR) *q*-value of ≤ 0.25 at least
465 in one of the timepoints are shown. *P*-values for each gene set are indicated next to the corresponding
466 bar.

467 (C) Heatmap of the log₂FC values of all 175 genes included in the “INFLAMMATORY RESPONSE”
468 GSEA gene set of control ER:STOP and *CASP4*-knockdown ER:RAS compared to control ER:RAS
469 after 5 days of 4OHT treatment. The top 25 differentially expressed signature genes in *RAS*^{G12V}-OIS
470 are zoomed in.

471 (D) *IL1A* mRNA relative expression levels were quantified by RT-qPCR after 5 days of 4OHT treatment
472 in ER:STOP and ER:RAS cells transfected with the indicated siRNA.

473 (E) *IL1B* mRNA relative expression levels were quantified by RT-qPCR after 5 days of 4OHT treatment
474 in ER:STOP and ER:RAS cells transfected with the indicated siRNA.

475 (F) *SAA1* mRNA relative expression levels were quantified by RT-qPCR after 8 days of 4OHT treatment
476 in ER:STOP and ER:RAS cells transfected with the indicated siRNA.

477 (G) SAA2 mRNA relative expression levels were quantified by RT-qPCR after 8 days of 4OHT treatment
478 in ER:STOP and ER:RAS cells transfected with the indicated siRNA.

479 (H) IMR90 ER:STOP/ER:RAS cells were transfected with control (NTP), *CASP1* or *CASP4*-targeting
480 siRNA and treated with 4OHT or not during 8 days as indicated. Lysates were subjected to
481 immunoblotting analyses with the indicated antibodies.

482 (I) IMR90 ER:STOP/ER:RAS cells were treated or not 8 days with 4OHT as indicated and secreted IL-
483 1β was quantified by ELISA.

484 Statistical significance in A-E was calculated using one-way analysis of variance (ANOVA). $**P < 0.01$,
485 and $*P < 0.05$. All error bars represent mean \pm s.e.m of 3 independent experiments.

486

487 **Figure 6**

488 **Caspase-4 contributes to the cell cycle arrest program in OIS**

489 (A) IMR90 ER:STOP and ER:RAS cells were transfected with control (NTP), two individual *CASP4*-
490 targeting siRNAs or a pool of 4 different siRNA sequences targeting *CASP4*, and treated with 4OHT or
491 not as indicated. BrdU incorporation was measured by IF 5 days after 4OHT addition.

492 (B) IMR90 ER:STOP/ER:RAS cells were stably transfected using retroviral shRNA vectors targeting
493 *CASP4* or *TP53*. Infection with the empty vector (vector) was used as control. On day 0, equal number
494 of cells were subjected to 4OHT treatment. Fifteen days after 4OHT addition, plates were fixed and
495 stained with crystal violet. Crystal violet was extracted and used to quantify cell content.

496 (C) Related to Figure 5A. DEG analysis between control ER:STOP and *CASP4*-knockdown ER:RAS
497 compared to control ER:RAS after 5 days of 4OHT treatment was performed. Heatmap of the log₂FC
498 values from the indicated genes.

499 (D) IMR90 ER:STOP and ER:RAS cells were transfected with control (NTP), *CASP1* or *CASP4*-
500 targeting siRNAs and treated with 4OHT during 4 days. Cell lysates were subjected to immunoblotting
501 analyses with the indicated antibodies.

502 (E) mRNA relative expression of the indicated genes in IMR90 ER:RAS cells transfected with control
503 (NTP) *CASP1* or *CASP4*-targeting siRNA was quantified by RT-qPCR after 5 days of 4OHT treatment.
504 Statistical significance in A and B was calculated using two-tailed Student's *t*-test. Statistical
505 significance in E was calculated using one-way analysis of variance (ANOVA). $***P < 0.001$, $**P < 0.01$,
506 $*P < 0.05$ and ns, not significant. All error bars represent mean \pm s.e.m of 3 independent experiments.

507 **Figure 7**

508 **Caspase-11 expression is induced in senescence *in vivo*.**

509 (A) Immunohistochemistry showing Ki-67 and caspase-11 staining in sections from *Pdx-cre WT* and
510 *Pdx-cre Kras^{G12D}* pancreas (left panels). Black arrows indicate acinar pancreatic cells, white arrows
511 indicate PanIN cells. Close up images showing PanINs with high and low expression of caspase-11
512 (Right panel).

513 (B) Quantification of Ki-67 positive cells of total PanIN cells from a total of 11 mice of 7 to 15 weeks of
514 age. PanINs were classified according to the expression of caspase-11 as indicated. The percentage
515 of Ki-67 positive cells was calculated scoring all cells of PanINs classified as high or low caspase-11
516 expression per mouse as indicated. Scatter plots were generated from total cells from high and low
517 caspase-11 expressing PanINs with individual points representing the mean Ki-67 percentage positivity
518 for each mouse, with horizontal lines representing group mean and s.e.m. Statistics: Mann Whitney U
519 test. ***p < 0.001

520 (C) Analysis of caspase-11 expression was conducted by immunohistochemistry in lung sections from
521 wild type (WT) or *nfk1* knock out mice (*nfk1^{-/-}*) at 9.5 months of age. 10-15 random images were
522 captured per mouse and average percentage positivity calculated for airway epithelial compartments.
523 Scatter plots represent mean percentage positivity for each animal with horizontal line representing
524 group median. Broad-band autofluorescence (an indicator of lipofuscin accumulation) was acquired
525 from paraffin-embedded sections excited at 458 nm with fluorescence emission captured above 475
526 nm using a fluorescence microscope (Leica DM550B). Fluorescence intensity was analyzed using
527 ImageJ. At least 10 small airways were analyzed per mouse and an average intensity calculated per
528 animal. Scatter plots represent average value per animal with the horizontal line representing group
529 median. Statistics: Mann Whitney U test. *p < 0.05, **p < 0.01 Representative images Casp4 staining
530 by immunohistochemistry in airway epithelial cells from wt and *nfk1^{-/-}* mice, captured using x40
531 objective.

532 (D) Analysis of caspase-11 expression by immunohistochemistry in lung sections of wt mice at 6.5
533 months of age (Young) and 24 months of age week (Old). Scatter plots were generated from 10-15
534 random images captured per animal with individual points representing mean percentage positivity for
535 each mouse with horizontal line representing group median. Statistics: Mann-Whitney U test. *p < 0.05.

536 Representative images of caspase-11 staining by immunohistochemistry (positive, brown; negative,
537 blue) in alveolar cells from wt mice 6.5 and 24 months of age, captured using x40 objective.

Figure 1

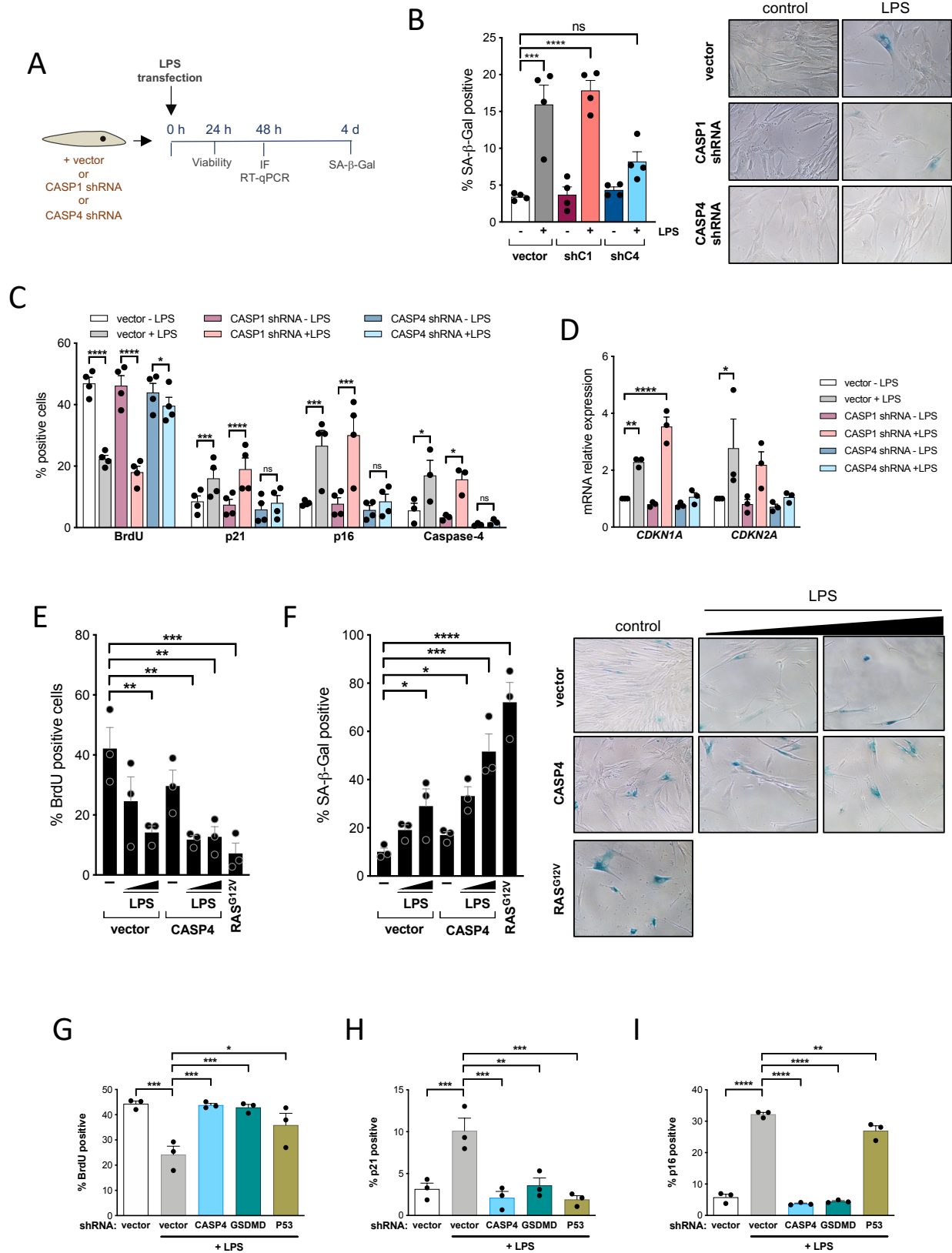


Figure 2

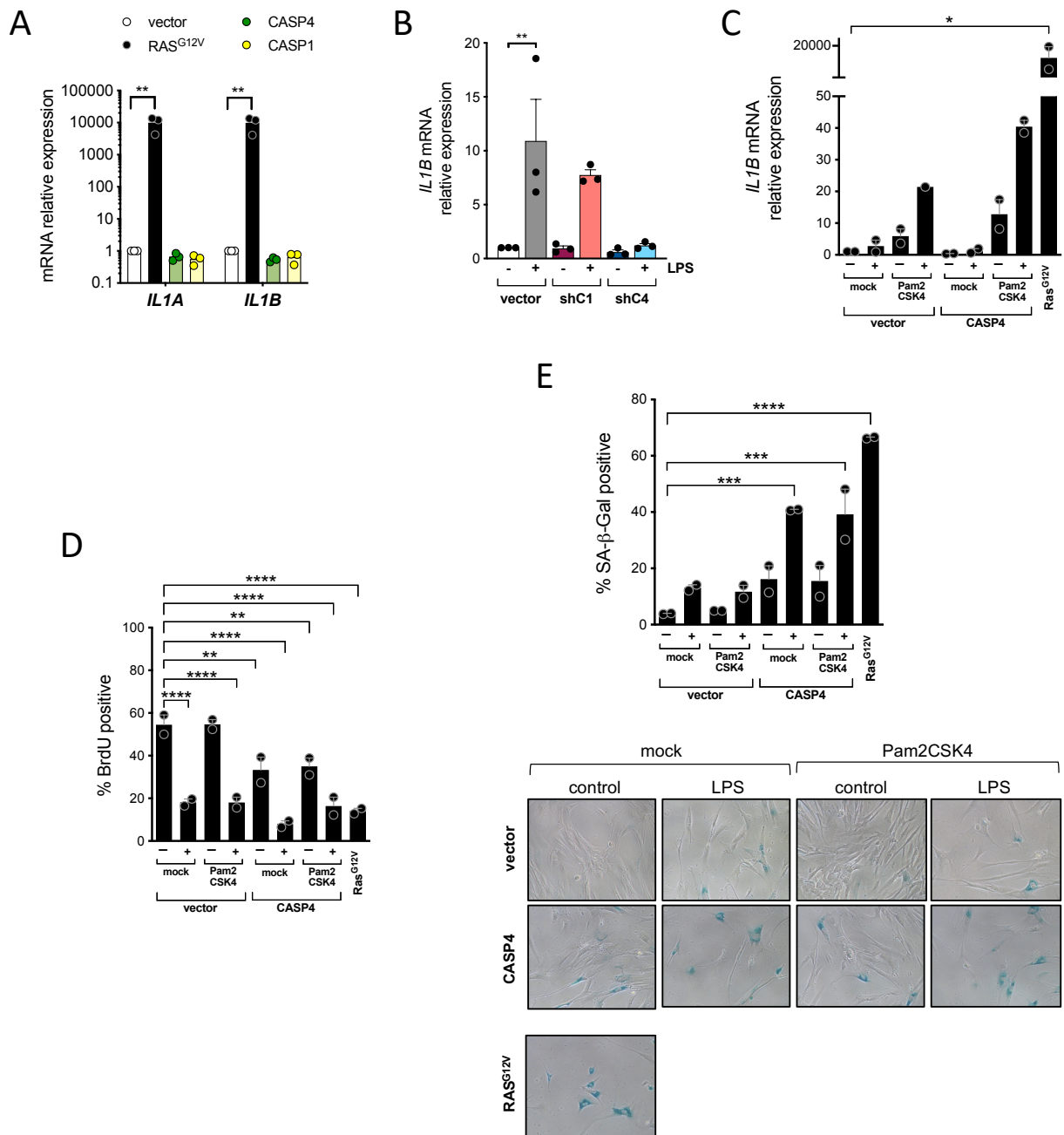


Figure 3

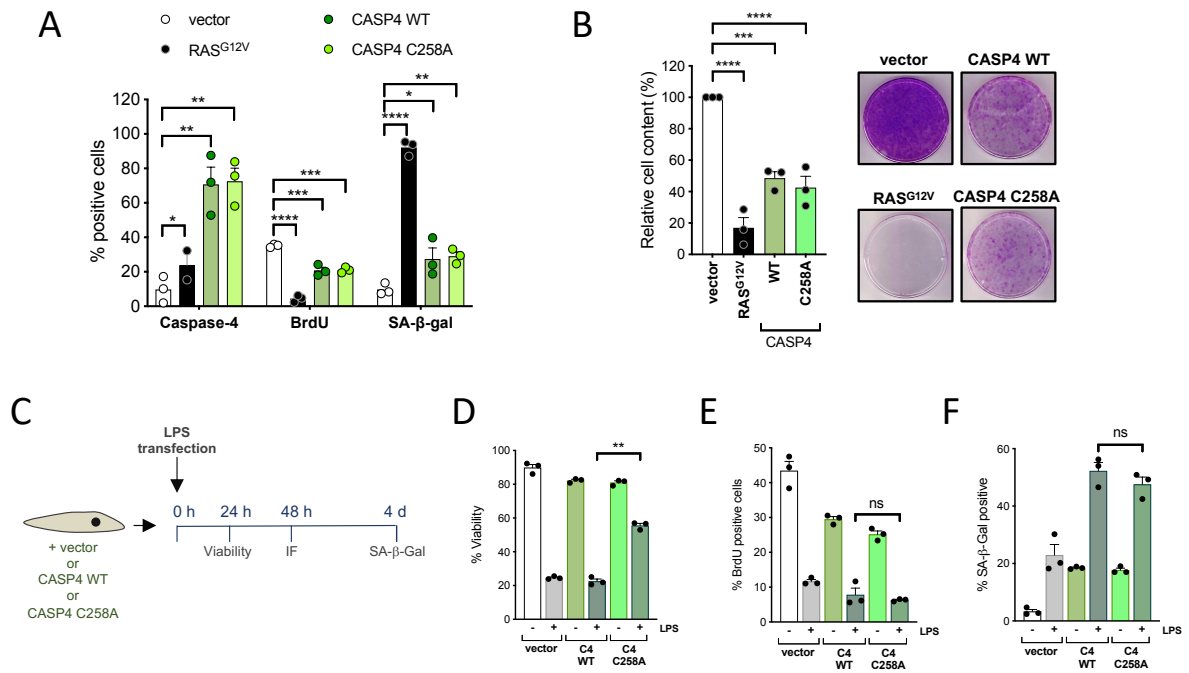


Figure 4

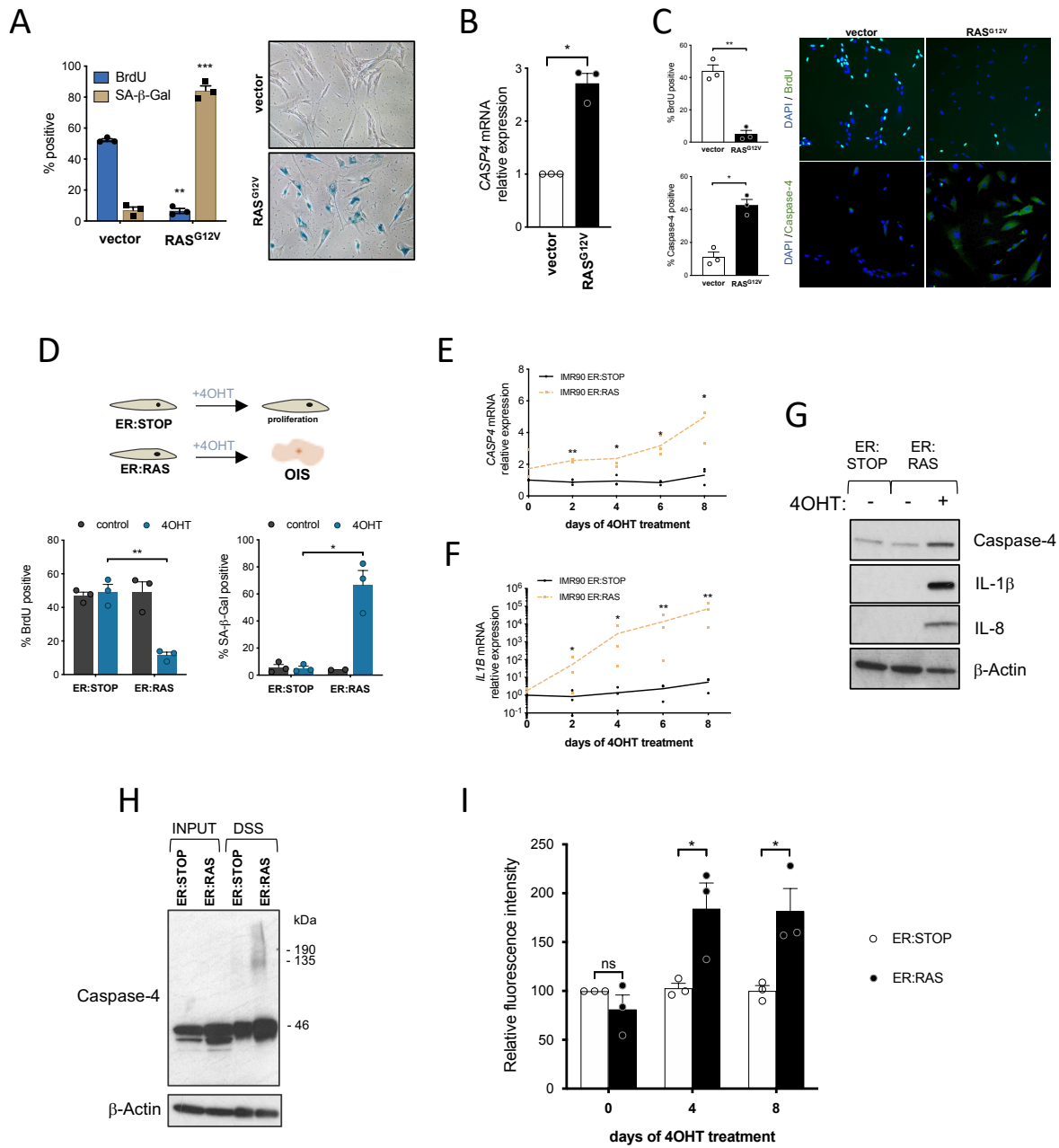


Figure 5

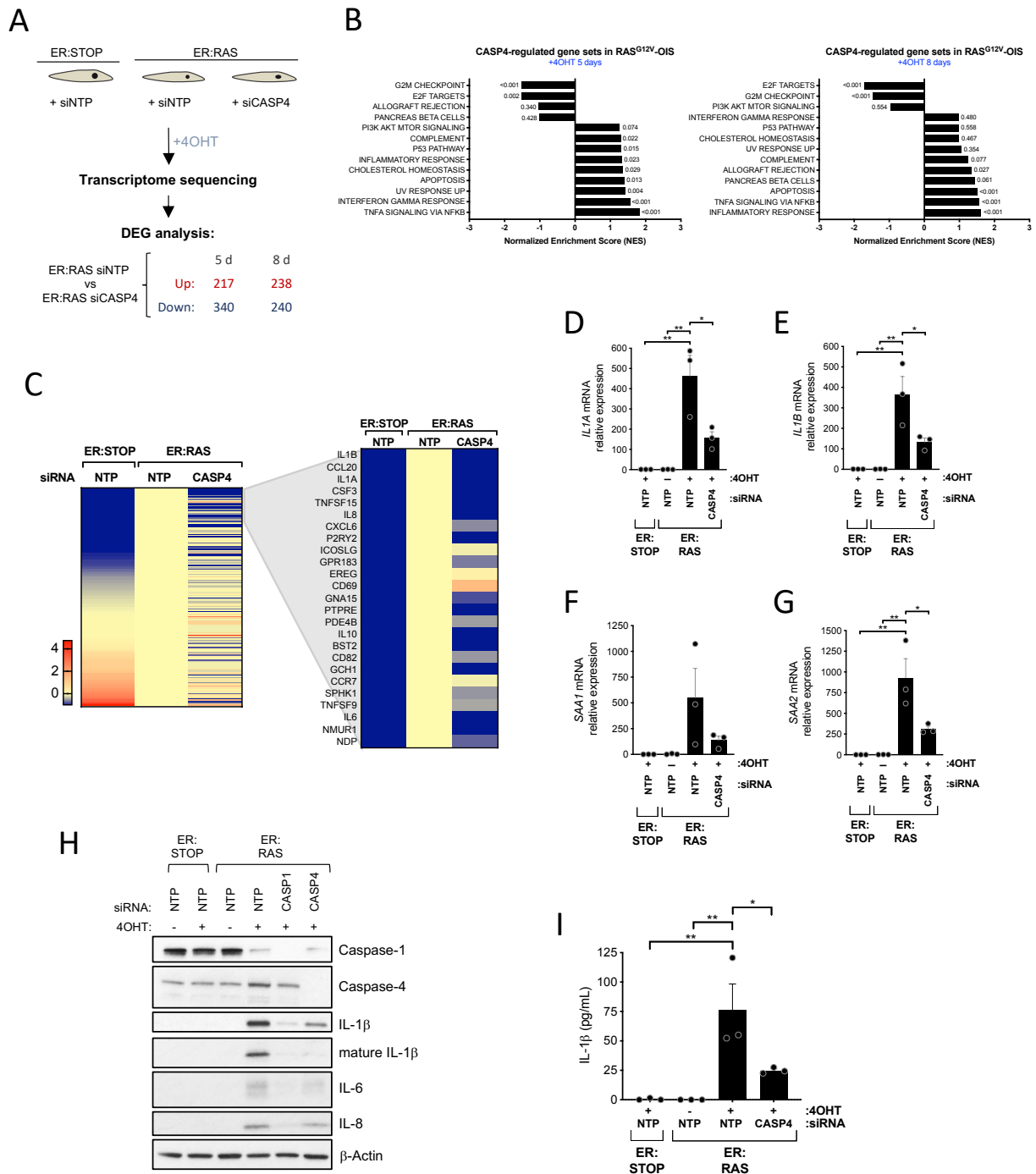


Figure 6

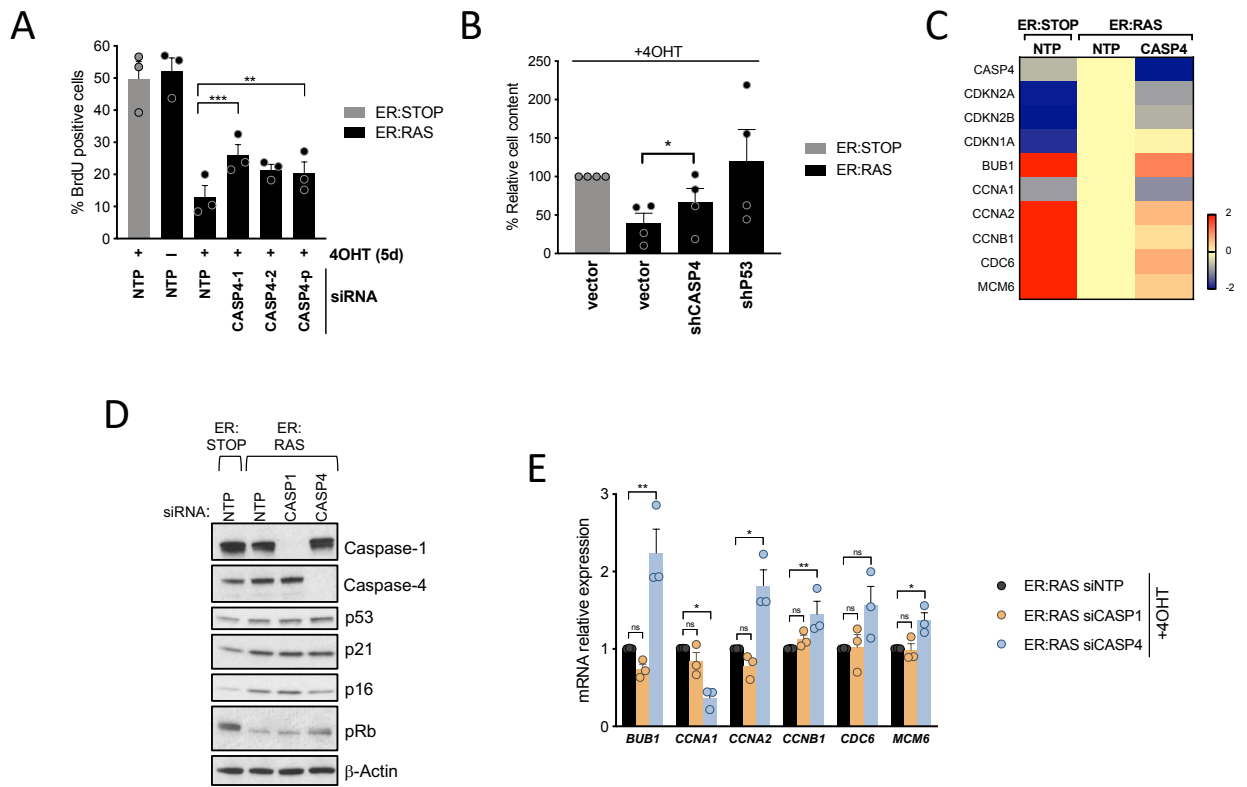
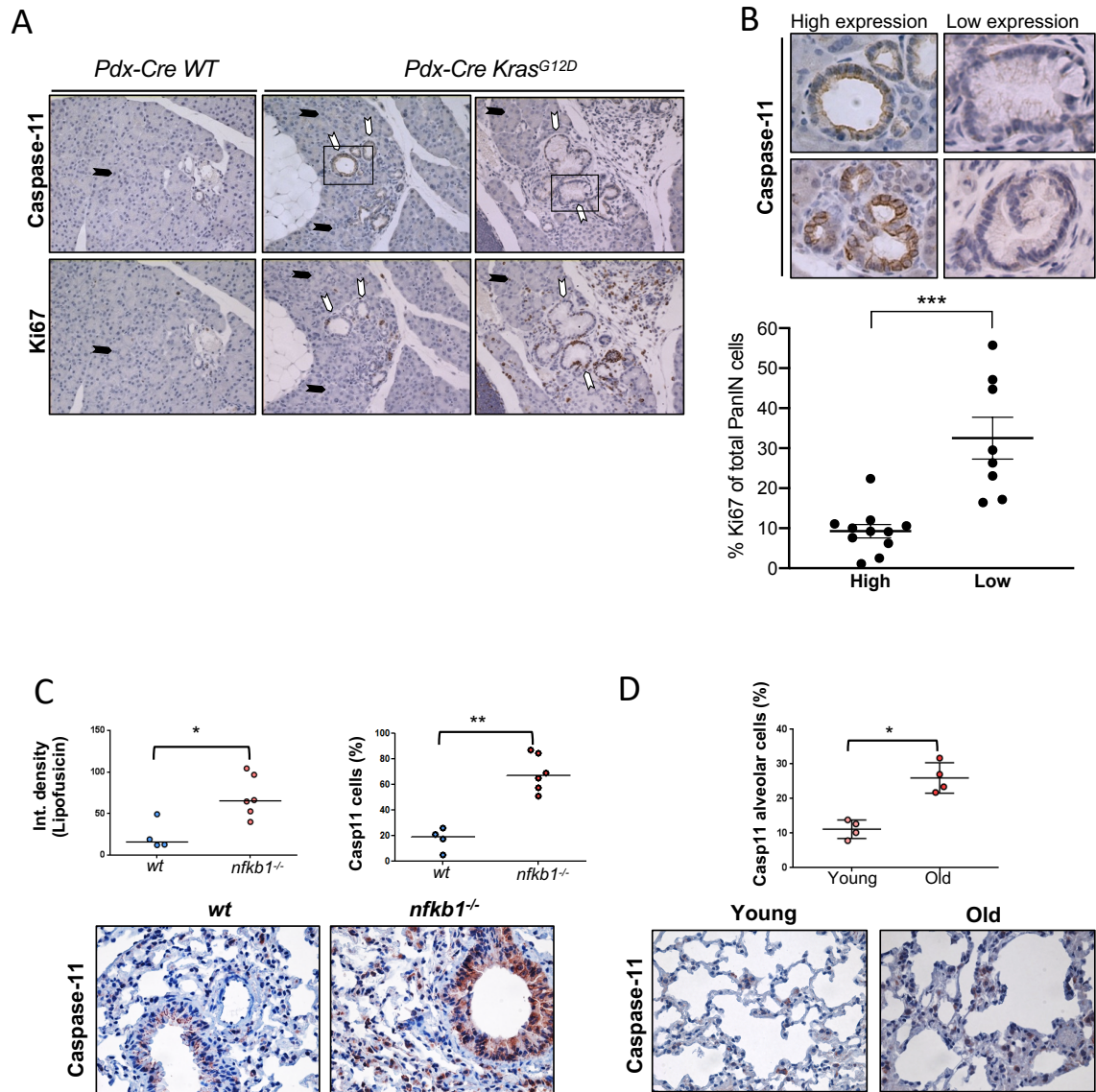


Figure 7



536 **Methods**

537

538 **RESOURCE AVAILABILITY**

539 **Lead Contact and Materials Availability**

540 Further information and requests for resources and reagents should be directed to and will be
541 fulfilled by the Lead Contact, Juan-Carlos Acosta (juan-carlos.acosta@igmm.ed.ac.uk).

542

543 **Data and Code Availability**

544 The transcriptomic data generated during this study is available at GEO.

545

546 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

547 HEK293T and IMR90 female human fetal lung fibroblast cells were obtained from American
548 Type Culture Collection. All cell lines were maintained in Dulbecco's Modified Eagle's Medium
549 (DMEM) (Sigma), supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher) and 1%
550 antibiotic-antimycotic solution (ThermoFisher). All cell lines were cultured at 37°C with 5%
551 CO₂ and tested for mycoplasma on a regular basis. All cell lines were regularly tested for
552 mycoplasma contamination using the Mycoalert Mycoplasma Detection Kit (Lonza). Cell
553 counting and viability were performed Muse® Count & Viability Assay Kit in a Muse Cell
554 Analyser (Merck Millipore).

555

556 **METHOD DETAILS**

557 **Chemical compounds and treatments**

558 OIS was induced by treating IMR90 ER:RAS cells with 100 nM 4OHT. IMR90 ER:RAS and
559 control ER:STOP were maintained in standard media supplemented with 200 µg/ml geneticin.
560 To induce oncogene induced senescence, IMR90-ER:RAS cells were treated
561 with 100 µM etoposide for 48 hours, followed by 5 days in normal culture media. For DNA
562 damage-induced senescence, IMR90 cells were treated with 10 µM Etoposide for 48 hours.
563 For non-canonical inflammasome activation and inflammasome priming experiments,

564 ultrapure lipopolysaccharide (LPS) from E. coli 111:B4 (Invivogen), muramyl dipeptide (MDP)
565 (Tocris), Pam2CSK4 (Tocris), Pam3CSK4 (Tocris), Recombinant Human Apo-SAA (A-
566 SAA)(Peprotech) and BSA (Sigma) were used. For priming time-course experiments, the
567 following concentrations were used: LPS (1 µg/ml), MDP (1 µg/ml), Pam2CSK4 (50 ng/ml),
568 Pam3CSK4 (500 ng/ml). To prime inflammasomes prior to LPS transfection, cells were treated
569 with Pam2CSK4 (1 µg/ml) A-SAA (10 µg/ml) or BSA (10 µg/ml) for 3 hours.

570

571 **Cell quantification and viability**

572 To determine viable cell concentration of cultures, cells were washed and incubated with
573 trypsin (ThermoFisher) for 5 min at 37 °C. Fully detached cells were collected by
574 centrifugation, resuspended in Muse Count & Viability Reagent (Merck Millipore), and counted
575 using the Muse Cell Analyzer (Merck Millipore). To determine cell viability, culture
576 supernatants and attached cells were pooled together before centrifugation. Pellets were
577 resuspended in Muse Count & Viability Reagent (Merck Millipore) and analysed using the
578 Muse Cell Analyzer (Merck Millipore).

579

580 **LPS transfection**

581 To electroporate LPS or MDP, the Neon Transfection System (Invitrogen, MPK5000) and the
582 Neon Transfection System 100 µL Kit (MPK10025) were used. Per each tip, 5×10^5 cells
583 were transfected with the indicated amount of LPS or MDP. Electroporation parameters were
584 set at 1500V, 30 ms and pulse number 1 for IMR90 cells and 1100V, 20 ms and pulse number
585 2 for HEK293T cells. Once electroporated, the tip content was unloaded into a clean
586 Eppendorf tube and tubes were centrifuged on a bench-top centrifuge at 3000 rpm 3 min.
587 Supernatant was removed to avoid any traces of MDP or LPS in the extracellular media prior
588 to plating.

589

590

591

592 **Conditioned medium for paracrine senescence transmission**

593 For the production of conditional medium (CM), IMR90 ER:STOP and ER:RAS cells were
594 cultured as previously described (Boumendil et al., 2019). IMR90 ER:STOP and ER:RAS cells
595 were cultured with DMEM supplemented with 100 nM 4OHT and 10% FBS for 4 days, and
596 with DMEM 100 nM 4OHT and 1% FBS for 4 additional days. CM was filtered using 0.2 µm
597 syringe filters (Millipore) and reconstituted with a solution of DMEM supplemented with 40%
598 FBS at a 3:1 ratio.

599

600 **Generation of plasmids**

601 Using standard retro-transcription procedures, total RNA extracted from IMR90 cells was
602 converted into cDNA generating a human coding sequence (CDS) library. CASP1 and CASP4
603 CDS were amplified from the obtained library and cloned into the pMSCV-puro vectors. The
604 caspase-4 catalytically dead C258A pMSCV-puro vector was generated from the wild-type
605 pMSCV-puro-CASP4 through site-directed mutagenesis by PCR using the Q5 Site-Directed
606 Mutagenesis Kit (New England Biolabs). The CASP4 and GSDMD-targeting lentiviral vectors
607 (pGIPZ) were purchased from Dharmacon. The CASP4-targeting retroviral vector (pRS-
608 shCASP4) was generated inserting the oligonucleotide (+)
609 GATCCCCCAACGTATGGCAGGACAAATTCAAGAGATTTGTCCTGCCATACGTTGTTTTTG
610 into the pRS empty backbone following the pSuper RNAi System manual (OligoEngine)
611 instructions. pLN-ER:RAS, LSXN-ER:Stop, MSCV-Ras^{G12V}, pCMV-VSVG, and pUMVC3-gag-
612 pol vectors have been described elsewhere (Acosta et al., 2013).

613

614 **Retroviral and lentiviral production and infection**

615 For retroviral production, 20 µg retroviral plasmid were cotransfected with 2.5 µg pCMV-VSVG
616 envelope plasmid and 7.5 µg pUMVC3-gag-pol helper vector using polyethylenimine linear
617 (Alfa Aesar) into HEK293T cells. For lentiviral production, 10 µg lentiviral plasmid were
618 cotransfected with 2.5 µg pCMV-VSVG and 7.5 µg psPAX2 using polyethylenimine linear (Alfa

619 Aesar) into HEK293T cells. Viral supernatant was collected from the HEK293T cells 2 days
620 after transfection and passed through a 0.45 μm syringe filter (ThermoFisher). The viral
621 supernatant was complemented with hexadimethrine bromide (Sigma) to a final
622 concentration of 4 $\mu\text{g}/\text{mL}$. When performing retroviral infections, IMR90s were treated with
623 fresh viral supernatant and subsequent viral supernatant collection and incubation of IMR90
624 cells was performed every 3 hours until three rounds of infection were performed. For lentiviral
625 infections, a single 3 hour incubation with 1:10 dilution of viral supernatant was performed. In
626 both cases viral supernatant was removed after the indicated rounds of infection, fresh media
627 was added to IMR90 cells and, 2 days later, selection with puromycin (1 $\mu\text{g}/\text{ml}$)
628 (ThermoFisher) was initiated. Before set-up, fresh standard media supplemented with the
629 selection agent was added for over a week or until no alive cells were observed in control cells
630 infected with a non-containing selection marker vector.

631

632 **siRNA transfection**

633 ON-TARGETplus siRNAs were obtained from Dharmacon. Sequences and IDs are detailed
634 in the Key Resources Table. For all transfections, 30 nM siRNA were incubated up to 1 hour
635 with Dharmafect 1 (Dharmacon, 1 $\mu\text{g}/\text{ml}$ final use concentration) to allow the formation of
636 siRNA:transfection agent complexes prior to transfection. On day 0, 200.000 IMR90 ER:STOP
637 and ER:RAS cells were plated in each T-6 well, 4OHT was added and siRNA reverse
638 transfections performed. Due to the transient nature of siRNA, cells were split 1:4 on day 3
639 and reverse transfections were repeated. To maintain the knockdown during 8 days, forward
640 transfections were performed again on day 5.

641

642 **Total RNA preparation and quantitative reverse transcription polymerase chain** 643 **reaction (qRT-PCR)**

644 Cell lysates were homogenized using QIAshredder (Qiagen) and RNA was extracted using
645 the RNeasy Plus Mini kit (Qiagen). RNA was transformed into cDNA using qScript cDNA
646 Supermix (Quanta Biosciences) following manufacturer's instructions. To perform quantitative

647 PCRs, samples were prepared in triplicates in 96-well plates. Each well contained 1 μ L of
648 cDNA, 200 nM forward primer, 200 nM reverse primer, 1x SYBR Select Master Mix (Applied
649 Biosystems) and up to 20 μ L of ultrapure DNase/RNase-free distilled water (ThermoFisher).
650 Plates were loaded into a StepOnePlus Real-Time PCR System (ThermoFisher) and the
651 following PCR cycling parameters were used: 10 min at 95 °C; 40 cycles of 15 s at 95 °C, 30
652 s at 60 °C and 15 s at 72 °C; 15 s at 95 °C. Data was analyzed using the double Delta Ct
653 method. The housekeeping gene ACTB was used to normalize data. Primers are specified in
654 the Table S1.

655

656 **Immunofluorescence and high-content microscopy**

657 Cells were fixed with 4% paraformaldehyde (FD NeuroTech) in PBS during 45 min. All
658 incubations were performed at room temperature and on an orbital shaker. To permeabilize
659 cells, cells were incubated with 0.2% Triton-X100 in PBS for 10 min. Cells were blocked with
660 immunofluorescence blocking buffer (1% Bovine Serum Albumin (BSA) and 0.2% Fish Gelatin
661 in PBS). Primary and secondary antibodies were diluted in immunofluorescence blocking.
662 Anti-BrdU primary solution was supplemented with 0.5 U / μ L DNase (Sigma) and 1 mM
663 MgCl₂ to improve anti-BrdU access to DNA-bound BrdU. Nuclei were stained with 1 μ g/mL
664 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Antibodies are listed in the Key
665 Resources Table. Immunofluorescence was analyzed using the high-content High-Content
666 Image Acquisition and Analysis software (Molecular Devices) as previously described
667 (Boumendil et al., 2019). One-wavelength images of the same frame were merged using the
668 software Fiji (ImageJ).

669

670 **Western blot analysis**

671 Whole cells were lysed in 1X Cell Lysis Buffer Cell (Cell Signalling) supplemented with
672 cComplete EDTA-free Protease Inhibitor Cocktail (Roche). Protein concentration was
673 determined by the Bradford assay using the Bradford reagent (Biorad) and BSA pre-set
674 standards (ThermoFisher) to construct a standard curve. To prepare samples for sodium

675 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 15 µg of protein were mixed
676 with 6 µL 6x reducing Laemmli SDS sample buffer (Alfa Aesar) in a final volume of 36 µL.
677 Samples were boiled 5 min at 95 °C and loaded in pre-cast Novex Tris-Glycine gels
678 (Invitrogen). Pre-cast gels were run in an XCell SureLock™ Mini-Cell Electrophoresis tank
679 (ThermoFisher) at 100 – 140 V. Proteins were transferred into nitrocellulose membranes using
680 the iBlot Gel Transfer Device (ThermoFisher). Membranes were blocked in Tris-buffered
681 saline (TBS) buffer (25 mM Tris-HCl + 137 mM NaCl + 2.7 mM KCl, pH7.4) supplemented
682 with 5% non-fat milk 1 h at room temperature on a rocking shaker. Primary and secondary
683 antibodies (described in the Key Resources Table) were diluted in TBS 5% milk buffer. To
684 visualize bands, membranes were incubated with enhanced chemiluminescence solution (GE
685 Healthcare) and exposed to X-ray films (GE Healthcare).

686

687 **Caspase-4 fluorometric activity assay**

688 To measure LEVD-AFC cleavage, the Caspase 4 Fluorometric Assay kit (Fluorometric) was
689 used following manufacturer's instructions. 2×10^6 IMR90 ER:STOP or ER:RAS cells were
690 lysed in 50 µL cell lysis buffer. The assay was conducted in black sterile 96-well polystyrene
691 plate (ThermoFisher). Fluorescence was measured (excitation filter: 400 nm; emission filter:
692 505 nm) using an Infinite® 200 PRO (Tecan) plate reader.

693

694 **Detection of caspase-4 oligomerization**

695 Fresh IMR90 ER:STOP or ER:RAS cell pellets were resuspended in 0.5 ml of ice-cold buffer
696 A (20 mM HEPES-KOH, pH 7.5; 10 mM KCl; 1.5 mM MgCl₂; 1 mM EDTA; 1 mM EGTA; 320
697 mM sucrose), lysed by shearing 10 times through a 25-gauge needle, and centrifuged 8 min
698 at 1.800 g at 4 °C. At this point, 30 µL of lysates were kept as input controls. Remaining
699 supernatants were diluted with 1 volume of CHAPS buffer (20 mM HEPES-KOH, pH 7.5; 5
700 mM MgCl₂; 0.5 mM EGTA; 0.1 mM PMSF; 0.1% CHAPS) and centrifuged 8 min at 5,000 x g.
701 Supernatants were discarded and pellets were resuspended in 50 µL of CHAPS buffer
702 containing 4 mM of disuccinimidyl suberate (DSS) during 30 min at room temperature to cross-

703 link proteins. Then, samples were centrifuged 8 min at 5,000 x g at 4 °C, supernatants
704 discarded and pellets resuspended in 60 µL of protein loading buffer (25 mM Tris-HCl, pH 6.8;
705 1% SDS; 10% glycerol; 6.25 mM EDTA; 0.01% bromophenol blue). Samples were heated for
706 2 min at 90 °C and 18 µL of resuspended cross-linked pellets were loaded onto a 4-12% pre-
707 cast Novex Tris-Glycine gels (Invitrogen). Further immunodetection of caspase-4 was
708 performed following standard western blotting procedures.

709

710 **Determination of IL-1 β content in conditioned media**

711 Conditioned media was collected, centrifuged 10 min at 1000 rpm at 4 °C and transferred to
712 a clean tube. Released IL-1 β was quantified using the Human IL-1 beta ELISA Ready-Set-
713 Go! Kit (ThermoFisher) following the manufacturer's instructions. Conditioned media IL-1 β
714 concentrations were deducted interpolating the data from the standard curve, as previously
715 described (Fernandez-Duran et al., 2019).

716

717 **Cell proliferation assays**

718 5-bromo-2'-deoxyuridine (BrdU) incorporation was used to measure the number of cells
719 actively replicating DNA. Cells were incubated with 10 µM BrdU (Sigma) for 16 to 18 hours.
720 Cells were stained for immunofluorescence and high-content microscopy as described.

721 To analyze long-term growth, low equal amounts of cells were plated in 10 cm diameter
722 dishes. Media was changed every 3 days and cells were fixed two weeks after initial seeding
723 with 0.5% glutaraldehyde (Sigma) in PBS for 20 min and left drying overnight. Dishes were
724 stained with 0.2% crystal violet for 3 hours, washed twice with tap water and dried. To quantify
725 cellular mass, cell-bound crystal violet was extracted in 10% acetic acid, equal amounts were
726 transferred to a spectrophotometer-compatible 96-well plate and absorbance was read at 595
727 nm.

728

729

730 **SA- β -Galactosidase assay**

731 5 x 10⁴ IMR90 cells per well were seeded in 6-well plates. Four days later, cells were fixed
732 with 0.5% glutaraldehyde (Sigma) in PBS during 10 min. Fixed cells were washed three times
733 with PBS 1 mM MgCl₂ pH 5.7, before adding to each well 2 mL of pre-warmed X-Gal staining
734 solution (2 mM MgCl₂, 5 mM K₄Fe(CN)₆ • 3H₂O, 5 mM K₃Fe(CN)₆, 1 mg/mL X-Gal solution
735 ready to use (ThermoFisher) in PBS). Plates were incubated for 2-24 h at 37 °C, washed and
736 imaged. SA- β -Gal activity positive and negative cells were quantified using FIJI/ImageJ.

737

738 **AmpliSeq transcriptome profiling**

739 RNA quality was assessed on the Bioanalyser 2100 Electrophoresis Instrument (Agilent) with
740 the RNA 6000 Nano Kit (Agilent). Samples were quantified using the Qubit 2.0 fluorometer
741 and the Qubit RNA Broad Range assay. 10 ng of RNA was reverse-transcribed to cDNA, and
742 target genes were amplified for 12 cycles of PCR using the Ion AmpliSeq Human Gene
743 Expression Core Panel (ThermoFisher). This panel contains a pool of 20,802 amplicons
744 (41,604 primers) of approximately 150 bases in length. Ion Torrent sequencing adapters and
745 barcodes Ion Xpress™ Barcode Adapters (Ion Xpress™ Barcode Adapters) were ligated to
746 the amplicons and adapter-ligated libraries were purified using AMPure XP beads. Libraries
747 were quantified by qPCR and diluted to 100 pM before being combined in equimolar pools of
748 8 per each Ion PI Chip Kit v3 (ThermoScientific). Sequencing was performed using the Ion PI
749 Hi-Q Sequencing 200 Kit (ThermoFisher). Sequence reads were mapped to the
750 hg19_AmpliSeq_Transcriptome_ERCC_v1.fasta reference. BAM files were generated using
751 the Torrent Suite software v 5.2.0 (ThermoFisher). Differentially expressed gene (DEG)
752 analysis was performed with the DESeq2 package v.1.20.0. Gene Set Enrichment Analysis
753 (GSEA) was performed using the Broad Institute GSEA software v3.0. DEG-obtained log₂FC
754 values were used as inputs for the GSEA. Molecular signatures were obtained from MSigDB
755 v.6.2.

756

757

758 **Experiments with mice**

759 Experiments were performed according to UK Home Office regulations. Mice carrying a
760 conditional *Pdx1-Cre Kras^{G12D/+}* allele were used and have been described previously (Morton
761 et al., 2010). Sections of formalin-fixed paraffin-embedded mouse pancreas from 6 to 14-
762 week-old mice were stained with antibody against Ki67 and Caspase-4. Caspase-4 signal was
763 used to classify high and low Caspase-4 expressing PanIN. Aging experiments were carried
764 out on male wild-type C57BL/6 mice or male *nfkb1^{-/-}* mice on a pure C57BL/6 background at
765 6.5, 9.5 and 24 months of age.

766

767 **Immunohistochemistry**

768 For the *Pdx1-Cre Kras^{G12D/+}* mice (PanIN), Using EnVision™ +Dual Link system-HRP (DAB+)
769 kit (K4065, Dako), sections of formalin-fixed paraffin-embedded mouse pancreas were stained
770 with antibody against Ki67 (ab21700, Abcam). The total number of Ki67 positive cells per
771 PanIN, and the total cells per PanIN were counted, and thus the percentage of Ki67 positive
772 cells per PanIN was calculated. The mean score for each mouse was calculated and these
773 scores were plotted scatter plot. Consecutive sections were stained with antibodies against
774 Caspase 4/11 (bs-6858R, Bioss). The stainings were examined and classified for high or low
775 expression of the respective antibodies, and each structure compared with the Ki67
776 percentage.

777 For *nfkb1^{-/-}* and aging mice analysis, sections were dewaxed in histoclear (5 min), rehydrated
778 through graded ethanol solutions (100, 90, and 70%) and washed in distilled H₂O.
779 Endogenous peroxidase activity was blocked by immersing sections in 0.3% H₂O₂ (Sigma,
780 H1009) diluted in H₂O for 30 min. To retrieve antigens, sections were boiled in 0.01 M citrate
781 (pH 6.0). Sections were blocked in normal goat serum diluted 1:60 in 0.1% BSA in PBS.
782 Sections were incubated with the primary antibody overnight at 4°C for Caspase 4/11 (bs-
783 6858R, Bioss). Biotinylated secondary antibody was added and detected using the rabbit
784 peroxidase ABC kit (Vector Laboratories, PK-4001), according to the manufacturer's
785 instructions. Substrate was developed using the NovaRed kit (Vector Laboratories, SK-4800).

786 Nuclei were counterstained with heamatoxylin, and sections were dehydrated through graded
787 ethanol solutions, cleared in xylene, and mounted in di-nbutylehthalate in xylene (Thermo
788 Scientific, LAMB-DPX). Staining was analysed with a NIKON ECLIPSE-E800 microscope, and
789 images were captured with a Leica DFC420 camera using the LAS software (Leica). 10-15
790 random images were captured per section and the percentage of positively stained cells
791 determined from total number of cells before an average per mouse was calculated.

792

793 **Broad-band autofluorescence (lipofuscin accumulation) analysis**

794 Broad-band autofluorescence was acquired from sections cut at 3 μm using X20 objective
795 (Leica DM550B). Sections were excited at 458 nm and fluorescence emission captured above
796 475 nm. Fluorescence intensity per airway epithelium was quantified using ImageJ software
797 and divided by background emission. At least 10 small airways per mouse was analysed.

798

799 **QUANTIFICATION AND STATISTICAL ANALYSIS**

800 GraphPad Prism 7 software was used for statistical analysis. Results were displayed as the
801 means \pm SEM and statistical significance was determined with Student's t tests, One-way
802 analysis of variance (ANOVA) or Two-way ANOVA.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Caspase-4	Santa Cruz	4B9
Anti-BrdU	BD Biosciences	555627
Anti-IL-1 α	R&D	MAB200
Anti-IL-1 β	R&D	MAB201
Anti-IL-6	R&D	AF206NA
Anti-IL-8	R&D	MAB208
Anti-p21	Sigma	P1484
Anti-p16 (IF)	ProteinTech	10883-1-AP
Mouse-Alexa Fluor 488	ThermoFisher	A-11029
Rabbit-Alexa Fluor 594	ThermoFisher	A-11037
Goat-Alexa Fluor 594	ThermoFisher	A-11058
Anti-Caspase-1	Adipogen	Bally-1
Anti-Gasdermin-D	Novus Biologicals	NBP2-33422
Anti-p16 (WB)	Santa Cruz	JC-8
Anti-p53	Santa Cruz	DO-1
Anti-pRb	BD Pharmigen	554136
Anti- β -Actin	Santa Cruz	I-19
Anti-Mouse-HRP	Sigma	A2554
Anti-Rabbit-HRP	Sigma	A0545
Anti-Goat-HRP	Sigma	2020
Anti-Mouse IgG (Fc specific)-Peroxidase	Sigma	Cat #A2554
Anti-Rabbit IgG (whole molecule)-Peroxidase	Sigma	Cat #A0545
Donkey anti-goat-HRP	Santa Cruz	Cat #sc-2020
Goat anti-Rabbit IgG Alexa Fluor 594	Thermo Fisher	Cat #A11037
Monoclonal anti-B-Actin-peroxidase (AC-15)	Sigma	Cat #A3854
Mouse monoclonal IgG2B Isotype Controls (20116)	R&D systems	Cat #MAB004
Rabbit polyclonal anti-Caspase 4 + Caspase 11 Biotin conjugated	Bioss	Cat #bs-6858R
Chemicals, Peptides, and Recombinant Proteins		
4-hydroxytamoxifen	Sigma	H7904
Geneticin	ThermoFisher	10131-027
Puromycin	ThermoFisher	A11138
Ultrapure lipopolysaccharide (LPS) from E. coli 111:B4	Invivogen	tlrl-3pelps
Muramyl dipeptide (MDP)	Tocris	tlrlmdp
Pam2CSK4	Tocris	4637
Pam3CSK4	Tocris	4633
Recombinant Human Apo-SAA	Peprtech	#300-13
5-bromo-2'-deoxyuridine (BrdU)	Sigma	858811
X-Gal solution ready to use	ThermoFisher	R0941
Critical Commercial Assays		
Neon Transfection System 100 μ L Kit	Invitrogen	MPK10025
Q5 site directed mutagenesis kit	New England Biolabs	E0554S
Caspase 4 Assay kit (Fluorometric)	Abcam	ab65658
Human IL-1 beta ELISA Ready-Set-Go! Kit	ThermoFisher	15581087
RNA 6000 Nano Kit	Agilent	5067-1511
Ion AmpliSeq Human Gene Expression Core Panel	ThermoFisher	A26325

Ion PI Hi-Q Sequencing 200 Kit	ThermoFisher	A26433
Deposited Data		
Ampliseq data	GEO	
Experimental Models: Cell Lines		
HEK293T	ATCC	N/A
IMR90 human fetal primary fibroblasts	ATCC	N/A
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	The Jackson Lab.	
Oligonucleotides		
ON-TARGETplus Non-targeting Pool	Dharmacon	D-001810-10
ON-TARGETplus CASP1-targeting Pool	Dharmacon	LQ-004401-00
ON-TARGETplus CASP4-targeting Pool	Dharmacon	LQ-004404-00
ON-TARGETplus CASP4-targeting #1	Dharmacon	J-004404-06
ON-TARGETplus CASP4-targeting #2	Dharmacon	J-004404-08
ON-TARGETplus GSDMD-targeting Pool	Dharmacon	LQ-016207-00
The sequences and description of primers used for RT-qPCR are provided in Table S1	N/A	N/A
Recombinant DNA		
pMSCV-puro-CASP1	This paper	Exp vector
pMSCV-puro-CASP4	This paper	Exp vector
pMSCV-puro-CASP4-C258A	This paper	Exp vector
pGIPZ-shCASP1	Dharmacon	V3LHS_392179
pGIPZ-shCASP4	Dharmacon	V3LHS_338745
pGIPZ-shGSDMD	Dharmacon	V3LHS_378066
pGIPZ shTP53	Dharmacon	V3LHS_333920
pRS-shCASP4	This paper	N/A
pRS-shTP53	(Acosta et al., 2008)	N/A
Software and Algorithms		
MetaXpress High-Content Image Acquisition and Analysis software (used for quantification of immunofluorescence microscopy images)	Molecular Devices	N/A
FIJI/ImageJ (used for merging of immunofluorescence microscopy images and SA- β -Gal quantification)	ImageJ	N/A
Torrent Suite software v.5.2.0	ThermoFisher	N/A
DESeq2 package v.1.20.0	(Love et al., 2014)	Bioconductor
GSEA software v.3.0	(Subramanian et al., 2005)	UC San Diego / Broad Institute
MSigDB v.6.2	(Subramanian et al., 2005)	UC San Diego / Broad Institute
GraphPad Prism 7	GraphPad Software	Statistics software

803 **Supplemental information**

804

805 **Supplemental figure legends**

806 **Figure S1**

807 **LPS-mediated caspase-4 activation induces a senescent phenotype in human primary**
808 **fibroblasts – related to Figure 1.**

809 (A) IMR90 fibroblasts were un-transfected (UnT), mock-transfected (mock), transfected with MDP (1 µg
810 / 5 x 10⁵ cells) or increasing concentrations of LPS (0.1 or 1 µg LPS / 5 x 10⁵ cells). Cell viability was
811 measured 2 h after transfection.

812 (B) Representative images of IMR90 cells mock-transfected (left) or transfected with 0.1 µg LPS / 5 x
813 10⁵ cells (middle) or 1 µg LPS / 5 x 10⁵ cells (right) under brightfield microscopy 24 h after transfection.

814 (C) IMR90 or 293T cells were un-transfected (mock) or transfected with 1 µg LPS / 5 x 10⁵ cells (LPS-
815 T). To confirm that pyroptosis is dependent on intracellular localization of LPS, IMR90 were also treated
816 with 1 µg LPS / 5 x 10⁵ cells without further transfection (LPS - UnT). Cell viability was measured 2.5
817 h after transfection. Data from a single representative experiment.

818 (D) Cells were treated as shown in Figure 1A. *CASP1* mRNA relative expression was quantified by RT-
819 qPCR 48 h after LPS transfection.

820 (E) Cells were treated as shown in Figure 1A. Cell viability was measured 24 h after LPS transfection.

821 (F) IMR90 cells were stably infected with an empty pRS vector (vector) or a pRS vector targeting either
822 *CASP4* (shC4), *GSDMD* (shGSDMD) or *TP53* (shP53) prior to transfection with 0.1 µg LPS / 5 x 10⁵
823 cells, and cell viability was measured 24 h after LPS transfection.

824 (G) IMR90 fibroblasts were mock-transfected, transfected with 1 µg MDP / 5 x 10⁵ cells, or with
825 increasing concentrations of LPS (0.01, 0.1 or 1 µg LPS / 5 x 10⁵ cells). Cell viability was measured
826 5, 24, 48 and 72 h after transfection.

827 (H) IMR90 fibroblasts were mock-transfected or transfected with increasing concentrations of LPS (0.1
828 or 1 µg LPS / 5 x 10⁵ cells). Cell viability was measured 2.5 h after transfection and viable cells were
829 replated and cultured for further 48 h before measuring cell viability again. Bars show a single
830 representative experiment.

831 (I-J) IMR90 fibroblasts were mock-transfected (control), transfected with 1 µg MDP / 5 x 10⁵ cells, or
832 with increasing concentrations of LPS (0.01, 0.1 or 1 µg LPS / 5 x 10⁵ cells). BrdU incorporation,

833 p21^{CIP1}, p16^{INK4a}, p21^{CIP1} and caspase-4 levels were measured by IF 48 h after transfection. SA-β-Gal
834 activity was determined 4 days after transfection. Representative pictures (left) of IF staining for
835 p16^{INK4a}, p21^{CIP1} and caspase-4 of mock-transfected (control) and cells transfected with LPS (0.01 LPS
836 / 5 x 10⁵ cells) are shown.

837 Statistical significance in A, D, E and J was calculated using one-way analysis of variance (ANOVA).
838 Statistical significance in G was calculated using using two-way analysis of variance (ANOVA).
839 Statistical significance in G was calculated using two-tailed Student's *t*-test. *****P* < 0.0001, ****P* <
840 0.001, ***P* < 0.01, and **P* < 0.05. ns, not significant. All error bars represent mean ± s.e.m of 3
841 independent experiments.

842

843 **Figure S2**

844 **LPS-mediated caspase-4 induced senescence is independent on inflammasome priming –**
845 **related to Figure 2**

846 (A-C) IMR90 were infected with *CASP4*, *CASP1*, *RAS*^{G12V} expression vectors or empty vector (vector)
847 control. (A) Protein amounts of *CASP4* and *CASP1* were analyzed by immunoblotting. (B) BrdU
848 incorporation and SA-β-Gal activity were determined 4 days after seeding equal number of cells. (C)
849 Relative cell content (left) was quantified 15 days after equal number of cells were seeded;
850 representative images (right) of crystal violet stained cells are shown.

851 (D) IMR90 cells were infected with *CASP4* expression vector or empty vector (vector) control and
852 transfected with LPS (0.1 μg LPS / 5 x 10⁵ cells). Caspase-4 and p16^{INK4a} protein levels were analyzed
853 by immunoblotting 48 h after transfection.

854 (E-F) Cells were treated as shown in Figure 1A. p53 (E) and caspase-4 (F) levels were measured by
855 IF 48 h after LPS transfection.

856 (G) IMR90 cells were treated with MDP (1 μg/mL), LPS, (1 μg/mL) Pam2CSk4 (0.05 μg/mL) and
857 Pam3CSk4 (0.5 μg/mL). *IL1B* mRNA relative expression was quantified at the indicated points. Data
858 from a single representative experiment.

859 (H) IMR90 cells infected with TLR2 expressing vector or control empty vector (EV) were primed with 10
860 μg/ml of A-SAA for 3 hours prior to electroporation with LPS (1 μg/mL) to activate *CASP4*. *IL1B* mRNA
861 relative expression was quantified 48 hours after LPS transfection.

862 (I) Samples from (H) were analyzed for *IL1A*, *IL6*, *IL8* mRNA relative expression 48 hours after LPS
863 transfection.

864 (J) SA- β -GAL staining was conducted 48 hours after treatment as in (H). Values represent the Mean \pm
865 SEM of 3 independent experiments.

866 (K) Proliferation capacity in experiment (H) was measured by BrdU incorporation assay.

867 (L) Analysis of *IL1B* mRNA expression by qRT-PCR in IMR90 cells infected with TLR2 expressing
868 vector or control empty vector (EV), primed with 1 μ g/ml Pam2CSK4 for 3 hours, followed by
869 electroporation with 1 μ g/mL LPS for 48 hours.

870 Statistical significance was calculated using one-way analysis of variance (ANOVA). **** $P < 0.0001$,
871 *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. ns, not significant. All error bars represent mean \pm s.e.m of 3
872 independent experiments.

873

874

875 **Figure S3**

876 **Caspase-4 mediated regulation of senescence is independent of its catalytical function – related** 877 **to Figure 3**

878 (A) Related to Figure 3D. Cells in culture under brightfield microscopy 24 h after transfection, at the
879 time of viability assessment.

880 (B) Related to Figure 3F. Representative images of SA- β -Gal stained cells 4 days after transfection.

881

882 **Figure S4**

883 **The caspase-4 non-canonical inflammasome is activated in Oncogene-induced senescence –** 884 **related to Figure 4**

885 (A) *CDKN1A* (p21^{CIP1}), *CDKN2A* (p16^{INK4a}) and *CDKN2B* (p15^{INK4b}) relative expression levels were
886 measured in IMR90 cells undergoing *RAS*^{G12V}-OIS and control cells.

887 (B) Representative images of caspase-4 stained cells by IF of IMR90 ER:STOP and ER:RAS cells
888 treated with the indicated siRNA 5 days after the addition of 4OHT.

889 (C) After 8 days of 4OHT treatment, conditioned media (CM) from IMR90 ER:STOP and ER:RAS cells
890 was collected and added to IMR90. After 48 h, BrdU incorporation (middle) and caspase-4 levels (right)
891 were measured by IF.

892 (D) IMR90 cells were treated with 10 μ M etoposide and 48 hour later BrdU incorporation (left) and
893 caspase-4 (right) levels were measured by IF. Data relative to BrdU incorporation belongs to a single
894 representative experiment.

895 (E) IMR90 ER:STOP and ER:RAS cells were treated with 4OHT for the indicated time, cells were
896 harvested and subjected to DSS-crosslinking. After SDS-PAGE separation, both DSS-crosslinked
897 samples and inputs were probed for caspase-4 following western blot procedures.

898 Statistical significance was calculated using two-tailed Student's *t*-test. *****P* < 0.0001, ****P* < 0.001,
899 ***P* < 0.01, and **P* < 0.05. ns, not significant. All error bars represent mean \pm s.e.m of 3 independent
900 experiments.

901

902

903 **Figure S5**

904 **Caspase-4 activation controls the proinflammatory SASP – related to Figure 5**

905 (A) *CASP4* mRNA relative expression levels were quantified by RT-qPCR after 5 days (left) and 8 days
906 (right) of 4OHT treatment in ER:STOP and ER:RAS cells transfected with the indicated siRNA.

907 (B) Top five differentially expressed genes upon *CASP4*-targeting in *RAS*^{G12V}-OIS identified by DEG
908 analysis 5 and 8 days after 4OHT treatment.

909 (C) Principal component analysis (PCA) of variance stabilized transformed data using a parametric fit
910 for the dispersion. Each dot corresponds to a sample replicate.

911 (D) Heatmap and hierarchical clustering of the 30 genes with highest variance across all samples based
912 on the total transformed data.

913 (E) Related to Figure 5B. Enrichment plots of the signature “INFLAMMATORY RESPONSE” upon
914 *CASP4*-targeting in *RAS*^{G12V}-OIS 5 (top) and 8 (bottom) days after 4OHT treatment are shown.

915 (F) IMR90 ER:STOP or ER:RAS cells were transfected with control (NTP), *CASP1* or *CASP4*-targeting
916 siRNA and treated with 4OHT during 8 days. IL-1 α , L-1 β , IL-6 and IL-8 levels were analyzed by IF 8
917 days after the addition of 4OHT. Representative images as used for the high content analysis are also
918 shown.

919 (G) After 8 days of 4OHT treatment, conditioned media (CM) from IMR90 ER:STOP and ER:RAS cells
920 was collected and added to IMR90. Concomitantly, IMR90 cells were transfected with control (NTP),

921 *CASP1* or *CASP4*-targeting siRNA. After 48 h, *IL1A*, *IL1B*, *IL8*, *IL6*, *CASP1* and *CASP4* mRNA relative
922 expression levels were measured by RT-qPCR.

923 Statistical significance in A and F was calculated using one-way analysis of variance (ANOVA).

924 Statistical significance in G was calculated using two-tailed Student's *t*-test. *****P* < 0.0001, ****P* <
925 0.001, ***P* < 0.01, **P* < 0.05 and ns, not significant. All error bars represent mean ± s.e.m of 3
926 independent experiments.

927

928

929 **Figure S6**

930 **Caspase-4 contributes to the cell cycle arrest program in OIS – related to Figure 6**

931 (A) *GSDMD* mRNA relative expression was quantified by RT-qPCR in IMR90 ER:STOP and ER:RAS
932 cells 0, 2, 4, 6 and 8 days after 4OHT addition.

933 (B) IMR90 ER:STOP/ER:RAS cells were treated with 4OHT for the indicated time. Caspase-4, full-
934 length (FL) and N-terminal (NT) Gasdermin-D, p21^{CIP1}, p16^{INK4a}, IL-1β and IL-8 levels were analyzed by
935 immunoblotting.

936 (C) IMR90 ER:STOP/ER:RAS cells were transfected with control, *CASP1*, *CASP4* or *GSDMD*-targeting
937 siRNAs. To analyze knockdown efficiency, *CASP1*, *CASP4* and *GSDMD* mRNA relative expression
938 levels were quantified by RT-qPCR after 5 days of 4OHT treatment.

939 (D) IMR90 ER:STOP and ER:RAS cells were transfected with control, *CASP1*, *CASP4* or *GSDMD*-
940 targeting siRNAs. *IL1A*, *IL1B* and *IL8* mRNA relative expression levels were quantified by RT-qPCR
941 after 5 days of 4OHT treatment.

942 (E) IMR90 ER:STOP and ER:RAS cells were transfected with the indicated siRNAs and secreted IL-1β
943 was quantified by ELISA 6,7, and 8 days after 4OHT addition. Statistical analysis was performed
944 comparing control senescent cells (ER:RAS siNTP) to the other conditions 8 days after 4OHT addition.

945 (F) IMR90 ER:STOP and ER:RAS cells were transfected with control (NTP), two individual *CASP4*-
946 targeting siRNAs or a pool of 4 different siRNA sequences targeting *CASP4*, and treated with 4OHT or
947 not as indicated. Caspase-4 levels were measured by IF 5 days after 4OHT addition.

948 (G) IMR90 ER:STOP and ER:RAS cells were transfected with control, or *CASP4*-targeting siRNAs and
949 SA-β-Gal activity was determined 8 days after the addition of 4OHT (left). Representative images for
950 SA-β-Gal activity are shown (right).

951 (H) Enrichment plots of the signatures “G2M CHECKPOINT” (left) and “E2F TARGETS” (right) upon
952 *CASP4*-targeting in *RAS*^{G12V}-OIS IMR90 cells 5 days after 4OHT treatment.

953 (I) IMR90 ER:STOP and ER:RAS cells were transfected with control (NTP), *CASP1* or *CASP4*- targeting
954 siRNAs. After 5 days of 4OHT treatment, caspase-4 proIL-1 β and pRb were analyzed by
955 immunoblotting.

956 (J) Catalytically inactive (C258A) *CASP4* was overexpressed in IMR90 cells prior to LPS transfection
957 (1 μ g LPS/5 x 10⁵ cells). 48 h after transfection, caspase-4 and pRb were analyzed by immunoblotting.

958 (K) IMR90 cells were infected with an empty pRS vector (vector) or a pRS vector targeting either *CASP1*
959 or *CASP4* prior to transfection with 0.1 μ g LPS / 5 x 10⁵ cells. *CCNA1*, *CDC6* and *BUB1* mRNA relative
960 expression were quantified by RT-qPCR 48 h after LPS transfection.

961 (L) IMR90 ER:STOP and ER:RAS cells were transfected with control, *CASP1*, *CASP4* or *GSDMD*-
962 targeting siRNAs. BrdU incorporation was measured by IF 5 days after 4OHT addition.

963 Statistical significance in A, G and L was calculated using two-tailed Student's *t*-test. Statistical
964 significance in C-F and K was calculated using one-way analysis of variance (ANOVA). *****P* < 0.0001,
965 ****P* < 0.001, ***P* < 0.01, and **P* < 0.05. ns, not significant. All error bars represent mean \pm s.e.m of 3
966 independent experiments.

967

968 **Table S1: Primers used for mRNA gene expression analysis**

Gene	Forward	Reverse
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
CASP4	GAGAAGCAACGTATGGCAGG	GGAATTCTTCATGAGGACAAAGC
IL1B	TGCACGCTCCGGGACTCACA	CATGGAGAACACCACTTGTGTCTCC
IL1A	AGTGCTGCTGAAGGAGATGCCTGA	CCCCTGCCAAGCACACCCAGTA
IL6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
IL8	GAGTGGACCACACTGCGCCA	TCCACAACCCTCTGCACCCAGT
CASP1	CAACTACAGAAGAGTTTGAGG	AACATTATCTGGTGTGGAAG
GSDMD	ATGGATGGGCAGATACAGGG	TGCTGCAGGACTTTGTGTTC
CDKN1A	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT
CDKN2A	CGGTCGGAGGCCGATCCAG	GCGCCGTGGAGCAGCAGCAGCT
CDKN2B	GAATGCGCGAGGAGAACAAG	CCATCATCATGACCTGGATCG
BUB1	ACACCATTCCACAAGCTT	CGCCTGGGTACTACTGTTT
CDC6	GTTCAATTCTGTGCCCGCAA	TAGCTCTCCTGCAAACATCCAG

CCNA1	CCATCGACCTCAGCAAGCA	TGGCTCCATGAGGGACACA
CCNA2	AGGAAACTTCAGCTTGTGGG	CACAACTCTGCTACTTCTGGG
CCNB1	TGTGTCAGGCTTTCTCTGATG	TTGGTCTGACTGCTTGCTCT
MCM6	ACTGTTCTGGACTTCTTGG	ACGAATCAGTTCCTCTGCT
SAA1	GAGCACACCAAGGAGTGATTT	GAAGCTTCATGGTGCTCTCT
SAA2	GCTGCAGAAGTGATCAGCAAT	CAGCGAGTCCTCCGCAC

Figure S1

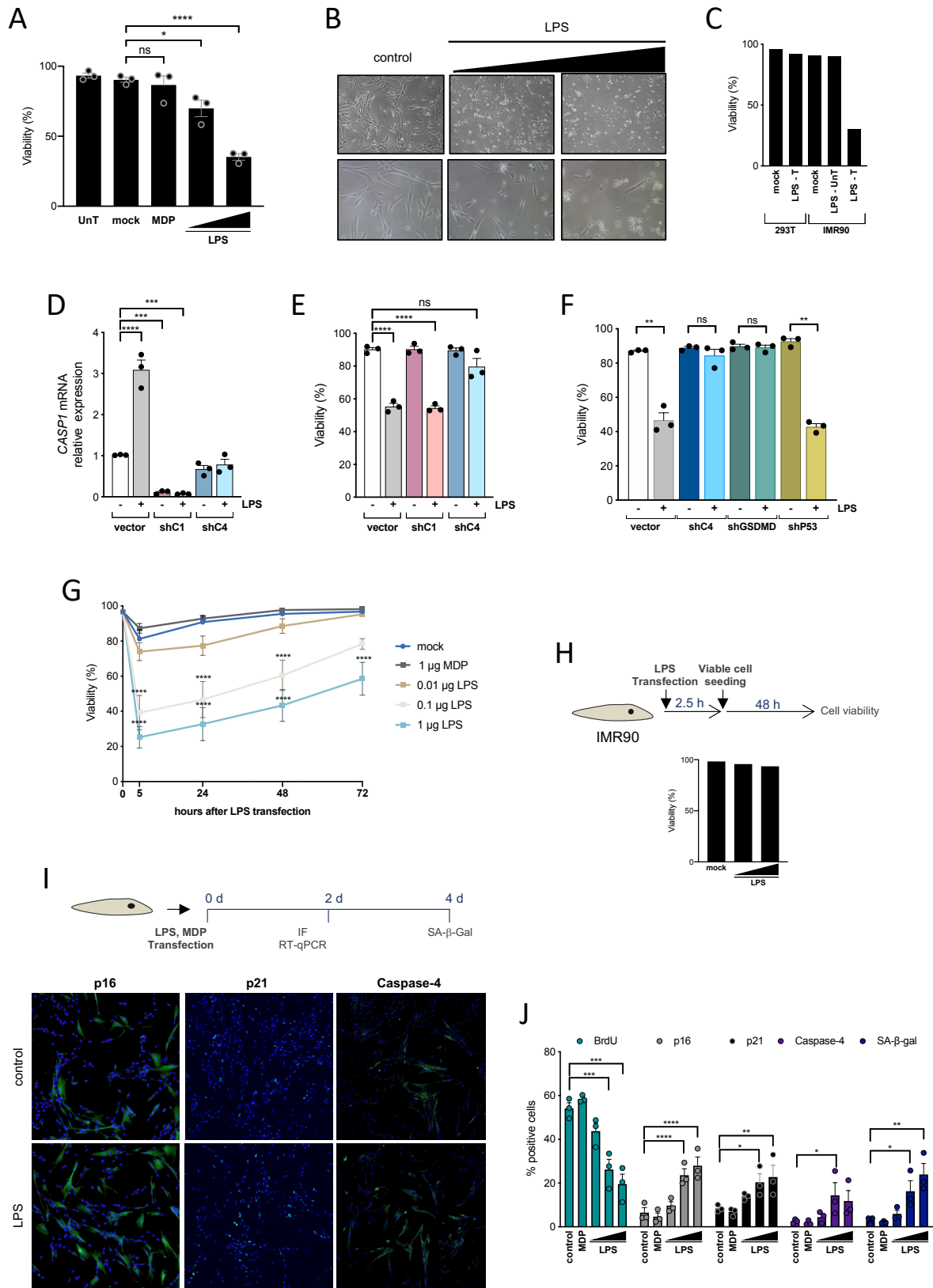


Figure S2

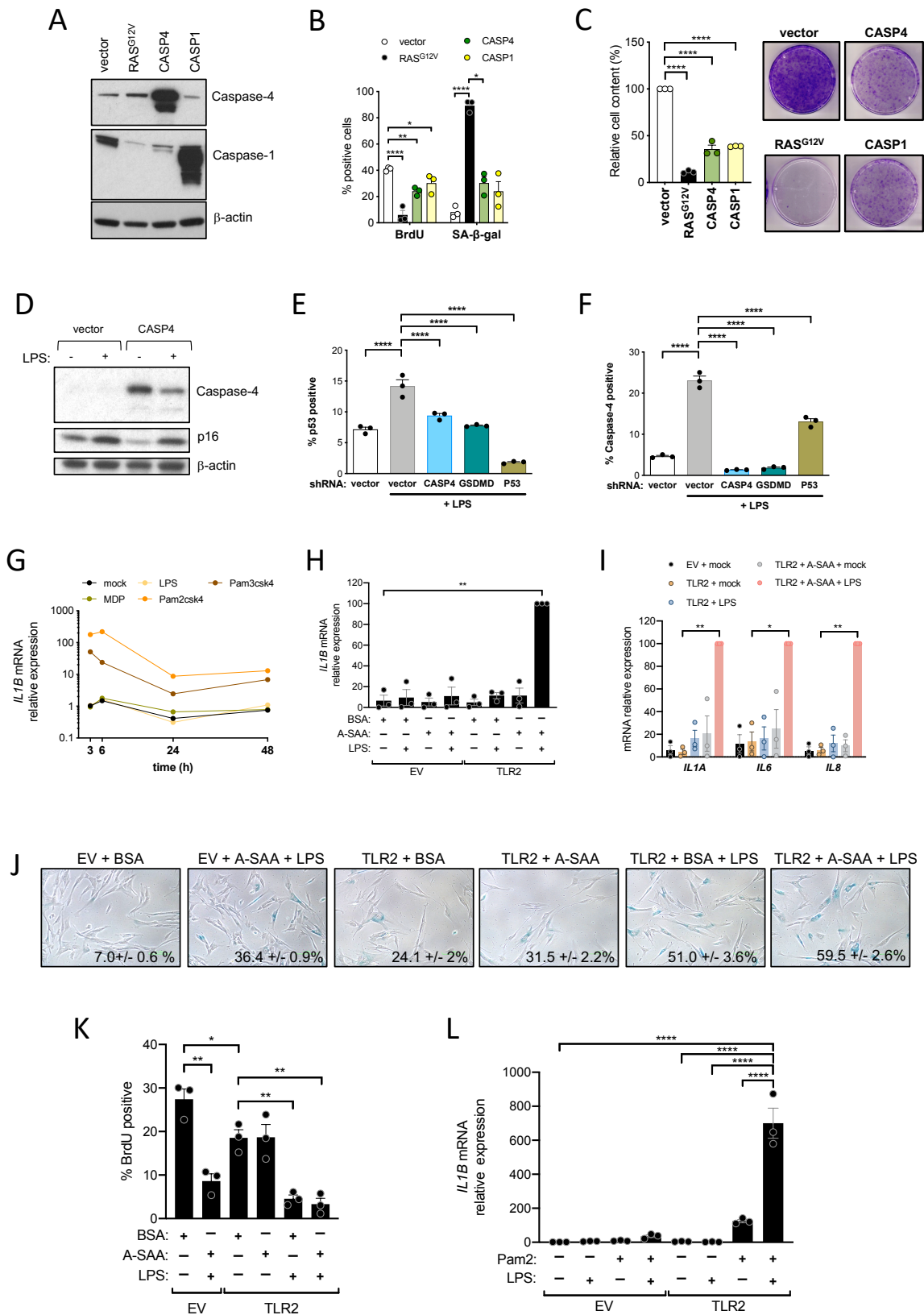
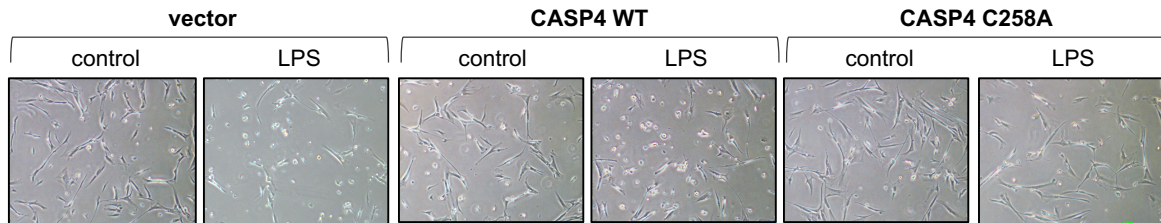


Figure S3

A



B

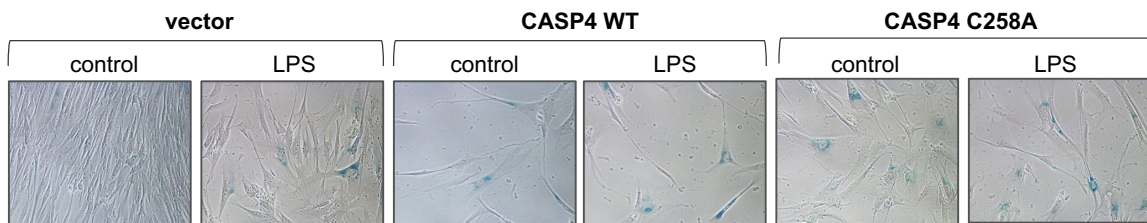


Figure S4

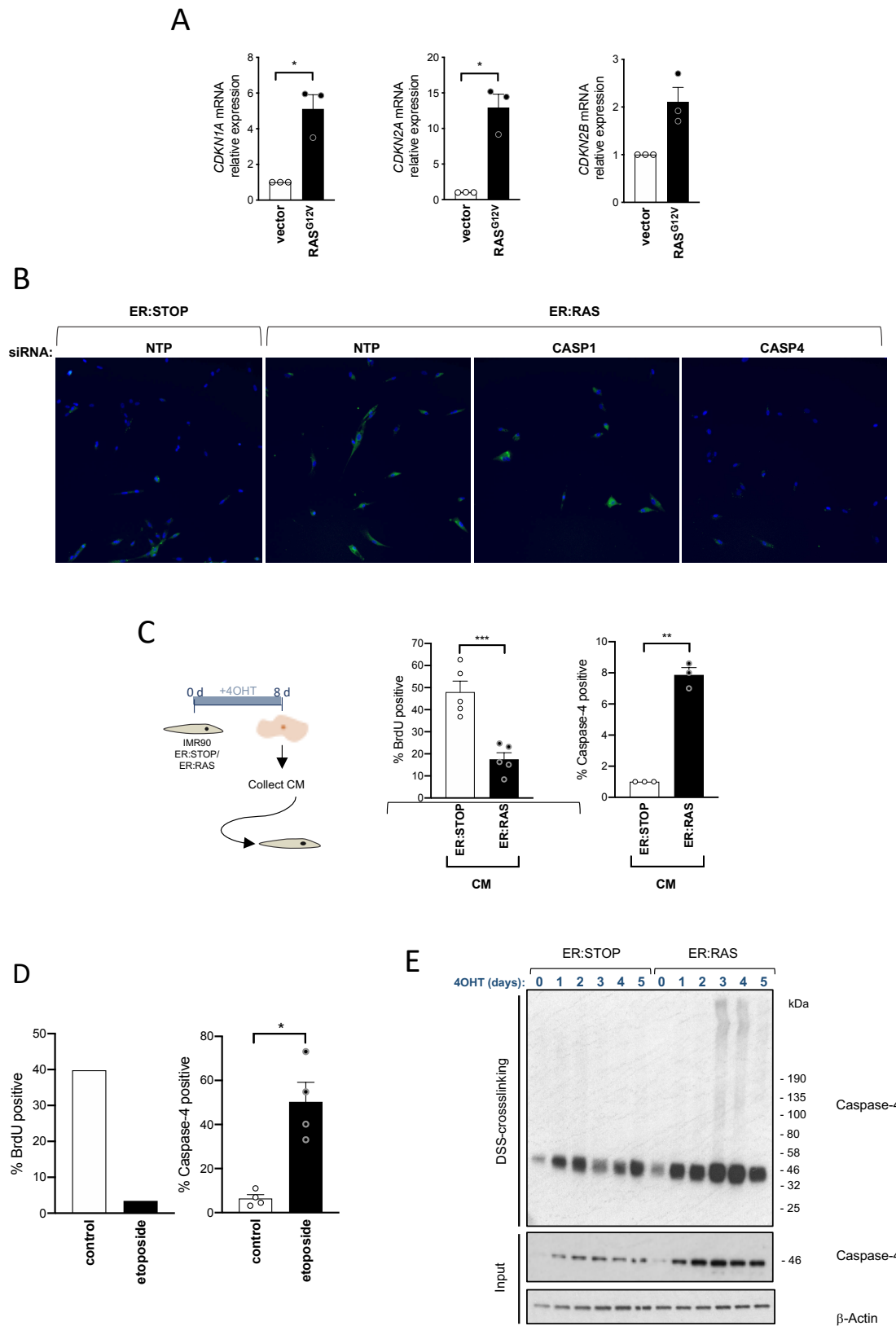


Figure S5

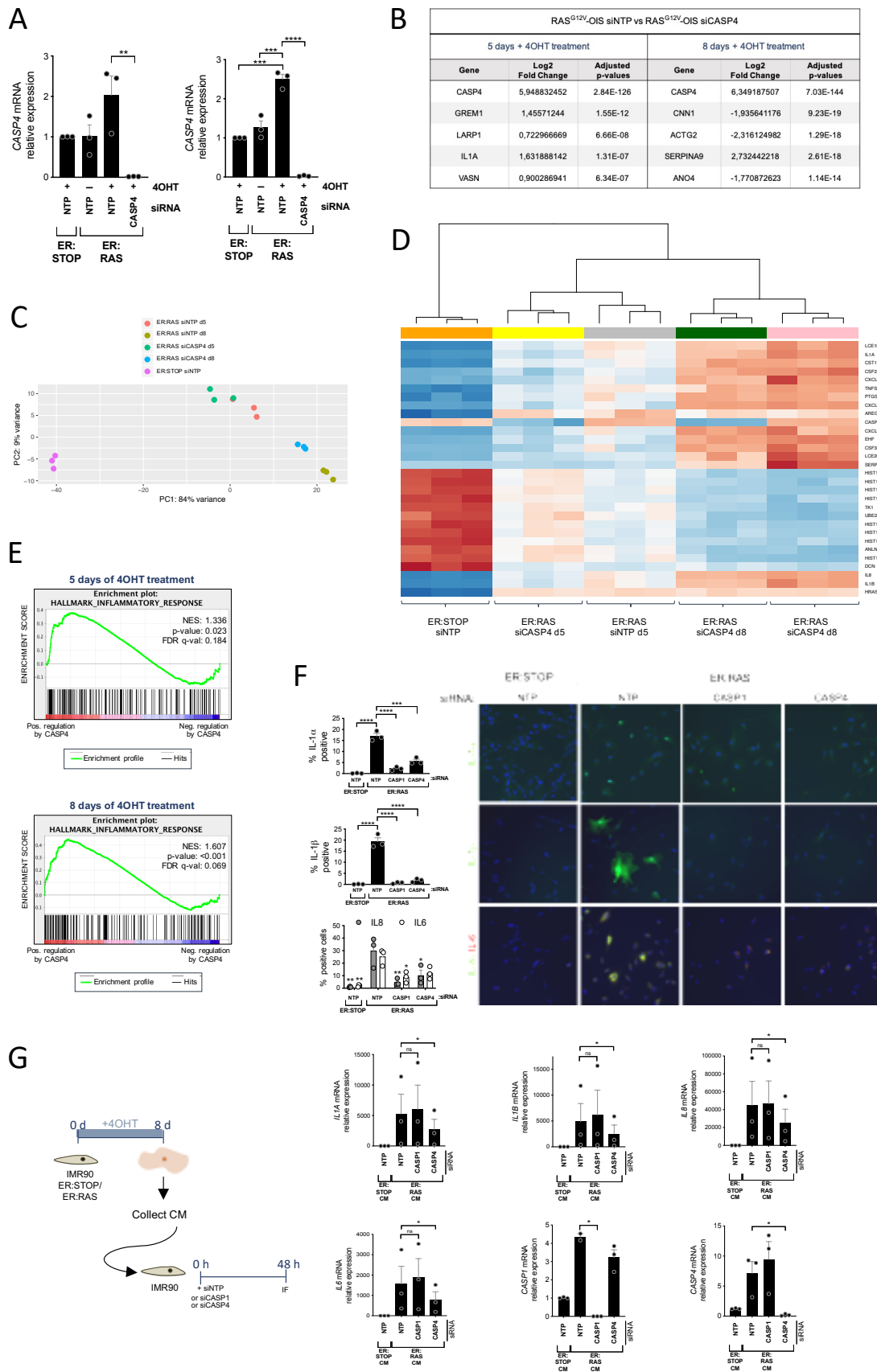
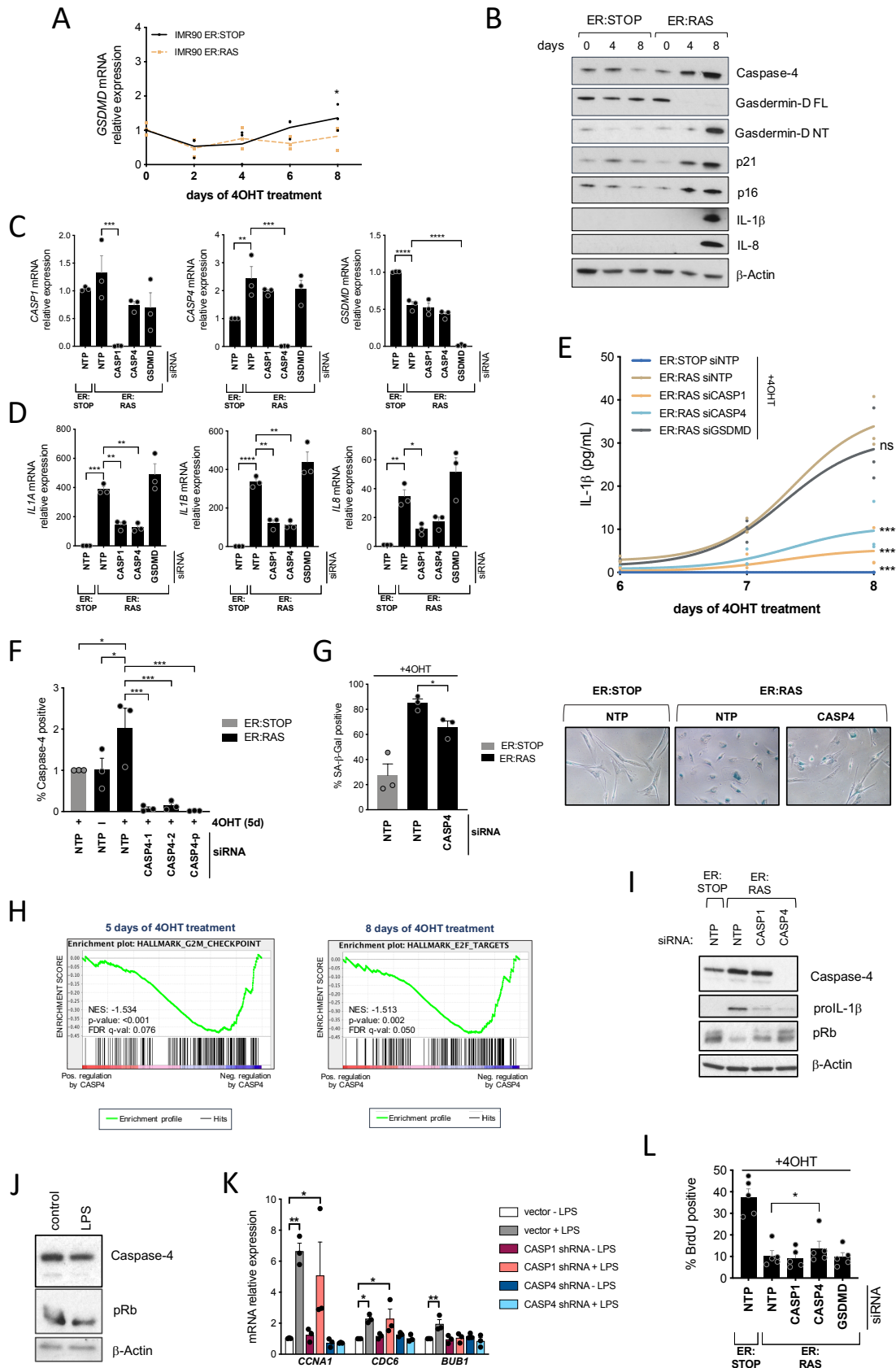


Figure S6



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