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1 **A cGAS-dependent response links DNA damage and senescence in alveolar**
2 **epithelial cells: A potential drug target in IPF**

3

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7

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19

20 **Running title:** The role of cGAS in alveolar epithelial cell senescence

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25

26 **ABSTRACT**

27 Alveolar epithelial cell (AEC) senescence is implicated in the pathogenesis of idiopathic
28 pulmonary fibrosis (IPF). Mitochondrial dysfunction including release of mitochondrial
29 DNA (mtDNA) is a feature of senescence, which led us to investigate the role of the DNA-
30 sensing GMP-AMP synthase (cGAS) in IPF, with a focus on AEC senescence. cGAS
31 expression in fibrotic tissue from lungs of IPF patients was detected within cells
32 immunoreactive for epithelial cell adhesion molecule (EpCAM) and p21, epithelial and
33 senescence markers respectively. Submerged primary cultures of AECs isolated from lung
34 tissue of IPF patients (IPF-AECs, n=5) exhibited higher baseline senescence than AECs from
35 control donors (Ctrl-AECs, n=5-7), as assessed by increased nuclear histone 2AX γ
36 phosphorylation, p21 mRNA and expression of senescence-associated secretory phenotype
37 (SASP) cytokines. Pharmacological cGAS inhibition using RU.521 diminished IPF-AEC
38 senescence in culture and attenuated induction of Ctrl-AEC senescence following etoposide-
39 induced DNA damage. Short interfering RNA (siRNA) knockdown of cGAS also attenuated
40 etoposide-induced senescence of the AEC line, A549. Higher levels of mtDNA were
41 detected in the cytosol and culture supernatants of primary IPF- and etoposide-treated Ctrl-
42 AECs when compared to Ctrl-AECs at baseline. Furthermore, ectopic mtDNA augmented
43 cGAS-dependent senescence of Ctrl-AECs, whereas DNase I treatment diminished IPF-AEC
44 senescence. This study provides evidence that a self-DNA driven, cGAS-dependent response
45 augments AEC senescence, identifying cGAS as a potential therapeutic target for IPF.

46

47 **Key words:** Alveolar epithelium, Idiopathic pulmonary fibrosis (IPF), cGAS, Mitochondrial
48 DNA (mtDNA) and Senescence

49

50 INTRODUCTION

51 Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal lung disease of unknown aetiology,
52 with few treatment options (6, 7, 27). Age is a risk factor and appears to be a major driver of
53 the disease; albeit through mechanisms that are not entirely understood (22). Cellular
54 senescence, a hallmark of ageing is characterized by irreversible cell cycle arrest, increased
55 resistance to apoptosis and the senescence-associated secretory phenotype (SASP) (37).
56 Senescence is an outcome of genomic DNA damage caused primarily by telomere shortening
57 and/or oxidative stress (ie as a consequence of mitochondrial dysfunction). There is
58 accumulating evidence that senescence contributes to IPF, including the linkage of mutations
59 that accelerate telomere attrition and the detection of senescent epithelial cells and fibroblasts
60 in lung from IPF patients (2, 14, 19).

61

62 The enzyme GMP-AMP synthase (cGAS) detects double stranded DNA (dsDNA) in the
63 cytoplasm to generate the cyclic dinucleotide cGAMP, a secondary messenger that elicits
64 pro-inflammatory and type I interferon (IFN) responses. Apart from sensing bacterial and
65 viral DNA in innate immunity, cGAS also binds and is activated by damaged endogenous
66 DNA released from the nucleus and mitochondria (39, 42). Notably, a number of recent
67 studies provide compelling evidence that cGAS activation by self-DNA is a driver of
68 inflammatory lung disease ie asthma, COPD and silicosis (reviewed by Ma *et al* (20)). cGAS
69 is also a crucial mediator of damaged DNA-induced cellular senescence (11, 42). The
70 contribution of cGAS in senescence involves activation of the stimulator of interferon genes
71 (STING) pathway and subsequent induction of the SASP, which perpetuates senescence via
72 autocrine and paracrine processes. Our group recently provided evidence that damaged
73 mitochondrial DNA (mtDNA) via the activation of cGAS augments lung fibroblast
74 senescence; a process which may contribute to the pathogenesis of fibrotic lung diseases such

75 as IPF (32). To our knowledge, the role of cGAS in alveolar epithelial cell (AEC) senescence
76 in the context of IPF pathology has not been previously evaluated (20).

77

78 MtDNA encodes essential protein subunits of the electron transport chain (ETC) which drive
79 mitochondrial respiration. In close proximity to the ETC where reactive oxygen species
80 (ROS) are formed, damage and subsequent mutations accumulate in mtDNA with age;
81 resulting in mitochondrial dysfunction and collapse in mitochondrial membrane potential (15,
82 16). DNA released by dysmorphic mitochondria is a potent damage-associated molecular
83 pattern (DAMP) that triggers inflammatory responses by binding pattern recognition
84 receptors (PRRs), including cGAS and toll-like receptor 9 (TLR9) (40). The increased
85 propensity for senescence of AECs and lung fibroblasts from IPF patients is associated with
86 alterations in mitochondrial homeostasis and function (6, 7, 31). Ryu and colleagues recently
87 showed that lung fibroblasts from IPF patients release increased amounts of mtDNA when
88 compared to age-matched controls and that higher levels of cell free mtDNA in the
89 bronchoalveolar lavage fluid (BALF) or serum of IPF patients predicts mortality (29). In
90 another study, increased levels of oxidized and damaged mtDNA were detected in lung tissue
91 of IPF patients as well as the supernatants of type 2 AECs (AEC2s) *in vitro* when subjected
92 to endoplasmic reticulum (ER) and oxidative stress (7). These studies and others suggest that
93 cell-derived, damaged mtDNA could play a key role in IPF pathology.

94

95 In this study, we investigated whether increased release of self (mt)DNA perpetuates AEC
96 senescence via cGAS. To address this, we used AECs isolated from IPF patients (IPF-LFs)
97 with high baseline senescence, or in cells from controls (Ctrl-AECs) that were induced to
98 become senescent using etoposide. The levels of cytosolic and extracellular mtDNA formed
99 by these cells were examined, as well as the effects of ectopic mtDNA and DNaseI on

100 senescence. Pharmacological intervention was used to determine the contribution of cGAS to
101 DNA-driven AEC senescence.

102 **MATERIALS AND METHODS**

103 *Lung tissue and primary cultures of AECs*

104 Unless stated otherwise, lung tissue was obtained from patients at the John Hunter Hospital
105 (New Lambton Heights, NSW, Australia) under ethical approval from the human research
106 ethics committees of both the hospital (16/07/20/5.03) and University of Newcastle (H-2016-
107 0325); following guidelines from the National Health and Medical Research Council
108 (NHMRC, Australia). All patients provided written, informed consent for their tissue to be
109 utilised in medical research, and were classified as either IPF or Control (Ctrl). Four primary
110 cultures of IPF-AECs were established from parenchymal tissue of lung obtained from
111 deceased IPF patients 1-2.5 h post-mortem, which were stored in Hank's balanced saline
112 solution (HBSS) at 4°C (between 4-18 h) before cell isolation and culture. The fifth culture
113 of IPF-AECs was established from lung of an IPF patient undergoing lung transplant at St
114 Vincent's Hospital (Sydney, NSW, Australia). Histological staining of lung sections from
115 these IPF patients show extensive pulmonary fibrosis (**Figure S1**, data supplement). For Ctrl-
116 AECs, macroscopically normal parenchymal lung tissue resections were obtained from
117 patients with no evidence of interstitial lung disease (ILD), mostly patients with cancer,
118 undergoing thoracic surgery. Demographic data of patients is provided in **table S1** (data
119 supplement).

120

121 AEC cultures were established using parenchymal tissue in a multi-step process involving
122 enzyme digestion, differential cell adhesion and cell sorting. Briefly, tissue (1-10 g) was
123 washed and minced before enzymatic digestion in 30 mL HBSS containing elastase (1.125
124 U), trypsin (750 U) and collagenase (1875 U) at 37°C for 60 min. The tissue digest was
125 neutralized by addition of an equal volume of Dulbecco's Modified Eagles Medium
126 (DMEM)-Hams F12 medium containing 20% v/v fetal calf serum (FCS) and DNase I

127 (15,000 U) before successive passage through 100 and 40 micron cell strainers. Dissociated
128 cells were pelleted by centrifugation before resuspension in adhesion medium comprised of a
129 1:1 ratio of bronchial epithelial cell growth medium (BEGM, Lonza) and DMEM-Hams F12
130 with 1% v/v FCS and DNase I (300 U/mL). Cells were then seeded into T75 flasks, which
131 were kept in an incubator at 37°C for 2 h to allow for macrophage and fibroblast adhesion.
132 Afterwards, unattached cells were removed, washed and resuspended in HBSS before
133 incubation with Anti-CD326 (EpCAM) conjugated magnetic MicroBeads (Miltenyi Biotec)
134 for 30 min to select epithelial cells. The cell suspension was pipetted onto a MiniMACS
135 column (Miltenyi Biotec) attached to a magnet and allowed to flow through the column.
136 After several washes with HBSS, the magnet was removed and the cells that were attached in
137 the magnetic field were eluted into BEGM with 1% v/v FCS, before being seeded into flasks.
138 The culture medium was replenished thereafter every 2-3 days. Flasks typically reached 80-
139 90% confluency within 2-4 weeks of seeding, after which they were sub-cultured.

140

141 *Cell culture conditions and treatments*

142 Submerged primary cultures of AECs, after the first or second passage, were grown in tissue
143 culture plates in BEGM at 37°C in air containing 5% CO₂. Treatments, including RU.521 (3
144 μM, Aobious), DNaseI (100 U/mL, Sigma) and ectopic mtDNA (0.1 μg/mL) isolated from
145 human lung fibroblasts were added to the culture medium 24 h after cells were seeded into 6,
146 12, 24 or 48 well plates (2 x 10⁴ cells/cm²) and re-added with each subsequent medium
147 change (every 2-3 days) over a 7 day period. For etoposide-induced senescence experiments,
148 Ctrl-AECs were treated once with etoposide (10 μM) or the appropriate volume of DMSO as
149 vehicle control. After 24 h incubation with etoposide, the medium was replenished with fresh
150 BEGM. RU.521 was added 30 min before etoposide, and re-added with each subsequent
151 medium change (every 2-3 days) for a total of 5 days after the initial addition of etoposide.

152 For experiments with the A549 tumor cell line (obtained from ATCC), cells were grown in
153 DMEM containing low glucose (1 g/L), L-alanyl-glutamine (4 mM), sodium pyruvate (1
154 mM), non-essential amino acids (1% v/v, Sigma) and FCS (1% v/v) at 37°C in air containing
155 5% CO₂. A549 cells were used between passages 16-21 and treated with etoposide and/or
156 RU.521 as described previously for the primary cultures of AECs. Rotenone (100 nM) or HT-
157 151 (100 nM), inhibitors of complex I of the mitochondrial respiratory chain and STING
158 respectively were also added to A549 cells in selected experiments (12, 31).

159

160 *siRNA transfection*

161 A549 cells grown in 6 or 24 well plates were transfected with 20 nM RNA siRNA duplex
162 oligonucleotides targeting cGAS using RNAiMax Lipofectamine (Invitrogen, CA, USA)
163 according to the manufacturer's instructions. Cells were transfected 24 h after seeding (2 x
164 10⁴ cells/cm²) in antibiotic-free DMEM containing FCS (10% v/v) by incubation with
165 siRNA-Lipofectamine complex for 4 h. The medium was then replaced with DMEM
166 containing 1% v/v FCS and antibiotics, before cells were maintained for an additional 5 days
167 in culture with subsequent medium changes every 2-3 days. Etoposide was added once, 24
168 hours following transfection. The sequences of cGAS and control siRNA are provided in
169 Schuliga *et al* (32).

170

171 *Immunofluorescence detection of cGAS in lung tissue*

172 Lung sections were co-stained for cGAS and epithelial cell adhesion molecule (EpCAM) or
173 p21 by immunofluorescence. Antigens were identified by rabbit polyclonal antibodies to
174 cGAS (#15102, Cell Signaling Technology), -phospho-STING (Ser366) (#19781, Cell
175 Signaling Technology) or p21 (#2947, Cell Signaling Technology) and monoclonal mouse
176 antibodies to EpCAM (#2929, Cell Signaling Technology) or p21 (#6246, Santa Cruz

177 Biotechnology). Primary antibodies bound to antigen were detected using Alexa Fluor 555
178 anti-rabbit-conjugate (Cell Signaling Technology) or Alexa Fluor 488 anti-mouse-conjugate
179 (Cell Signaling Technology). All primary antibodies were used at a 1 in 50 dilution, whereas
180 secondary antibodies were used at a 1 in 1000 dilution. Tissues were mounted under
181 coverslips using Prolong Gold AntiFade with DAPI (Molecular Probes, Cell Signaling
182 Technology) and fluorescent images were captured at 200 X magnification using a Nikon
183 Eclipse Ti-U fluorescence microscope.

184

185 *Immunofluorescence detection of cells in culture*

186 Phosphorylated histone 2AX (H2AX γ), EpCAM, cGAS and surfactant protein A (SP-A) were
187 detected in cells in culture by immunofluorescence. Cells grown in 48-well plates were fixed
188 with 4% w/v formaldehyde in PBS for 10 min before blocking and permeabilization with
189 0.15% v/v Triton X-100, 10% v/v goat serum and 1% w/v BSA in PBS for 10 min. Cells
190 were then incubated with anti-H2AX γ (Ser139) (#9718, Cell Signaling Technology), -cGAS
191 (#15102, Cell Signaling Technology) or -SP-A (#sc13977, Santa Cruz Biotechnology) rabbit
192 polyclonal IgG or anti-EpCAM (#2929, Cell Signaling Technology) mouse monoclonal IgG
193 overnight at 4°C. After washing, cells were incubated with Alexa Fluor 555 anti-rabbit-
194 conjugate and/or Alexa Fluor 488 anti-mouse-conjugate (Cell Signaling Technology) for 1 h
195 at room temperature. All antibodies were used at a 1 in 300 dilution. Cells were
196 counterstained with DAPI (1 μ g/mL, Sigma) and mounted in 70% v/v glycerol. Fluorescent
197 images of cells were taken at 100 X magnification using a Nikon Eclipse inverted Ti-U
198 fluorescence microscope. For H2AX γ quantitation, the fluorescence intensity of the red
199 (H2AX γ) and blue (DAPI) channels for each image were pseudo-colored and merged using
200 Fiji software (NIH). Quantitation was achieved using macro plugins to specifically measure
201 the percentage area of nucleus associated with fluorescence from H2AX γ .

202

203 *Immunohistochemistry (IHC)*

204 Serial sections of parenchymal lung tissue from IPF patients embedded in paraffin were
205 immunohistochemically stained for cGAS, p21 and EpCAM. Antigens were identified by
206 rabbit polyclonal antibodies to cGAS (#15102, Cell Signaling Technology), p21 (#2947, Cell
207 Signaling Technology) or EpCAM (#15102, Cell Signaling Technology). Antibody staining
208 was completed using the Dako EnVision anti-rabbit kit as appropriate (Dako Corp.,
209 Carpinteria, CA, USA) and 3,3'-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA);
210 where sections were counterstained with hematoxylin.

211

212 *Purification of mtDNA*

213 Primary cultures of human lung fibroblasts established as previously described and grown to
214 confluence in T75 tissue culture flasks were harvested by trypsinization (32). Mitochondria
215 were isolated using the Mitochondrial Isolation Kit for Cultured Cells (ThermoScientific)
216 according to the Manufacturer's instruction. DNA was extracted from mitochondria using
217 QIAamp DNA mini spin columns (Qiagen). mtDNA was isolated from fibroblasts rather than
218 epithelial cells because high numbers of cells were required to isolate enough mtDNA for
219 experimentation.

220

221 *Senescence-associated β -galactosidase detection*

222 For senescence-associated β -galactosidase (SA- β -Gal) staining, cell cultures in 12 or 24 well
223 plates were fixed and stained using a commercial kit (Cell Signaling Technology) according
224 to the manufacturer's instructions. Cells were imaged using an Olympus IX51 inverted
225 microscope. SA- β -Gal staining was used qualitatively in the assessment of senescence, with
226 images being representative of 2-3 separate experiments.

227

228 *Preparation of cytosol extracts*

229 Cells were harvested by trypsinization before resuspension in 1mL PBS. The cells in 900 μ L
230 suspension were pelleted by centrifugation (300 g) before sub-cellular fractionation using the
231 Mitochondrial Isolation Kit for Cultured Cells (ThermoScientific) according to the
232 Manufacturer's instruction. The kit allows for the isolation of a cytosol fraction free of nuclei
233 and mitochondria (32). The remaining 100 μ L of cell suspension was combined with 700
234 μ L of 50 μ M NaOH before heating at 100°C for 30 min, then neutralization by the addition of
235 200 μ L 1M Tris-HCl pH 8. Denatured whole cell extracts allow levels of mtDNA detected in
236 the cytosol fraction (and medium) to be normalized to total mtDNA in the cells.

237

238 *ELISA*

239 Levels of IL-6, interferon- β (IFN- β) and transforming growth factor- β 1 (TGF- β 1) in
240 conditioned medium were measured by specific sandwich enzyme-linked immunosorbent
241 assays (ELISA) using commercial kits (DuoSets, RnDSystems, MN, USA) as according to
242 the manufacturer's instructions.

243

244 *PCR analysis*

245 Levels of nucleic acids were analyzed by real time polymerase chain reaction (PCR). RNA
246 and DNA were purified from cells and/or from subcellular fractions (ie cytosol and
247 conditioned media) using RNeasy and QIAamp DNA mini spin columns (Qiagen)
248 respectively. RNA was reverse transcribed into cDNA using the iScript Advanced cDNA kit
249 (BioRad). DNA was amplified by qPCR using the iTaq Universal SYBR Green Supermix
250 (BioRad) in an ABI Prism 7500HT sequence detection system (Applied Biosystems) with the
251 relevant PCR primers (sequences provided in Schuliga *et al* (32)). For RNA quantitation, the

252 threshold cycle (CT) value determined for each gene of each sample was normalized against
253 that obtained for 18S rRNA, used as an internal control. The level of mRNA for a particular
254 gene is proportional to $2^{-(\Delta CT)}$, where ΔCT is the difference between the CT values of the
255 target gene and 18S rRNA. Relative levels (2^{-CT}) of mtDNA and nuclear DNA (nDNA) in
256 the cytosol fraction and media were measured using PCR primers for the mitochondrial and
257 nuclear genes, tRNA^{Leu(UUR)} and β 2-microglobulin (B2M) respectively, expressed as a
258 percentage of their levels detected in denatured whole cell extracts.

259

260 *Statistical analysis*

261 Grouped data are graphed as box and whisker plots. For experiments with primary cultures, n
262 represents individual experiments conducted using AECs from separate IPF patients or
263 controls. For A549 cells, two separate experiments with three biological replicates per
264 treatment group per experiment were conducted. Comparisons between two groups were
265 analyzed by the non-parametric Wilcoxon matched pairs signed rank or Mann-Whitney U
266 tests (Graphpad Prism 5.0, Graphpad, San Diego, CA) as appropriate. A value of $P < 0.05$ was
267 considered to be statistically significant.

268

269 **RESULTS**

270 *cGAS is associated with senescent epithelial cells in the fibrotic lung of IPF patients*

271 Immunofluorescence (IF) detection of cGAS in serial sections of lung parenchymal tissue
272 from three separate IPF patients shows cGAS is localized within cells that are positive for the
273 epithelial marker, EpCAM (**Fig. 1** & **Fig. S2**, data supplement). cGAS was also detected in
274 EpCAM positive epithelial cells of lung parenchyma from control donors, albeit expression
275 appeared less intense than in IPF lung (**Fig. 1a**). Dual-labelling IF of serial sections suggests
276 that cGAS positive regions of epithelium in IPF lung also express phosphorylated STING
277 (pSTING), the downstream mediator of DNA-bound cGAS, and the senescence marker, p21
278 (**Fig. 1b & 1c**). Although some non-specific fluorescence (green channel) was detected in
279 the staining controls, this fluorescence was extracellular and attributable to auto-fluorescence
280 from ECM protein (**Fig. 1**). Immunohistochemistry of serial sections of lung tissue from an
281 IPF patient provides additional evidence that cGAS and p21 are present within the same
282 populations of epithelial cells (**Fig. 2**).

283

284 *AECs from IPF patients exhibit high baseline senescence in vitro*

285 Submerged cultures of IPF- and control (Ctrl)-AECs contained EpCAM positive cells, with
286 >85% also exhibiting a pericellular distribution of SP-A, signifying type 2 pneumocytes
287 (AEC2s) (**Fig. 3a-b**). Larger EpCAM⁺, SP-A⁻ cells were likely to be type I pneumocytes
288 (AEC1s). IPF-AECs (n=5) exhibited a higher baseline senescence than Ctrl-AECs (n=5-7),
289 as measured by increased levels of nuclear H2AX γ , p21 mRNA and production of IL-6 and
290 interferon- β (IFN- β) (P<0.05) (**Fig. 3c-f**). The levels of mRNA encoding IL-6 and two other
291 SASP cytokines, CCL2 and IL-8 were also higher in IPF- than Ctrl-AECs (**Fig. S3**, data
292 supplement). Furthermore, SA- β -Gal staining appeared more intense for IPF-AECs, when
293 compared to Ctrl-AECs (**Fig. 3g**).

294

295 *Pharmacological cGAS inhibition diminishes IPF-AEC senescence*

296 Pharmacological cGAS inhibition with RU.521 diminished IPF-AEC senescence in culture
297 for 7 d. The exposure of IPF-AECs from five separate patients during exponential growth to
298 RU.521 reduced the following markers of senescence: levels of nuclear H2AX γ , p21 mRNA,
299 and IL-6 and IFN- β protein (**Fig. 4a-d**). The levels of mRNA transcripts for SASP mediators
300 (IL-6, CCL2 and IL-8) were also lower when compared to the vehicle control (**Fig. S4**, data
301 supplement). Cytochemical staining also suggested that RU.521 reduced the levels of SA- β -
302 Gal (**Fig. 4e**).

303

304 *cGAS drives DNA damage-induced AEC senescence*

305 The role of cGAS in the acquisition of AEC senescence was also examined using Ctrl-AECs.
306 This phenotype exhibited heightened senescence following treatment with the DNA
307 damaging agent, etoposide (10 μ M); in a manner sensitive to pharmacological cGAS
308 inhibition using RU.521 (**Fig. 5 & Fig. S5** data supplement) ($P < 0.05$, $n = 5-6$). Comparable
309 effects of RU.521 were observed in cultures of the AEC2 cell line, A549 following treatment
310 with etoposide (**Fig. S6** data supplement) ($P < 0.05$, $n = 5-6$). Additionally, transfection of A549
311 cells with cGAS-selective siRNA attenuated etoposide-induced increases in senescent
312 markers (**Fig. 6 & Fig. S7** online supplement) ($P < 0.05$, $n = 5-6$). Furthermore, the
313 pharmacological inhibition of STING in A549 cells using HT-151 (0.1 μ M) also attenuated
314 etoposide-induced increases in senescence markers (**Fig. S8**, data supplement) ($P < 0.05$, $n = 6-$
315 7).

316

317 *mtDNA release is higher in senescent AECs*

318 The release of mtDNA and nuclear DNA (nDNA) by AECs were assessed by qPCR using
319 primers for tRNA^{Leu(UUR)} and β 2-microglobulin (B2M), respectively (28). Levels of mtDNA
320 in the cytosol and conditioned medium (CM) of IPF-AECs (expressed as % of total cellular
321 mtDNA) were higher than Ctrl-AECs (**Fig. 7a**) ($P < 0.05$, $n = 5-6$). Etoposide-induced Ctrl-
322 AEC senescence corresponded with an increase in the release of mtDNA into the cytosol and
323 extracellular space (**Fig. 7b**) ($P < 0.05$, $n = 6$). Levels of nDNA were also higher in the CM of
324 IPF-AECs and senescence-induced Ctrl-AECs when compared to baseline levels of Ctrl-
325 AECs (**Fig. 7c-d**) ($P < 0.05$, $n = 5-6$). However, no differences in the levels of nDNA in the
326 cytosolic fractions were observed ($P > 0.05$, $n = 5-6$).

327

328 *Rotenone induces mtDNA release and AEC senescence in a cGAS-dependent manner*

329 Increased levels of mtDNA in the cytosol of IPF- and etoposide-treated Ctrl-AECs suggests
330 mtDNA damage and release contributes to the senescence phenotype. To delineate the
331 specific contribution of endogenous mtDNA in cGAS activation and senescence, AECs cells
332 were treated with rotenone; an inducer of mitochondrial stress and subsequent mtDNA
333 damage (31). Incubation of A549 cells with rotenone (100 nM) evoked mtDNA release as
334 shown by increases in the levels of mtDNA detected in both the cytosol and supernatant (**Fig.**
335 **8a**) ($P < 0.05$, $n = 6$). Such increases corresponded with increases in markers of senescence
336 including increased levels of nuclear H2AX γ , p21 mRNA and IL-6, IFN- β and TGF- β
337 cytokine production (**Fig. 8b-f**) ($P < 0.05$, $n = 5-7$). These rotenone-induced increases were
338 sensitive to cGAS inhibition using RU.521 (**Fig. 8b-f**) ($P < 0.05$, $n = 5-7$).

339

340 *Extracellular DNA augments AEC senescence in a cGAS-dependent manner*

341 We next examined whether extracellular DNA contributes to AEC senescence. The addition
342 of the endonuclease, DNase I (100 U/mL, 7d) reduced the levels of all senescence markers,

343 except CCL2 mRNA, evaluated in cultures of IPF-AECs from 5 separate donors ($P < 0.05$,
344 $n = 5$); suggesting that increased levels of extracellular self-DNA may mediate secondary
345 senescence in autocrine and/or paracrine manners (**Fig. 9 & Fig. S9** data supplement).
346 Furthermore, mtDNA derived from human lung fibroblasts and added to the medium of Ctrl-
347 AECs induced increases in senescent markers, including p21 expression (**Fig. 10 & Fig. S10**
348 data supplement) ($P < 0.05$, $n = 5$). The effect of ectopic mtDNA on Ctrl-AEC senescence was
349 sensitive to cGAS inhibition using RU.521 ($P < 0.05$, $n = 5$).

350

351

352

353 **DISCUSSION**

354 This study examined the roles of self-DNA and cGAS in AEC senescence, a feature of IPF
355 pathology (14, 19, 23, 33). cGAS and pSTING were detected in epithelial cells of lung
356 parenchymal sections from IPF patients, including cells positive for the senescent marker,
357 p21. Using submerged primary cultures of AECs comprised predominantly of AEC2s, IPF-
358 AECs were shown to release higher levels of mtDNA into the cytosolic- and extracellular-
359 spaces than Ctrl-AECs and this corresponded to higher baseline senescence. Increased levels
360 of mtDNA detected in the cytosol and conditioned medium of Ctrl-AECs following treatment
361 with etoposide provides additional evidence that increased mtDNA release is a feature of
362 AEC senescence. Pharmacological inhibition of cGAS in IPF-AECs *in vitro* diminished
363 senescence, implicating cGAS as a sensor of cytosolic damaged DNA in AEC senescence. In
364 support, targeting cGAS attenuated etoposide-induced Ctrl-AEC and A549 senescence.
365 Additionally, pharmacological STING inhibition also attenuated etoposide-induced A549
366 senescence. Furthermore, ectopic mtDNA augmented Ctrl-AEC senescence in a cGAS-
367 dependent manner and DNaseI attenuated IPF-AEC senescence, suggesting a contribution of
368 extracellular DNA to autocrine and/or paracrine senescence. Collectively, our data indicates
369 that a cGAS-dependent response links DNA damage and AEC senescence in IPF. Our
370 findings are potentially important, particularly as AEC senescence is proposed to be the crux
371 of the aberrant wound healing response of IPF; possibly by hindering re-epithelialization
372 and/or by the activation of resident fibroblasts (8).

373

374 Cytosolic cGAS binds dsDNA irrespective of sequence to generate cGAMP and a subsequent
375 inflammatory/interferon-like response via the activation of the STING pathway. Besides from
376 its role in innate immunity, cGAS is an essential mediator of damaged DNA-induced cellular
377 senescence (11, 42). Whilst there is accumulating evidence that AEC senescence is crucial in

378 IPF pathology, little is known about the underlying contributions of self-DNA and/or cGAS
379 to this cellular process. In the current study, cGAS antigen was detected in epithelial cells of
380 lung parenchymal tissue from IPF patients. cGAS immunoreactivity in epithelial cells of
381 fibrotic lung appeared to be more intense than in parenchymal lung tissue from control
382 donors. cGAS was also detected in senescent (p21 expressing) epithelial cells in IPF lung.
383 Targeting cGAS using RU.521 diminished IPF-AEC senescence *in vitro* and attenuated
384 etoposide-induced Ctrl-AEC senescence as assessed by concomitant decreases in a range of
385 parameters widely used in the evaluation of cellular senescence (11, 19, 31, 41, 42). One of
386 those markers, H2AX γ is a surrogate of nuclear DNA damage, being phosphorylated at
387 dsDNA breaks in the nucleus as part of the DNA damage response (DDR) that initiates
388 senescence (31). It is likely that the effects of targeting cGAS on AEC senescence involves
389 the attenuation of NF- κ B-regulated SASP cytokine expression. NF- κ B is activated
390 downstream of the STING signaling pathway and is a pivotal regulator of the SASP
391 secretome (11). The latter comprises an array of cytokines, chemokines and proteases that
392 can mediate secondary senescence via autocrine and paracrine processes. In this study, the
393 impact of targeting cGAS on the SASP was evidenced by decreases in the production of IL-6
394 (the archetypal SASP cytokine), TGF- β and expression of the IL-6, CCL2 and IL-8 genes in
395 primary AECs and/or A549s. However, other SASP mediators were not analysed in this
396 study, nor was the causal involvement of NF- κ B in regulating the SASP and secondary
397 senescence. Despite these shortcomings, our current data provides evidence that cGAS is an
398 important mediator of AEC senescence, with a potential role in IPF pathology.

399

400 The sequential activation of TANK-binding kinase-1 (TBK1) and interferon regulatory
401 factor-3 (IRF3), downstream mediators of STING, leads to the induction of interferon I and
402 III gene expression. In this study, levels of the type I interferon, IFN- β were also increased in

403 the medium of senescent AECs in a cGAS-dependent manner. However, a causal role of
404 IFN- β in mediating AEC senescence was not evaluated. Whilst IFN- β is not a universal
405 component of the SASP (nor are most SASP components), there is growing evidence that
406 type I interferons contribute to cellular senescence. For example, prolonged treatment of
407 fibroblasts with IFN- β induces nuclear DNA damage and a subsequent DDR leading to
408 senescence (34). However, the mechanisms by which IFN- β and other type I interferons may
409 evoke cellular senescence is not yet well understood (10).

410

411 Damage to mtDNA such as base oxidation and deletions increases in frequency with
412 oxidative stress and accumulates in ageing and diseased tissue (17). These modifications
413 reduce the physical association of mtDNA with mitochondrial packaging proteins, leading to
414 the leakage of mtDNA and its accumulation in the cytosol (39). Other mechanisms may also
415 contribute to increases in cytosolic mtDNA including diminished mitophagy, as observed in
416 primary cultures of AEC2s derived from IPF patients, and AEC2s of control donors/A549
417 cells subject to ER and oxidative stress (6). Cytosolic self-DNA, whether of mitochondrial or
418 nuclear origin, elicits cGAS-dependent cell-autonomous inflammatory responses (ie sterile
419 inflammation) (1, 40). Recently, Han *et al* reported that cytosolic levels of self dsDNA in the
420 airway epithelium of mice are elevated following challenge with allergen and that cGAS
421 knockout specifically in airway epithelial cells attenuates allergen-induced airway
422 inflammation (13). Furthermore, the targeting of cGAS and/or STING has been shown to
423 attenuate lung inflammation induced by acute exposure to either cigarette smoke (CS) or
424 silica microparticles (3, 24). However, a recent study using the murine bleomycin lung
425 fibrosis model, showed that whilst bleomycin evoked increases in the extracellular release of
426 self DNA and expression of STING and cGAS in the lungs, STING gene deletion augmented
427 fibrosis (30). This effect of STING, which was independent of cGAS and IFN I/III expression

428 was mediated by a dysregulated immune response involving exacerbated neutrophilic
429 inflammation. This immunomodulatory effect of STING potentially involves alternative
430 pathways mediated by DNA sensors such as DDX41 and IFI16, including a recently
431 identified non-canonical, cGAS-independent pathway that leads to prominent NF- κ B
432 activation, but only modest IRF3 expression (9, 43).

433

434 As mentioned previously, innate immune sensing of cytosolic DNA by cGAS also promotes
435 senescence. Whilst earlier studies have implicated damaged nuclear DNA (nDNA) as an
436 activator of cGAS-dependent senescence, it is likely that mtDNA contributes as well (11, 41,
437 42). Our data that shows increased levels of mtDNA in both the cytosol and extracellular
438 space of senescent AECs implicates mtDNA as a potential driver of IPF-AEC senescence.
439 To provide additional evidence that mitochondrial dysfunction is the underlying cause of
440 mtDNA release in AEC, we induced mitochondrial stress in A549 cells using rotenone, an
441 agent that targets complex I of the inner mitochondrial membrane to induce superoxide
442 formation. Rotenone not only induced an increase in the release of mtDNA, but also
443 senescence in a manner sensitive to cGAS inhibition. Furthermore, ectopic mtDNA was
444 shown to induce AEC senescence in a cGAS-dependent manner. We should also make note,
445 that levels of nDNA were also increased in the medium, albeit not cytosol of IPF-AECs and
446 etoposide-treated Ctrl-AECs when compared to Ctrl-AECs at baseline. It is plausible and
447 highly likely that nDNA may also contribute to AEC senescence involving cGAS.
448 Collectively our data support a contribution of self-DNA in AEC senescence involving
449 cGAS. The involvement of other PRRs such as TLR9 or absent in melanoma 2 (AIM2) were
450 not assessed in this study.

451

452 Previous investigations with AECs from IPF patients have shown that they are prone to both
453 senescence and mitochondrial dysfunction (6-8, 19, 23). Our studies with lung fibroblasts,
454 including those from IPF patients (ie IPF-LFs) provide evidence that both processes are
455 linked as part of a vicious circle involving increased production of mitochondrial-derived
456 ROS (5, 31, 38). Our recent findings with IPF-LFs and current data with IPF-AECs also
457 identify mtDNA and cGAS as potential mediators of senescence. We propose that in IPF,
458 damaged mtDNA leaked into the cytoplasmic and extracellular spaces of AECs by aging,
459 dysfunctional mitochondria activates cytosolic cGAS to evoke a ‘sterile’ inflammatory
460 response that perpetuates secondary senescence (**Fig. 11**). Senescent AECs do not self-renew
461 following injury but produce more cytokines in a failed attempt to re-epithelialize. The spill
462 over of SASP mediators by senescent AECs may contribute to a fibrotic niche that influences
463 neighboring lung fibroblasts, including their transition into a senescence phenotype (*ie* the
464 bystander effect’ (25)). In support, the culture of ‘naïve’ human lung fibroblasts with
465 conditioned medium from senescent AECs induces senescence in the former (Waters *et al*, In
466 press). Interestingly, lung fibroblast senescence is also associated with increased expression
467 of α -smooth muscle actin and collagen type I alpha, markers of the myofibroblast phenotype
468 (31, 41). The bioactive molecules that mediate the transfer of senescence from AECs to lung
469 fibroblasts and/or their activation is yet to be determined, but could involve ROS, cytokines
470 (*ie* IL-6), growth factors (*ie* TGF- β) and/or DAMPs including mtDNA (4, 26, 31, 32).
471 Indeed, Bueno *et al* recently showed that mtDNA induces TGF- β release in primary human
472 lung epithelial cells as well as activation of human lung fibroblasts, as measured by increased
473 expression of α -smooth muscle actin (7). Overall, our current findings have added
474 significance in understanding IPF pathology following the recent discovery that higher levels
475 of extracellular mtDNA occur in IPF and are predictive of all-cause mortality (29).

476

477 Targeting cGAS-STING signalling may be a potential treatment option for IPF because AEC
478 senescence is thought to contribute to the aberrant wound repair response that drives fibrosis
479 (33). Whilst RU.521 is a highly selective cGAS inhibitor, its potency is substantially
480 diminished for DNA substrates of shorter length (<100 bp), possibly limiting its therapeutic
481 potential *in vivo* (18, 21, 36, 44). The recent discovery of small molecular drugs with
482 improved efficacy for inhibiting human cGAS activated by shorter DNA substrates, provides
483 greater opportunity for the clinical use of pharmacological cGAS inhibitors (18). Other drugs
484 such as the recently discovered highly selective inhibitor of STING, H-151 may also have
485 therapeutic potential, as would agents that preserve mtDNA integrity (ie MitoTEMPO) or
486 recombinant DNAses that target cell free DNA (12, 35). Further exploration of
487 pharmacological cGAS-STING signalling pathway inhibitors and ectopic DNAses in pre-
488 clinical models of lung fibrosis is clearly warranted.

489

490 In conclusion, we show that cGAS inhibition diminishes IPF-AEC senescence and chemical-
491 induced Ctrl-AEC senescence. We also provide evidence that AEC senescence is associated
492 with an increase in the release of DNA, which reinforces senescence in a cGAS-dependent
493 manner. Overall, our study suggests that damaged, self-DNA activates cGAS to perpetuate
494 AEC senescence and aging in IPF and other fibrotic lung diseases. We propose that cGAS is
495 potentially a central player in IPF pathology; connecting mitochondrial stress, sterile
496 inflammation (involving cGAS activation by self-DNA) and senescence.

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503

504 **AUTHORS CONTRIBUTION** Concept and design: DAK, MS; acquisition, analysis and
505 interpretation of data: MS, AK, JR, CT, CG, KECB, AJ; and drafting the manuscript for
506 intellectual content: DAK, MS, CG, JKB, CMP, SEM and NB.

507

508 **CONFLICTS OF INTEREST**

509 The authors confirm that there are no conflicts of interest.

510

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512

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645

646

647

648 **FIGURE LEGENDS**

649 **Figure 1.** *cGAS* antigen is detected within senescent epithelial cells of IPF lung. **(a, top**
650 **panel)** Sections of lung parenchymal tissue from a patient with IPF (patient 1) and control
651 donor were immunostained for cGAS (red) and EpCAM (green) and counterstained for DAPI
652 (blue). Arrows show cGAS within EpCAM positive cells. **(b, top)** Section of IPF lung
653 (patient A) immunostained for cGAS (red) in combination with p21 (green). Arrows show
654 cGAS and p21 co-localisation in epithelial cells. **(c)** Serial sections of lung from another
655 patient with IPF (patient 3) immunostained for EpCAM (green) in combination with cGAS
656 (red, **top left panel**), p-STING (red, **bottom left panel**) and p21 (red, **top right panel**). **(a-**
657 **c, bottom (right) panel)** Primary antibodies were omitted in corresponding serial sections
658 designated as staining controls. Scale bar = 50 micron.

659

660 **Figure 2.** *Immunohistochemical detection of cGAS in senescent epithelial cells of IPF lung.*
661 Serial sections from the same region of lung from a patient with IPF (patient 1) stained with
662 hematoxylin (blue/purple) and cGAS (brown, **top left panel**), EpCAM (brown, **top right**
663 **panel**) and p21 (brown, **bottom left panel**). Primary antibodies were omitted in
664 corresponding serial section designated as staining control (**bottom right label**). Scale bar =
665 50 micron.

666

667 **Figure 3.** *IPF-AECs exhibit high baseline senescence.* **(a-b)** Immunofluorescence and
668 phase-contrast images of submerged primary cultures of Ctrl-AECs **(a-b)** and IPF-AECs **(b)**.
669 Cells were immunostained for EpCAM (green) and SP-A (red). **(c-f)** The senescence of IPF-
670 AECs (n=5) and Ctrl-AECs (n=5-6) at baseline was assessed by measuring levels of nuclear
671 H2AX γ (with representative fluorescence images) **(c)**, p21 mRNA **(d)** and IL-6 **(e)** and IFN- β
672 **(f)** protein. **(g)** Representative images of SA- β -Gal cytochemical stains of IPF and Ctrl-

673 AECs. Quantitative data were analyzed by a Mann Whitney test (*P<0.05). Scale bar = 50
674 micron.

675

676 **Figure 4.** *Targeting cGAS diminishes IPF-AEC senescence.* (a-e) The effect of RU.521 (3
677 μ M) on the senescence phenotype of IPF-AECs (n=5) after 7 d, as measured by changes in
678 the levels of nuclear H2AX γ (a), p21 mRNA (b) and IL-6 (c) and IFN- β (d) protein. Included
679 are representative images of H2AX γ immunofluorescence (b) and SA- β -Gal cytochemical
680 staining (e). Differences between vehicle and treatment groups were assessed by a Wilcoxon
681 matched pairs signed rank test (*P<0.05, n=5). Scale bar = 50 micron.

682

683 **Figure 5.** *cGAS inhibition attenuates etoposide-induced Ctrl-AEC senescence.* (a-e) The
684 effect of RU.521 (3 μ M) on the senescence phenotype of Ctrl-AECs 5 d after the addition of
685 etoposide (10 μ M). Included are representative fluorescence images of H2AX γ and
686 cytochemical staining for SA- β -Gal. Data were analyzed by Friedman's analysis of variance
687 (ANOVA) with differences between two treatment groups assessed by a Wilcoxon matched
688 pairs signed rank test (*P<0.05, n=5-6). Scale bar = 50 micron.

689

690 **Figure 6.** *cGAS knockdown attenuates A549 senescence.* (a) Levels of cGAS mRNA
691 (*P<0.05, n=5) and immunoreactivity (images representative of three replicates) in A549
692 cells, 1 d post siRNA transfection. (b-f) The effect of cGAS siRNA on A549 senescence 5 d
693 after addition of etoposide. Quantitative data were analyzed by Friedman's ANOVA with
694 differences between two treatment groups assessed by a Wilcoxon matched pairs signed rank
695 test (*P<0.05, n=5). Scale bar = 50 micron.

696

697 **Figure 7.** *IPF- and senescent-induced Ctrl-AECs release increased amounts of mtDNA.* (a)
698 Baseline levels of mtDNA in the cytosol and medium of IPF- and Ctrl-AECs as detected by
699 qPCR using primers for the mitochondrial gene, mt-tRNA^{Leu(UUR)}. Levels of mtDNA are
700 expressed as a percentage of total mtDNA detected in cells. Data were analyzed by a Mann
701 Whitney test (*P<0.05, n=5-6). (b) Levels of mtDNA in the cytosol and medium of
702 etoposide-treated Ctrl-AECs (10 μM, 3 d). Differences between two treatment groups were
703 analyzed by a Wilcoxon matched pairs signed rank test (*P<0.05, n=6). (c, d) Levels of
704 mtDNA, as detected using primers for B2M in the cytosol and media of IPF- and Ctrl-AECs at
705 baseline, and etoposide-treated Ctrl-AECs (*P<0.05, n=5-6).

706

707 **Figure 8.** *Targeting cGAS attenuates rotenone-induced A549 senescence.* (a) Levels of
708 mtDNA in the cytosol and medium of rotenone-treated A549 cells (0.1 μM, 2 d). (b-f) The
709 effect of RU.521 (3 μM) on the senescence phenotype of rotenone-treated cells after 5 d, as
710 measured by changes in the levels of nuclear γH2AX, p21 mRNA and IL-6, IFN-β and TGF-
711 β protein in conditioned media. Differences between two treatment groups were analyzed by
712 a Mann Whitney test, *P<0.05 (n=5-6). Scale bar in images = 50 micron.

713

714 **Figure 9.** *DNaseI diminishes the escalation of IPF-AEC senescence.* (a-e) IPF-AECs (n=5)
715 in culture treated with DNaseI (50 U) for 7 d were evaluated for markers of senescence,
716 including levels of nuclear γH2AX (a), p21 mRNA (b), and IL-6 (c) and IFN-β (d) protein .
717 Representative fluorescence images of γH2AX and cytochemical staining for SA-β-Gal (e)
718 are shown. Scale bar = 50 micron.

719

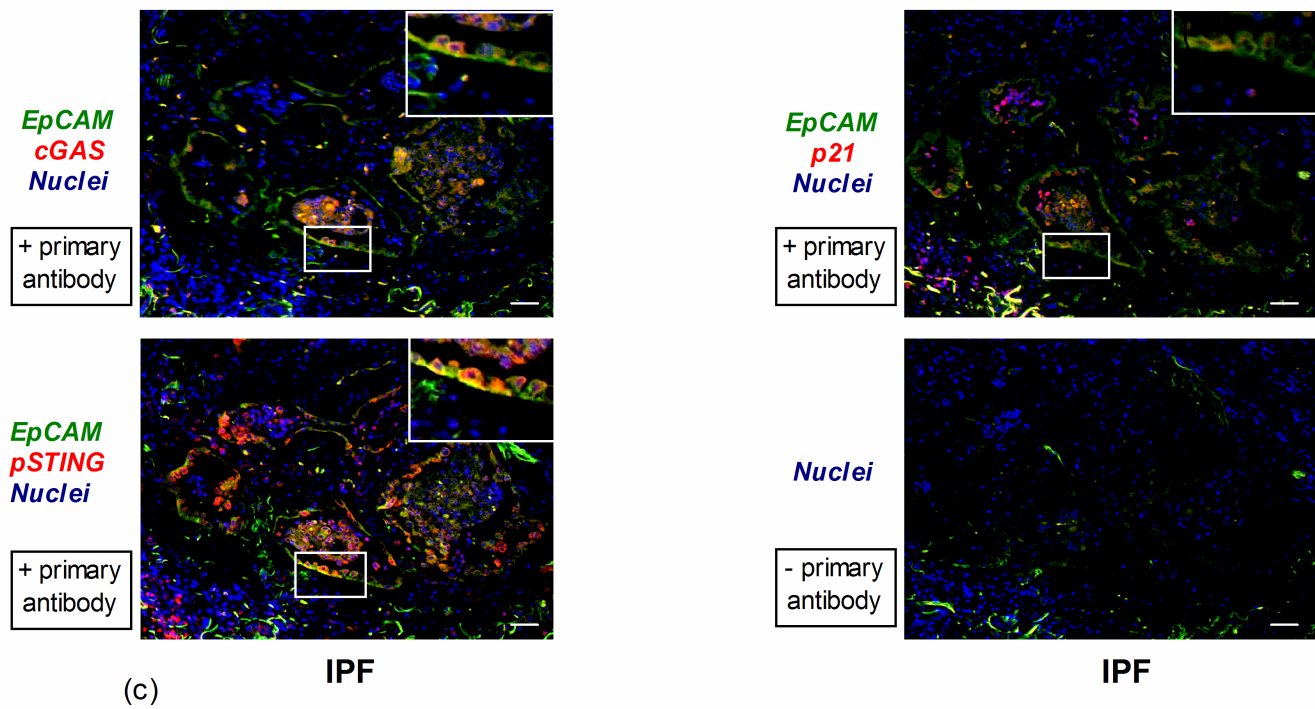
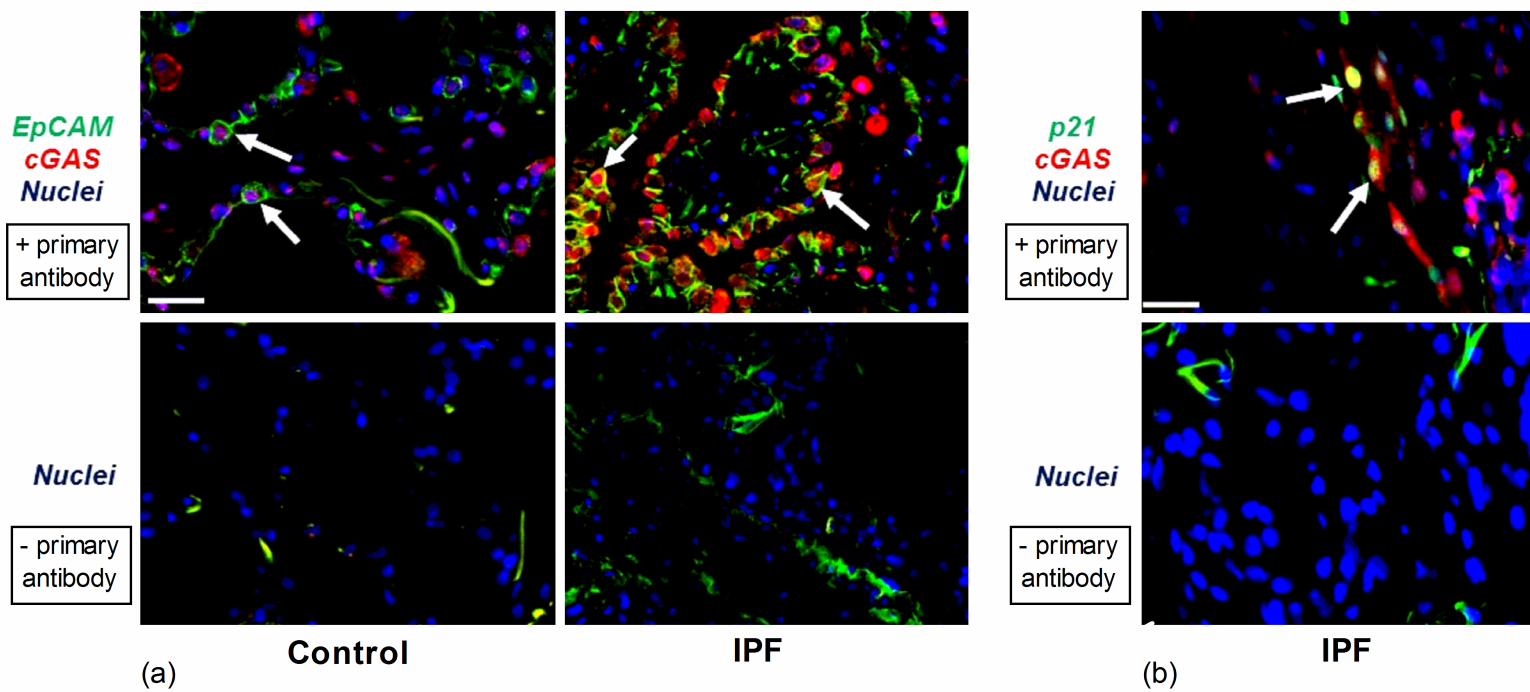
720 **Figure 10.** *Ectopic mtDNA induces Ctrl-AEC senescence in a cGAS-dependent manner.* (a-
721 e) The combined effects of ectopic mtDNA (0.1 μg/mL) and RU.521 (3 μM) on Ctrl-LF

722 senescence, as measured by increases in nuclear γ H2AX (a), p21 mRNA (b) and IL-6 protein
723 (c). Included are representative fluorescence images of γ H2AX and cytochemical staining for
724 SA- β -Gal (d). Quantitative data were analyzed by Friedman's ANOVA with differences
725 between two treatment groups assessed by a Wilcoxon matched pairs signed rank test
726 (*P<0.05, n=5). Scale bar = 50 micron.

727

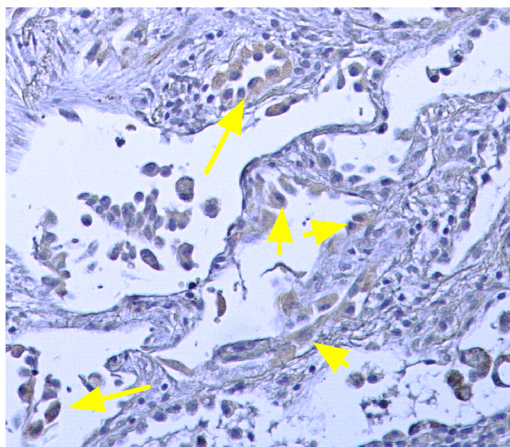
728 **Figure 11. cGAS in AEC senescence.** We propose in IPF that damaged mtDNA released
729 into the cytosol of AECs by dysfunctional mitochondria, elicits innate immune responses by
730 binding to PRRs. In particular, cGAS activation in the cytosol by self-DNA evokes a 'sterile'
731 inflammatory response that induces secondary senescence.

732



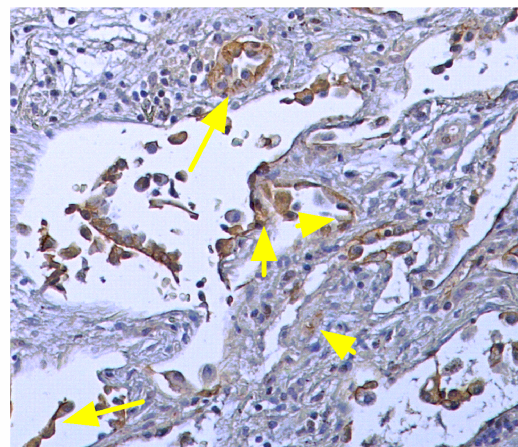
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haematoxylin

+ primary
antibody



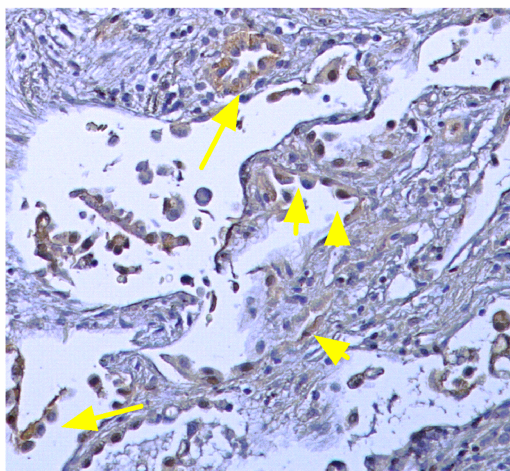
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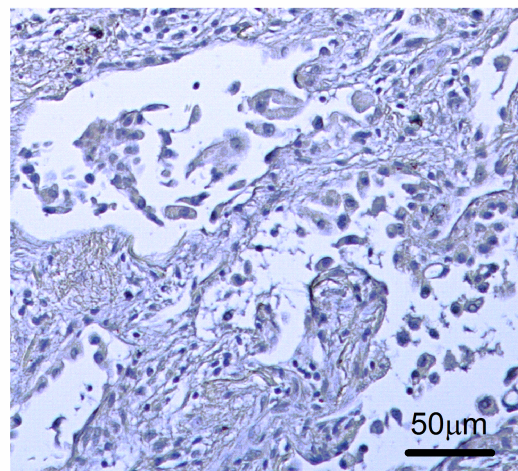
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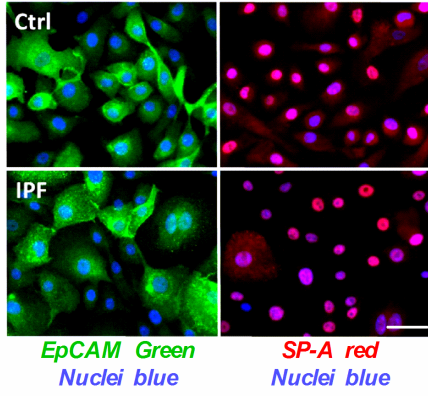
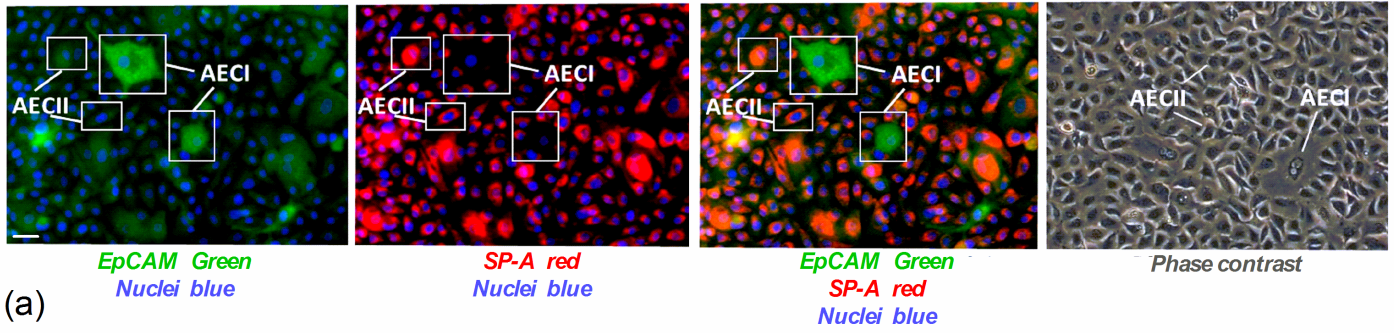
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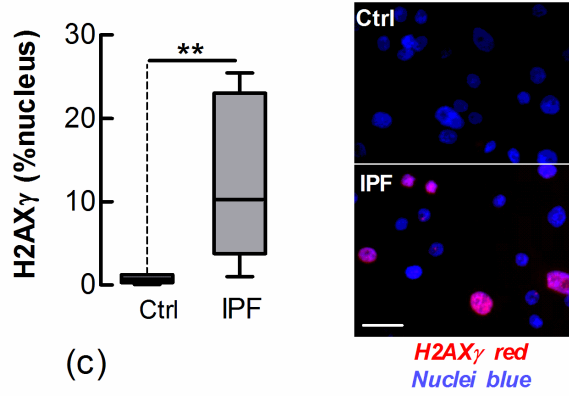
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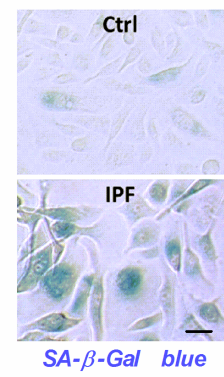
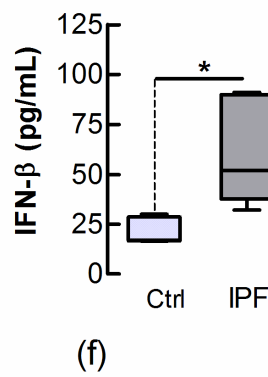
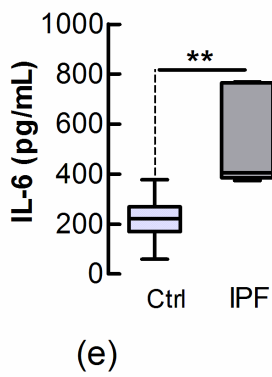
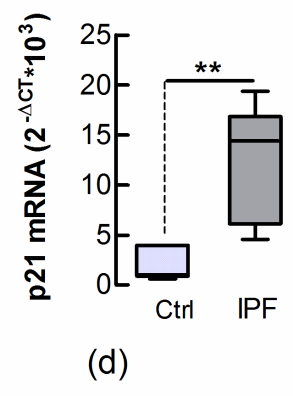


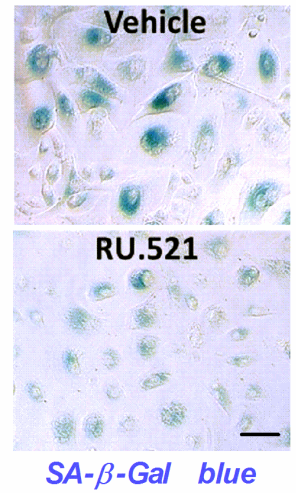
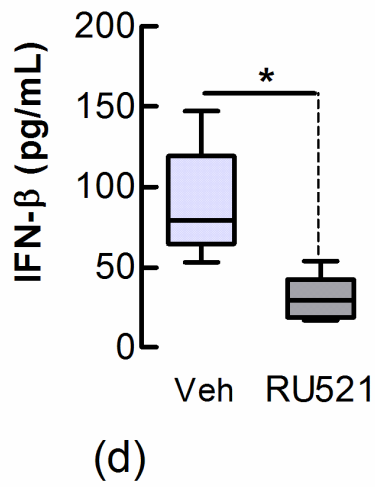
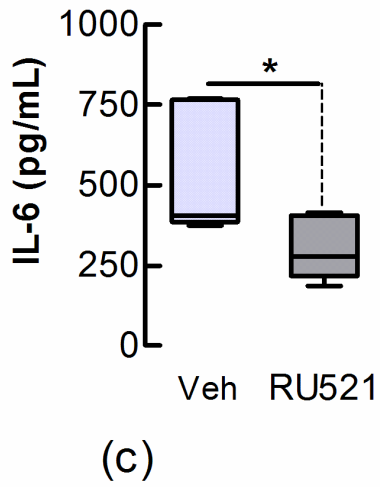
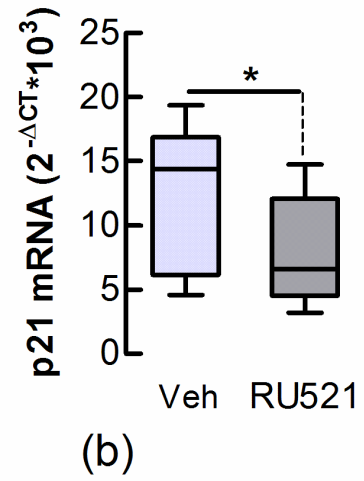
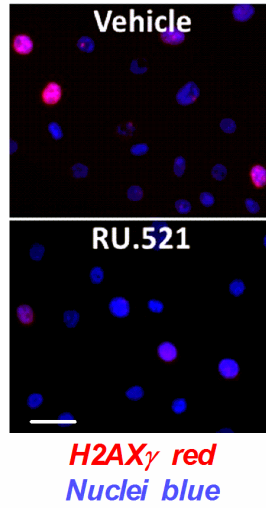
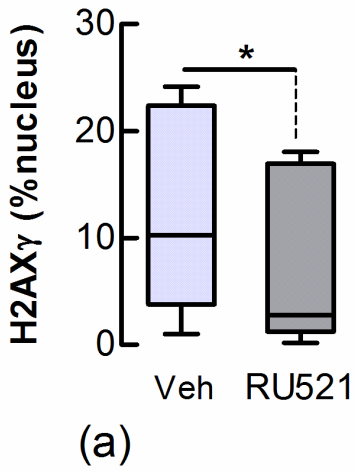


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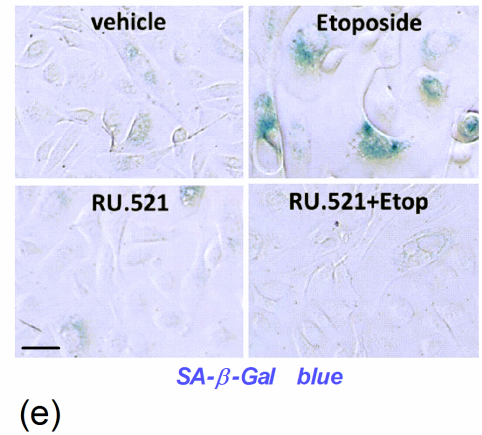
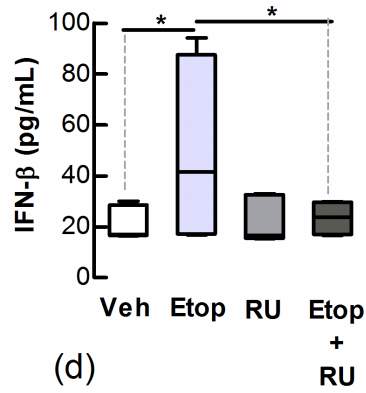
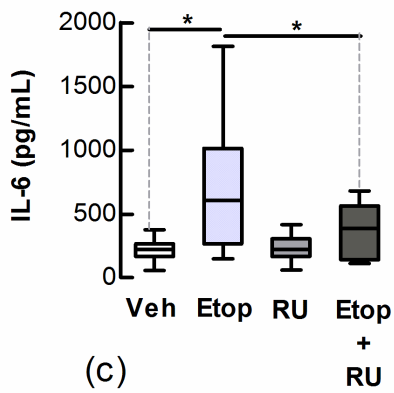
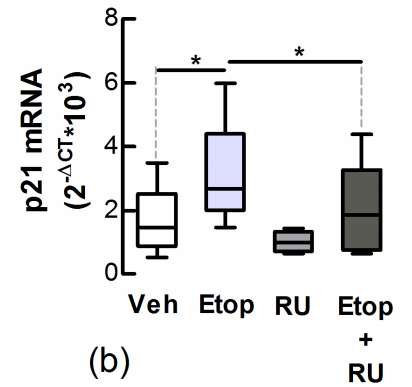
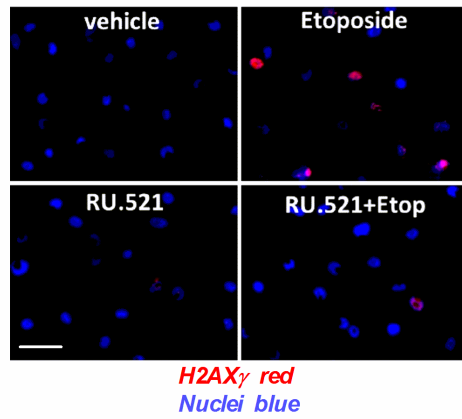
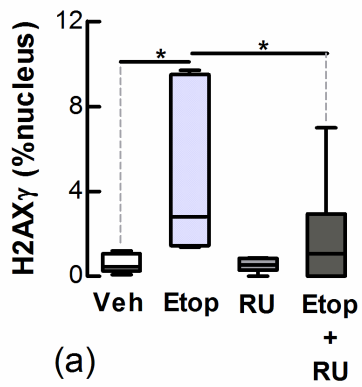


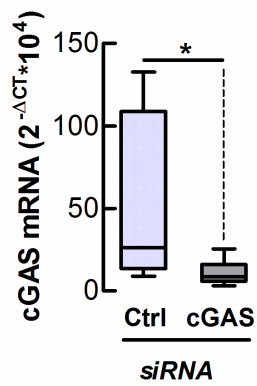
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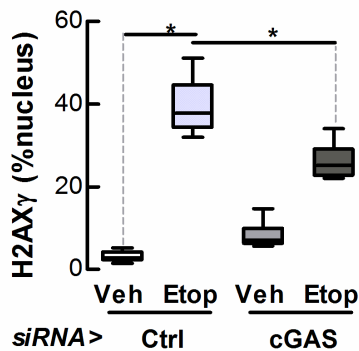
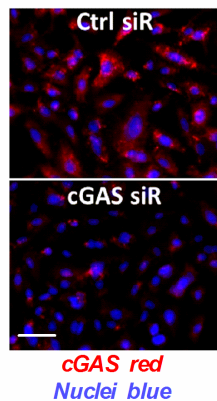


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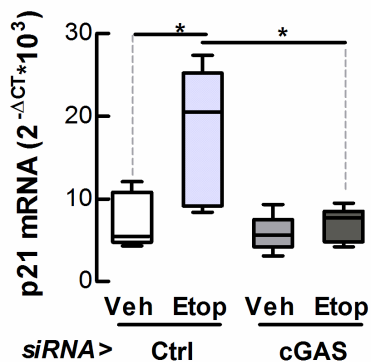
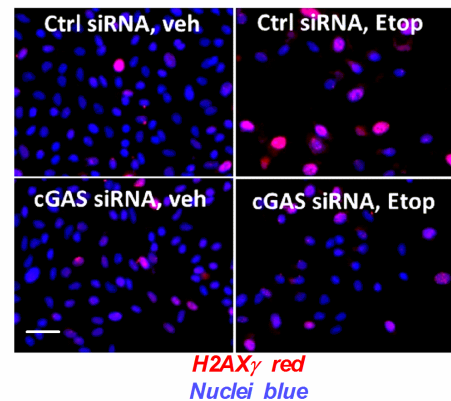




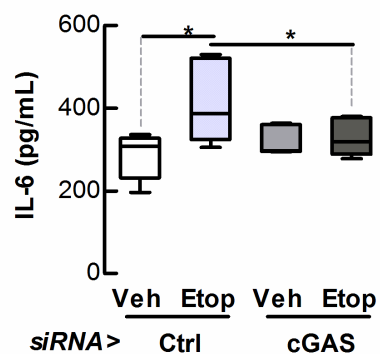
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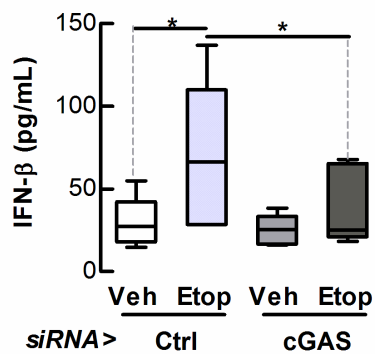
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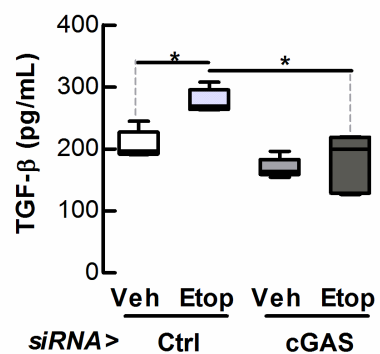
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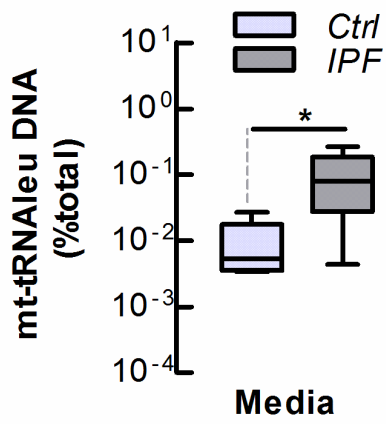
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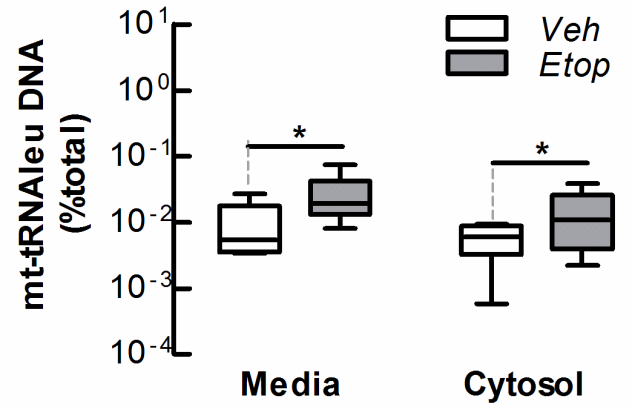
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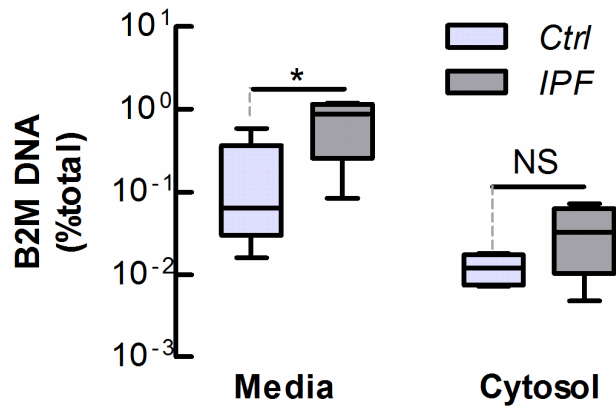
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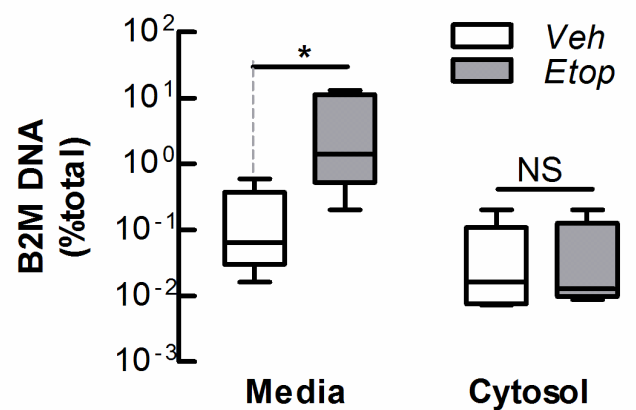
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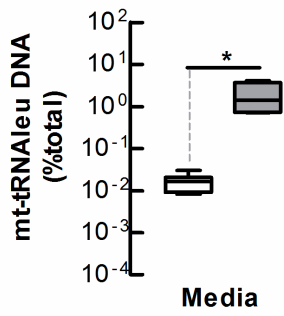
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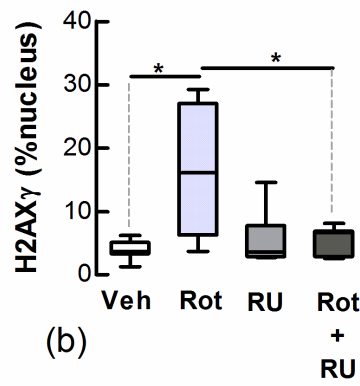
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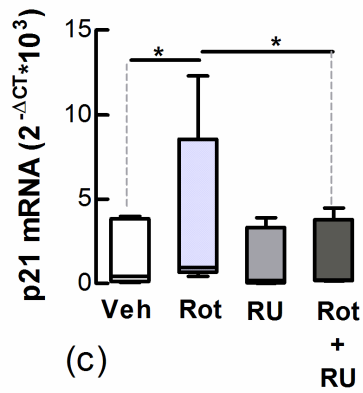
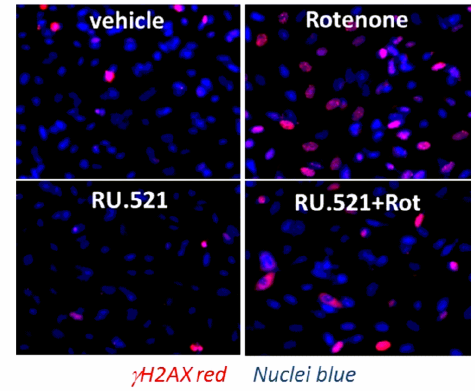
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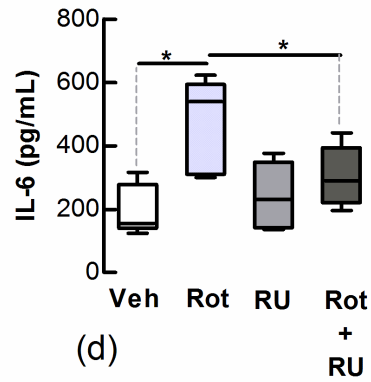
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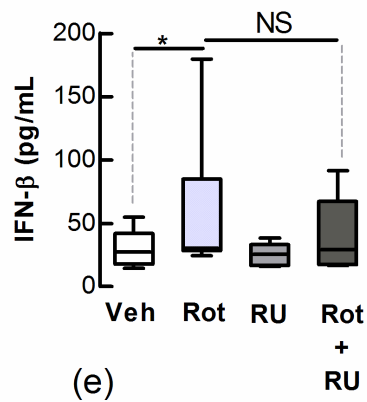
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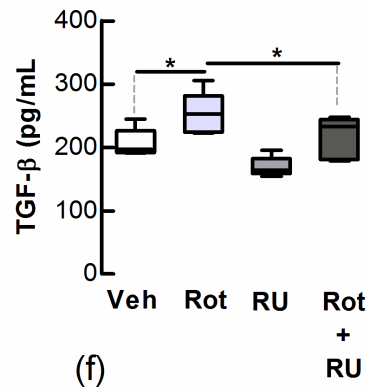
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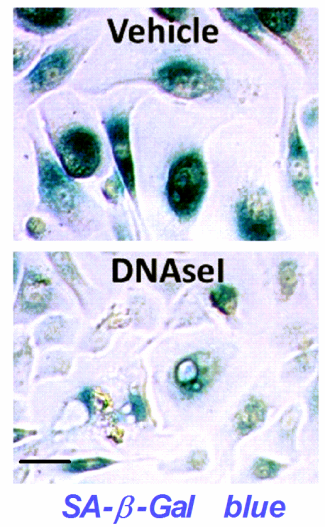
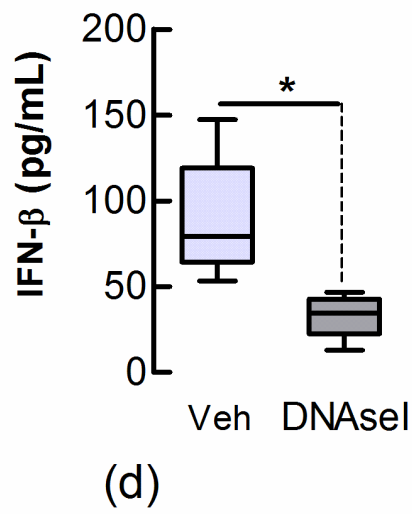
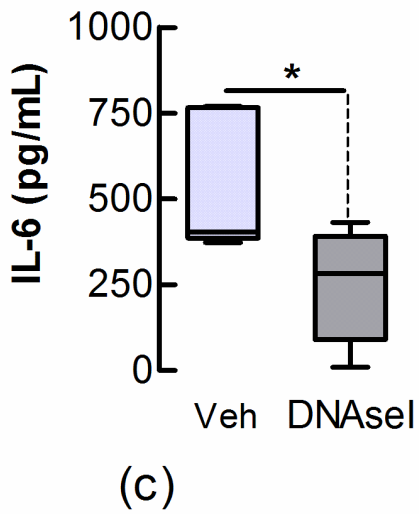
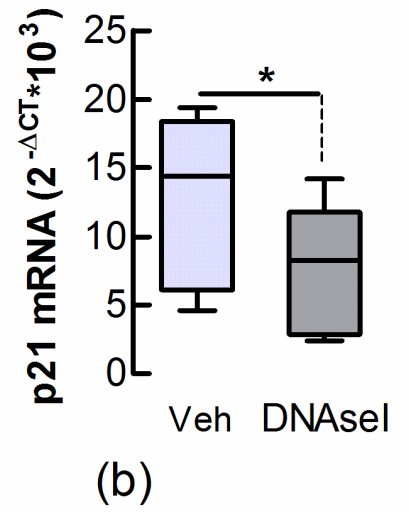
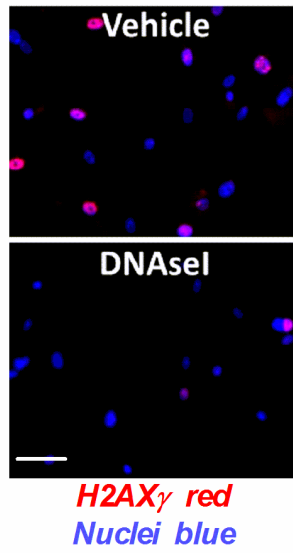
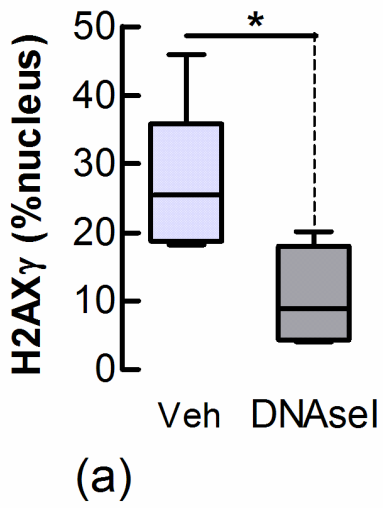
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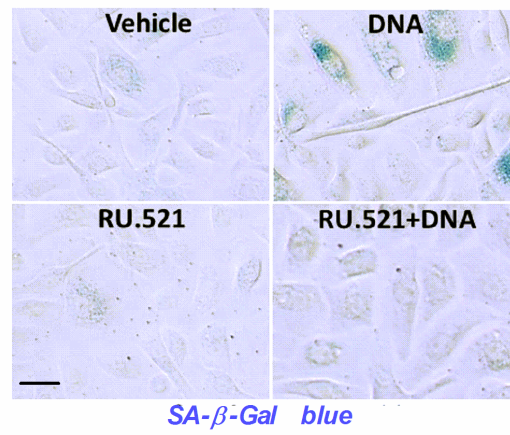
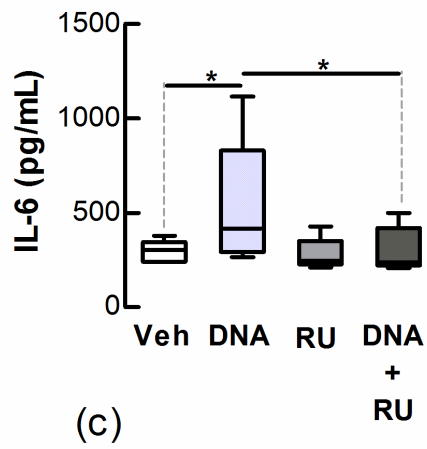
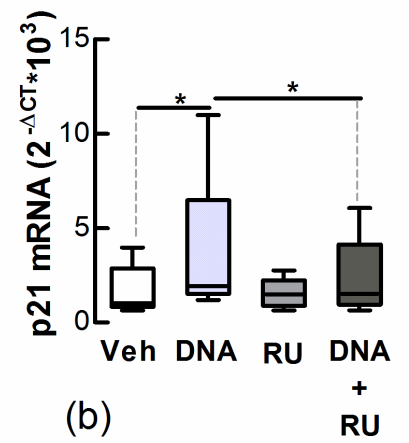
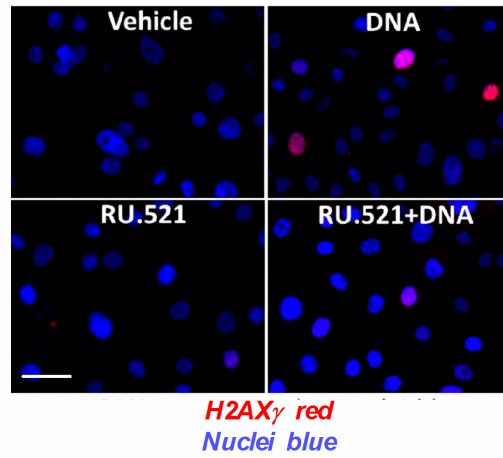
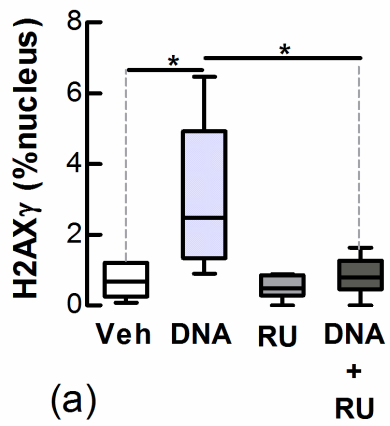
(e)



(f)



(e)



(d)

