1 **Chronic WNT/β-catenin signaling induces cellular senescence in lung epithelial**
2 **cells**

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21 Highlights

22 - **Aged ATII cells show increased cellular senescence and decreased**
23 **progenitor cell potential**
24 - **Aged ATII cells display increased WNT/β-catenin activity**
25 - **Chronic activation of WNT/β-catenin signaling induces cellular**
26 **senescence in ATII cells**
27 - **Chronic WNT/β-catenin signaling induces profibrotic changes in ATII**
28 **cells**

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Abstract

The rapid expansion of the elderly population has led to the recent epidemic of age-related diseases, including increased incidence and mortality of chronic lung diseases, such as Idiopathic Pulmonary Fibrosis (IPF). Cellular senescence is a major hallmark of aging and has a higher occurrence in IPF. The lung epithelium represents a major site of tissue injury, cellular senescence and aberrant activity of developmental pathways such as the WNT/β-catenin pathway in IPF. The potential impact of WNT/β-catenin signaling on alveolar epithelial senescence in general as well as in IPF, however, remains elusive. Here, we characterized alveolar epithelial cells of aged mice and assessed the contribution of chronic WNT/β-catenin signaling on alveolar epithelial type (AT) II cell senescence. Whole lungs from old (16-24 months) *versus* young (3 months) mice had relatively less epithelial ($EpCAM^+$) but more inflammatory ($CD45^+$) cells, as assessed by flow cytometry. Compared to young ATII cells, old ATII cells showed decreased expression of the ATII cell marker *Surfactant Protein C* along with increased expression of the ATI cell marker *Hopx,* accompanied by increased WNT/β-catenin activity. Notably, when placed in an organoid assay, old ATII cells exhibited decreased progenitor cell potential. Chronic canonical WNT/β-catenin activation for up to 7 days in primary ATII cells as well as alveolar epithelial cell lines induced a robust cellular senescence, whereas the non-canonical ligand WNT5A was not able to induce cellular senescence. Moreover, chronic WNT3A treatment of precision-cut lung slices (PCLS) further confirmed ATII cell senescence. Simultaneously, chronic but not acute WNT/β-catenin activation induced a profibrotic state with increased expression of the impaired ATII cell marker Keratin 8. These results suggest that chronic WNT/β-catenin activity in the IPF lung contributes to increased ATII cell senescence and reprogramming. In the fibrotic

55 environment, WNT/β-catenin signaling thus might lead to further progenitor cell
56 dysfunction and impaired lung repair.
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59 ## 1. Introduction

60 Physiological lung aging contributes to changes in lung function and susceptibility to
61 a wide range of chronic lung diseases (CLD), such as chronic obstructive pulmonary
62 diseases (COPD) or idiopathic pulmonary fibrosis (IPF) [1, 2]. Several aging
63 hallmarks are observed in CLDs, however, our current knowledge of the main
64 similarities and/or differences between normal lung aging and CLD pathogenesis is
65 limited and needs to be extended to further identify potential therapeutic options in
66 CLDs that target aging-associated mechanisms.

67 The distal lung epithelium consists of different airway and alveolar epithelial cells,
68 which are essential for homeostasis and proper function of the alveolus. Notably,
69 alveolar epithelial type (AT) II cells secrete surfactant proteins such as Surfactant
70 Protein C (*Sftpc*/ SP-C) and serve as progenitor cells for ATI cells, which are
71 indispensable for gas exchange [3, 4]. Injury and loss of distal lung epithelial cells are
72 major hallmarks of many CLDs, including IPF. IPF is thought to result from aberrant
73 and continuous activation of injured distal lung epithelial cells leading to alterations in
74 the cellular phenotype that contributes to a failure in repair and regeneration (also
75 referred to as “reprogramming”) [4, 5]. More recently, several aging mechanisms
76 have been implicated in ATII cell reprogramming [2, 6], however, the underlying
77 molecular mechanisms contributing to the aging phenotype in IPF, remains largely
78 unexplored.

79 Ten hallmarks of the aging lung were described and in particular stem cell exhaustion,
80 cellular senescence, and extracellular matrix dysregulation have been shown to
81 contribute to the aging phenotype [2]. Cellular senescence is characterized by
82 irreversible cell cycle arrest due to augmented levels of cell cycle inhibitors p16INK4a
83 and p21CIP1 [7, 8], high activity of senescence-associated β-galactosidase (SA-β-

84 gal) as well as secretion of senescence-associated secretory phenotype (SASP), by
85 which senescent cells significantly impact the (micro-)environmental niche [9]. While
86 cellular senescence is a physiological process, required for the regulation of
87 embryogenesis [10, 11] and prevention of tumor cell proliferation [8], aberrant
88 accumulation of senescent cells has further been demonstrated to exhibit deleterious
89 effects on tissue homeostasis [8, 12], for example by contributing to stem/progenitor
90 cell exhaustion [13].

91 Increased senescent epithelial cells and their associated SASP have been linked to
92 IPF [14, 15]. Different stressors can induce cellular senescence [8, 12]. Senescence
93 is triggered by a persistent DNA damage response that is initiated by extrinsic (UV
94 damage, chemotherapeutic drugs, γ-irradiation) or intrinsic (telomere attrition,
95 oxidative stress, hyperproliferation) insults. In addition, oncogene-induced
96 senescence (OIS) is a specific type of premature senescence, which is classically
97 triggered by hyperactivation of oncogenes such as Ras or BRAF [16] but also
98 activation of WNT/β-catenin signaling can result in OIS [17, 18].

99 The WNT signaling pathway regulates a number of cellular process, including cellular
100 migration, proliferation and differentiation. WNT proteins are secreted, cysteine-rich
101 glycosylated proteins that can activate the β-catenin-dependent (canonical) WNT
102 pathway (such as WNT3A) or the β-catenin-independent (non-canonical) WNT (such
103 as WNT5A) pathway, by binding to various transmembrane receptors (Frizzled 1-10).
104 In both developing and adult lung, WNT/β-catenin signaling controls progenitor cell
105 function and regulates tissue homeostasis [19-23]. Importantly, aberrant WNT/β-
106 catenin signal activity has been demonstrated in human and experimental lung
107 fibrosis [19, 24-26] and linked to distal lung epithelial cell dysfunction [27-29].
108 Moreover, increased WNT/β-catenin activity has been demonstrated to lead to

109 accelerated aging [18, 30]. Its role in the aging lung, however, is still under-
110 investigated [31]. Here, we aimed to elucidate the role of WNT/β-catenin signaling in
111 the process of normal lung aging and its contribution to cellular senescence and
112 reprogramming of ATII cells.

113

114 ## 2. Materials and Methods

115 ### Animals

116 Young or old pathogen-free C57BL/6N or J mice were obtained from Charles River or
117 Jackson Laboratory and housed in rooms with constant humidity and temperature
118 with 12h light cycles and free access to water and rodent chow. Mice were sacrificed
119 and lungs were used for collection of whole lung tissue, ATII cells or PCLS. For all
120 experiments in Fig 3 - 6 C57BL6/N mice were used, for experiments in Fig 1
121 C57BL6/N or J mice were used as indicated. Specific ages of mice were as follows:
122 Fig1A 16-24 months 6-20 weeks, Fig S1A 16-21 months 6-20 weeks, Fig 1B 16-24
123 months, 6-12 weeks, Fig 1C 16-24 months, 6-12 weeks, Fig 1D 16-24 months, 6-21
124 weeks, Fig 1 E/F 14-24 months, 21-24 weeks, Fig 1 H 20-24 months 10-20 weeks,
125 Fig 2 14-18 months, 8-16 weeks, Fig 3-6: 6-12 weeks.

126 TCF/Lef:H2B/GFP (TCF-GFP, The Jackson Laboratory, 013752) mice of 56-80
127 weeks were used for aging analysis. Young adult TCF-GFP mice of 8-16 weeks were
128 used as control. All animal experiments were performed according to the institutional
129 and regulatory guidelines of University of Colorado Institutional Animal Care and Use
130 Committee.

131 ### Isolation of primary murine alveolar epithelial cell type II (pmATII) cells

132 The pmATII cells were isolated as previously described [28, 32] with slight
133 modifications. In brief, lungs were filled with dispase (Corning, New York, NY, USA)
134 and low gelling temperature agarose (Sigma Aldrich, Saint Louis, MO, USA) before
135 tissue was minced and the cell suspension was filtered through 100-, 20-, and 10-µm
136 nylon meshes (Sefar, Heiden, Switzerland). Negative selection of fibroblasts was
137 performed by adherence on non-coated plastic plates. Macrophages and white blood

138 cells were depleted with CD45 and endothelial cells were depleted with CD31
139 specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to
140 the manufacturer's instructions. Cell purity was assessed routinely by analysis of
141 endothelial (CD31), mesenchymal (α-SMA, CD90), epithelial (EpCAM, panCK and
142 proSP-C), and hematopoietic cell (CD45) markers by immunofluorescence or flow
143 cytometry.

144 For the analysis of WNT-GFP epithelial cells and for the organoid experiments,
145 isolation was performed as described above. No depletion of fibroblasts was
146 performed, the CD45 and CD31 depleted single cell suspension was further enriched
147 for epithelial cells by positive selection using EpCAM (CD326) Microbeads (Miltenyi
148 Biotec, Bergisch Gladbach, Germany).

149 **Flow cytometry**

150 A single cell suspension was generated by dispase treatment, mincing and serial
151 filtering as described above. Cells were washed once in FACS buffer, stained with
152 anti-mouse CD326 (Ep-CAM), APC (Biolegend 118214), anti-mouse CD45, PE
153 (Biolegend 103106) or respective IgG controls (Biolegend 400512, 400608) for
154 20mins at 4°C in FACS buffer, washed once and analyzed. FACS-based detection of
155 SA-β-galactosidase was performed as previously described [14, 33]. Briefly, pmATII
156 or MLE12 cells were incubated with Bafilomycin A1 (100 nM, Enzo Life Sciences,
157 Farmingdale, NY; USA) and C_{12}FDG (33µM, Life technologies, Carlsbad, CA; USA)
158 for 1 and 2 h, respectively, directly after isolation or at day 2 of culture. Cells were
159 trypsinized and washed. Stained cells were analyzed with a FACS LSRII (BD
160 Bioscience, San Jose, CA; USA). Positive populations were quantified by FlowJo
161 software (Tomy Degital Biology Co., Ltd., Tokyo, Japan)

162 Cells from old or young adult WNT-GFP mice were stained by anti-mouse EpCAM
163 conjugated with APC (Biolegend, 118214) or APC rat IgG2a isotype control for
164 EpCAM (BioLegend 400511) in dark for 15 min at room temperature, followed by
165 PBS washing and centrifuge at 300g, 15°C for 5 min. Then the cells were
166 resuspended in PBS with 1% FBS and 25mM HEPES. DAPI (4',6-Diamidino-2-
167 Phenylindole, Dihydrochloride, final concentration 2μg/ml) was added to the cell
168 suspension before analysis or sorting. GFP reporter activity in the $EpCAM^+$
169 population was assessed based on fluorescence intensity using FACSDiva software
170 (BD Bioscience). The analysis was performed by FACS Fortessa cell analyzer (BD
171 Bioscience).

172 **Senescence-associated (SA)-β -galactosidase staining**

173 pmATII cells or precision-cut lung slices (PCLS) were prepared from C57BL6/N
174 wildtype (WT) mice as previously described [25] (see supplemental information for
175 additional details) and cultured in multi-well plates. Cytochemical staining for SA-β-
176 galactosidase was performed using a staining Kit (Cell Signaling Technology,
177 Danvers, MA), according to the manufacturer´s instructions. Images were acquired
178 using a Zeiss Axiovert40C microscope. The percentage of senescent cells was
179 determined by counting of total and SA-β-galactosidase-positive cells in 3 random
180 microscopic fields per condition (100× magnification).

181 **Preparation of WNT-conditioned medium (CM)**

182 Mouse fibroblasts-like L-cells stably expressing WNT-3A or WNT-5A were used to
183 obtain WNT-CM according to a standardized protocol [34]. Parental L-cells (control:
184 ATCC CRL-2648), L-WNT-3A cells (ATCC CRL-2647) L-WNT-5A cells (ATCC CRL-
185 2814), were cultured in DMEM/F12 medium supplemented with 10% (vol/vol) FCS,

186 100 mg/l streptomycin, and 100 U/ml penicillin. WNT CM or control CM was prepared
187 according to the ATCC guidelines and as previously published [34]. In short,
188 confluent L-cell cultures were split 1:10 and cultured for 4 d in supplemented
189 DMEM/F12 medium in 10-cm culture dishes. After 4 d, the medium was collected
190 and the cells were cultured for another 3 d in fresh DMEM/F12 medium with
191 supplements. The second batch of medium was collected after 3 d and mixed with
192 medium of day 4 (ratio 1:1). The combined medium, which is referred to as CM, was
193 filtered and stored at –20°C till further use.

194 **Cell culture**

195 In experiments using pmATII cells, cells were seeded, cultured for 48h in ATII cell
196 medium (DMEM (Sigma Aldrich), 2 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL
197 streptomycin (both Life Technologies, Carlsbad, CA), 3.6 mg/ml glucose (Applichem
198 GmbH, Darmstadt, Germany) and 10 mM HEPES (PAA Laboratories) containing
199 10% FCS (PAA Laboratories, Pasching, Austria). Then the ATII cells were treated
200 with ATII cell medium supplemented with 5% FCS and containing DMSO, 1µM CHIR
201 99021 (CHIR) or 100ng/ml recombinant mouse WNT3A (RnD Systems, 1324-WN,
202 Minneapolis, MN, USA dissolved in 0.1% BSA in PBS) or treated with WNT3A
203 conditioned medium mixed with ATII cell medium (1:1; final FCS concentration 5%).
204 MLE12 cells were purchased from ATCC (CRL-2110) and maintained in DMEM/F12
205 (Gibco®, USA) medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL
206 streptomycin. Cells were seeded at 25 000 cells per well in a 6-well plate and allowed
207 to adhere for 24 h. Cells were then treated every 48h with DMSO, 1µM CHIR 99021
208 (CHIR) and 2 µM CHIR in DMEM/F12 supplemented with 5% FCS or treated with
209 WNT3A conditioned medium mixed with DMEM/F12 (1:1; final FCS concentration
210 5%).

211 **Organoid culture**

212 Organoids were cultured as previously described [3, 35]. Briefly, MLg (ATCC CCL-
213 206) mouse lung fibroblasts were proliferation-inactivated with 10µg/ml mitomycin C
214 (Merck, Darmstadt, Germany) for 2 hours. 20.000 primary mouse ATII cells were
215 resuspended in 50 µl media and diluted 1:1 with 20.000 MLg cells in 50µl growth
216 factor reduced Matrigel (Corning, New York, USA). Cell mixture was seeded into 24-
217 well plate 0,4 µm trans-well inserts (Corning, New York, USA). Cultures were treated
218 at day 0 and every 2nd or 3rd day in DMEM/F12 containing 100 U/ml
219 penicillin/streptomycin, 2mM L-alanyl-L-glutamine, Amphotericin B (Gibco), insulin-
220 transferrin-selenium (Gibco), 0.025µg/ml recombinant human EGF (Sigma Aldrich, St
221 Louis, USA), 0.1µg/ml Cholera toxin (Sigma Aldrich, St Louis, USA), 30µg/ml bovine
222 pituitary extract (Sigma Aldrich, St Louis, USA), and 0.01µM freshly added all-trans
223 retinoic acid (Sigma Aldrich, St Louis, USA). 10 µM Y-27632 (Tocris) was added for
224 the first 48 hours of culture. Microscopy for organoid quantification at day 14 was
225 performed using a LSM710 system (Zeiss) containing an inverted AxioObserver.Z1
226 stand.

227 **Generation and treatment of PCLS**

228 Precision-Cut Lung Slices (PCLS) were generated as previously described [14, 20].
229 Briefly, lungs were flushed through the heart with sterile sodium chloride solution and
230 filled with low gelling temperature agarose (2%, A9414; Sigma) in precision-cut lung
231 slices (PCLS) medium (DMEM/Ham's F12 supplemented with 100 U/mL penicillin,
232 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B (Sigma Aldrich)). Next, lobes
233 were cut with a vibratome (Hyrax V55; Zeiss, Jena, Germany) to a thickness of 300
234 µm (speed 10–12 µm·s−1, frequency 80 Hz, amplitude of 1 mm). PCLS were treated
235 with WNT3A or control conditioned medium mixed with PCLS medium (1:1; final FCS

236 concentration 5%). RNA was isolated and gene expression was measured by qRT-
237 PCR.

238 **Immunofluorescence staining**

239 PCLS were fixed with 4% paraformaldehyde (PFA) for 20 minutes, then blocked with
240 5% normal goat serum (Abcam) for 1h. After incubation with primary antibody (p21
241 1:200 (ab188224, Abcam, Cambridge, UK)); (at 4°C overnight and secondary
242 antibody at room temperature for 1h, staining was evaluated via confocal microscopy
243 (LSM 710; Zeiss, Oberkochen, Germany). For immunofluorescence staining
244 experiments, ATII cells were seeded on poly-l-lysin treated coverslips. Cells were
245 stopped at day 2 and fixed with ice-cold acetone-methanol (1:1) for 10 min and
246 washed 3 times with 0.1% BSA in PBS. Next, cells were permeabilized with 0.1%
247 Triton X-100 solution in PBS for 20 min, blocked with 5% BSA in PBS for 30 min at
248 room temperature and incubated with primary antibodies (proSP-C 1:100 (AB3786,
249 Millipore, Darmstadt, Germany), E-Cadherin 1:200 (610181, BD, Franklin Lakes, NJ,
250 USA), Cytokeratin 1:500 (Dako, Glostrup, Denmark), followed by secondary
251 antibodies, 1 h each. DAPI (Roche, Basel, Switzerland) staining for 10 min was used
252 to visualize cell nuclei. Next, coverslips were fixed with 4% PFA for 10 min, mounted
253 with fluorescent mounting medium (Dako, Glostrup, Denmark) and visualized with an
254 Axio Imager microscope (Zeiss, Oberkochen, Germany). Cyto Spins were obtained
255 by centrifugation of freshly isolated pmATII cells (10min 300g, 100.000cells/spin).
256 Cells were fixed with 4% PFA, and blocked with 5% goat Serum (Abcam, ab7481) for
257 30 min. Cells were subsequently incubated with the respective primary antibody at
258 RT for 2 h in PBS containing 0.1% BSA, (proSP-C (Merck Millipore, AB3786,
259 Darmstadt, Germany), p21 (Abcam, ab188224) followed by incubation with the
260 fluorescently labeled secondary antibody (goat anti-rabbit Alexa Fluor 555, Life

261 Technologies). DAPI staining (Life Technologies, 62248) was used to visualize cell
262 nuclei and cytospins were analyzed using an Axio Imager microscope (Zeiss,
263 Oberkochen, Germany).

264 **Cytotoxicity assay**

265 Cytotoxicity of CHIR99021 (4423-Tocris) was evaluated using an LDH-cytotoxicity
266 detection kit (Roche 11644793001) according to manufacturer´s instructions. MLE12
267 cells were cultured in 24 plates in 10% DMEM for 7 days and were treated with
268 CHIR99021 every second day (2µM). After day 6 the medium was changed to DMEM
269 containing 0.1% FCS containing CHIR and supernatant was collected at day 7 and
270 incubated with reaction mixture. TritonX-100 was used as a positive control and
271 DMEM as a negative control. Cytotoxicity was calculated as % of the positive control.

272

273 **RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR,**
274 **qPCR)**

275 Total RNA was extracted using the PEQLAB Total RNA extraction Kit (PEQLAB,
276 Erlangen, Germany) according to the manufacturer's instructions. For PCLS, RNA
277 was extracted as previously described [14, 20, 36], with minor variations. Briefly, 3
278 pieces of PCLS each sample were snap frozen in liquid nitrogen and kept at -80 °C
279 until isolation was done. Frozen PCLS was homogenized using Tissue Lyser II
280 (QIAGEN, Hilden, Germany) and then incubated with triazol reagent (Sigma, St Louis,
281 USA) on ice for 30 min. Cell debris were removed by centrifuging samples at 1000 xg
282 for 5min and the supernatant were cleaned by PerfectBind RNA Columns (peqGOLD
283 Total RNA Kit, Erlangen, Germany) and DNase I (Applichem, Darmstadt, Germany).
284 Cleaned RNA was eluted from column using RNase-free Water and stored at -80 °C.

285 cDNAs were generated by reverse transcription using SuperScriptTM II (Invitrogen,
286 Carlsbad, CA, USA). Quantitative (q)RT-PCR was performed using Light Cycler 480
287 detection system and SYBR Green (Roche Diagnostics, Mannheim, Germany).
288 Hypoxanthine phosphoribosyltransferase (HPRT) was used as a reference gene.

289 Relative gene expression is presented as ΔCt value (ΔCt =[Ct Hprt]-[Ct gene of
290 interest]). Relative change in transcript level upon treatment is expressed as ΔΔCt
291 value (ΔΔCt= ΔCt of treated sample-ΔCt of control).

292 Primers:

Gene	forward primer	reverse primer
mCdkn2a	CGGGGACATCAAGACATCGT	GCCGGATTTAGCTCTGCTCT
mCdkn1a	ACATCTCAGGGCCGAAAACG	AAGACACACAGAGTGAGGGC
mAxin2	AGCAGAGGGACAGGAACCA	CACTTGCCAGTTTCTTTGGCT
mKrt8	ACATCGAGATCACCACCTACC	GGATGAACTCAGTCCTCCTGA
mHprt	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
mGdf15	TCGCTTCCAGGACCTGCTGA	TGGGACCCCAATCTCACCTCT

293

294 **Western blotting**

295 Cold RIPA buffer plus protease and phosphatase inhibitor (Roche Diagnostics,
296 Mannheim, Germany) was added to the cells which were washed twice by PBS. The
297 plate with cells was kept on ice for 30 minutes, swirled occasionally for uniform
298 spreading. Then, cells were scraped and the lysate was collected to a
299 microcentrifuge tube. The tube was centrifuged at ~14,000 g for 15 minutes to pellet
300 the cell debris. The supernatant was transferred to a new tube and the protein
301 concentration was quantified using Pierce™ BCA Protein Assay Kit (Pierce, Thermo

302 Fisher Scientific). Equal amounts of protein were loaded with 4× Laemmli loading
303 buffer (150 mM Tris HCl [pH 6.8], 275 mM SDS, 400 nM dithiothreitol, 3.5% (w/v)
304 glycerol, 0.02% bromophenol blue) and subjected to electrophoresis in 17%
305 polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked
306 with 5% non-fat dried milk solution in TRIS-buffered saline containing 0.01% (v/v)
307 Tween (TBS-T) (Applichem, Darmstadt, Germany) for 1h and incubated with primary
308 antibodies (anti p21, MAB88058, Merck Millipore (Billerica, MA, USA); anti β-actin,
309 A3854) at 4°C overnight. Next, blots were incubated for 1 h at RT with secondary,
310 HRP-conjugated, antibodies (GE-Healthcare) prior to visualization of the bands using
311 chemiluminescence reagents (Pierce ECL, Thermo Scientific, Ulm, Germany),
312 recording with ChemiDocTMXRS+ system and analysis using Image Lab 5.0
313 software (Biorad, Munich, Germany).

314 **Gene set enrichment analysis (GSEA)**

315 Gene set enrichment analysis (GSEA) was performed using the GSEA software [37,
316 38] on a previously published single-cell RNA sequencing dataset from IPF and
317 control human isolated ATII cells (GSE94555) [39]. A pre-ranked gene list was
318 generated from normalized data and based on log2 fold change. Enrichment of a
319 gene set in one distinct phenotype was considered significant with a false discovery
320 rate (FDR) q-value < 0.05 and a nominal p-value < 0.05. Five different gene set lists
321 were used: Wnt target genes (https://web.stanford.edu/group/nusselab/cgi-
322 bin/wnt/target_genes, The Wnt Homepage – Wnt target genes, last accessed 23
323 September 2019), Canonical Wnt signaling (gene ontology ID GO: 0060070), Aging
324 (gene ontology ID GO:0007568), Senescence [40] and SASP [9].

325 **Statistical analysis**

326 All data is presented as mean ± s.d. and was generated using GraphPad Prism 8.
327 The number of biological replicates is indicated in each experiment. Statistical
328 significance was evaluated with either Wilcoxon signed-rank test, Mann-Whitney U
329 test or repeated-measures one-way ANOVA followed by Newmann-Keuls multiple
330 comparison test, with one-sample t-tests in comparison to a hypothetical value of 0 or
331 100 or two-way ANOVA followed by Sidak´s multiple comparison test where
332 appropriated. Differences were considered to be statistically significant when $P <$
333 0.05.

334 ## 3. Results

335 ### 3.1. Old lung epithelial cells are senescent and exhibit impaired progenitor cell
336 function

337 We aimed to investigate lung epithelial cells in lungs of young (3 months) compared
338 to old (16-24 months) mice. Analysis of a single cell suspension of the whole lung
339 revealed a relative decrease of the epithelial (EpCAM$^+$) cell population, while the
340 percentage of CD45$^+$ cells was significantly increased in the old mice (Fig S1A),
341 which is consistent with recent reports demonstrating lung "inflammaging" [6]. We
342 used well-established protocols to isolate ATII cells from the single cell suspension
343 and observed less cell numbers in old animals compared to young ones (Fig 1A).
344 This finding was irrespective of size and bodyweight of the animals (Suppl. Fig 1B).
345 Old ATII cells exhibited increased activity of the senescence marker senescence-
346 associated β-galactosidase (SA-β-gal) as assessed by flow-cytometry (Fig 1B,
347 5.69±2.64% senescent cells in young mice; 12.90±0.94% senescent cells in old mice;
348 $p<0.05$) or conventional light microscopy, with SA-β-gal high cells stain in blue (Fig
349 1C). Furthermore, we observed significantly increased *Cdkn2a* and *Gdf15* gene
350 expression levels, indicative of increased cellular senescence in old ATII cells (Fig
351 1D). In contrast, we observed reduced gene expression of *Surfactant Protein C*
352 (*Sftpc)* and *Surfactant Protein A (Sftpa)* in old ATII cells compared to young ones (Fig
353 1D).The transcript level of *Hopx*, a protein implicated in bipotent ATII/ATI progenitors,
354 was increased in old ATII cells (Fig 1D). The upregulation of P21 protein expression
355 as well as the downregulation of proSP-C protein expression was confirmed by
356 immunofluorescence (Fig 1E; F respectively). These data support the idea that ATII
357 cells are exhausted in old lungs. To further determine the progenitor cell potential of
358 these cells, we placed primary ATII cells in an organoid assay (Fig 1G) [3, 35, 41].

359 Notably, old primary ATII cells formed significantly fewer organoids as compared to
360 cells isolated from young animals (Fig 1H). Altogether, these data indicate that the
361 aged lung contains ATII cells with increased cellular senescence and reduced
362 progenitor cell potential.

363 **3.2. Increased WNT/β-catenin activity in old ATII cells**

364 WNT/β-catenin signaling has been implicated in lung epithelial cell progenitor
365 function [27-29, 42] and aberrant ATII cell reprogramming in IPF [18, 30, 31]. Thus,
366 we wondered if WNT/β-catenin signaling contributes to lung aging and potentially
367 cellular senescence. In order to assess WNT/β-catenin activity in ATII cells from
368 young or old mice, we used a reporter mouse line that expresses GFP under the
369 control of multimerized TCF/Lef DNA binding sites, thus faithfully recapitulating
370 WNT/β-catenin-signaling activity (WNT-GFP mice) [43]. We observed increased
371 WNT/β-catenin activity in old ATII cells as compared to the young mice (Fig 2A and B;
372 10.50±8.30% GFP^+ cells in young mice versus 26.3±9.23% GFP^+ cells in old mice;
373 $p<0.01$).

374 **3.3. Chronic WNT/β-catenin signaling induces cellular senescence in ATII cells**

375 We next asked whether increased WNT/β-catenin-activity results in ATII cell
376 senescence. To this end, we activated WNT/β-catenin-signaling chronically with
377 CHIR99021 (CHIR), a GSK3-β inhibitor that leads to direct β-catenin-accumulation, a
378 key feature of WNT/β-catenin pathway activation [20]. Prolonged CHIR treatment for
379 7 days in a murine ATII cell line (MLE12 cells) induced a strong, dose-dependent
380 induction of WNT/β-catenin signaling, as measured by the gene expression of the
381 bona fide WNT target gene *Axin2* (Fig 3A). No cytotoxicity of CHIR was observed
382 (Fig S2A). At the same time, chronic WNT/β-catenin activation led to increased SA-β-
383 gal activity as assessed by flow cytometry as well as conventional light microscopy

384 (Fig 3B, C). In addition, we observed increased *Cdkn1a (p21)* transcript (Suppl Fig
385 S2B) as well as P21 protein levels (Fig 3D). Similarly, we treated primary ATII cells,
386 which expressed high levels of proSP-C, E-cadherin and Cytokeratin (Fig 4A), with
387 CHIR and found increased *Axin2* and *Cdkn2a (p16)* expression (Fig 4B). In order to
388 investigate whether specific WNT ligands exhibit similar effects, we treated primary
389 mouse ATII cells with WNT3A, a WNT ligand, which is increased in IPF [24, 27]. We
390 used either conditioned medium from L-cells overexpressing WNT3A [44] (Fig 4C, E,
391 F) or recombinant WNT3A (Fig 4D). Consistently, WNT3A induced transcript level of
392 senescence markers *Cdkn2a* and *Cdkn1a* accompanied by *Axin2* (Fig 4C, D). Both
393 WNT3A and CHIR led to increased SA-β-gal activity after 7 days of treatment (Fig 4E,
394 F). Notably, gene expression of *Cdkn2a* was induced rapidly after 24h of WNT3A
395 treatment, whereas *Cdkn1a* expression as well as SA-β-gal was induced only by
396 chronic stimulation after 7 days. Notably, stimulation of ATII cells with conditioned
397 medium containing a non-canonical WNT ligand, WNT5A, did not induce signs of
398 cellular senescence (Fig S3B). Co-treatment of cells with both WNT ligands revealed
399 that the non-canonical ligand WNT5A reduced the ability of WNT3A to induce
400 senescence. Gene expression of *Axin2*, *Cdkn2a* and *Cdkn1a* (Fig S3C) as well as
401 SA-β-gal activity (Fig S3D) was significantly reduced, thus further confirming that
402 canonical WNT/β-catenin signaling induces ATII cell senescence. Finally, we aimed
403 to investigate whether WNT3A is able to induce cellular senescence in a 3D lung
404 environment and subjected precision-cut lung slices (PCLS) from young mice to
405 chronic WNT stimulation. CHIR or WNT3A treatment led to increased gene
406 expression of senescence markers *Cdkn2a* and *Cdkn1a* (Fig 5A, B). Importantly,
407 prolonged WNT3A treatment also resulted in P21 protein expression, primarily in E-
408 cadherin^{+} epithelial cells, as monitored by immunofluorescence staining (Fig 5C).

409

410 **3.4. Chronic WNT/β-catenin stimulation induces a fibrotic phenotype in distal**
411 **epithelial lung cells**

412 WNT/β-catenin activity has been linked to a fibrotic epithelial cell phenotype by
413 several studies [27-29, 42] and our data further indicate that WNT/β-catenin
414 contributes to cellular senescence. Thus, we next aimed to investigate the potential
415 overlap of WNT/β-catenin signaling and cellular senescence in the fibrotic epithelium
416 of IPF. In a published dataset from primary human ATII cells isolated from Donor and
417 IPF patients (GSE94555 [39]), we found a concomitant and significant gene set
418 enrichment of both, WNT signaling (gene list from WNT/β-catenin GO: 0060070 and
419 https://web. stanford. edu/group/nusselab/cgi - bin/wnt / target_genes, Fig 6A) as well
420 as aging, cellular senescence and senescence associated secretory phenotype
421 (SASP) (gene lists from: aging (GO:0007568), Senescence [40], or senescence
422 associated secretory phenotype (SASP) [9]), in fibrotic ATII cells (Fig 6B). In line with
423 these findings, we found a recently described marker of fibrotic epithelial cells,
424 Keratin 8 [32, 45], to be induced by chronic, but not acute WNT3A stimulation in
425 pmATII cells (Fig 6C, D, corresponding senescence and *Axin2* expression in Fig 4C).

426

427 **4. Discussion**

428 Aging is a major risk factor for the development of IPF, however, which aging
429 mechanisms contribute to IPF development remains under-investigated and how
430 these are regulated, is largely unknown [2]. Here, we provide evidence of cellular
431 senescence and ATII progenitor cell exhaustion in the aging mouse lung, which
432 might predispose to CLD development. There is increasing evidence suggesting that

433 senescent cells accumulate in aging tissues and organs, thereby impairing
434 physiological repair and regenerative processes, thus leading to organismal aging [8,
435 12]. ATII cell reprogramming, including cellular injury and hyperplasia is a central
436 phenotype observed in IPF [4, 5, 46]. Aged mice exhibit a higher susceptibility to lung
437 fibrosis development, which correlated with the burden of senescent cells upon injury
438 [15, 47]. In accordance, ATII cells have been shown to exhibit cellular senescence
439 and SASP secretion in IPF and further show signs of telomere attrition and oxidative
440 stress [14, 46]. It remains unclear, however, which signaling pathways drive the
441 aging phenotype in (impaired) ATII cells.

442 In this study we observed that WNT/β-catenin signaling is increased in aged lungs,
443 which is consistent with findings in other organs [18, 30]. Moreover, we demonstrate
444 that active WNT/β-catenin signaling contributes to ATII cell senescence, potentially
445 leading to progenitor cell exhaustion. Similarly, increased WNT/β-catenin has also
446 been linked to cellular senescence in other organs and conditions, including normal
447 embryogenesis [10, 11]. Aberrant activity of WNT/β-catenin is well described in IPF
448 [19, 24-26] and plays a critical role for ATII progenitor cell function [21, 23]. Notably,
449 we observed that chronic WNT/β-catenin stimulation robustly induced cellular
450 senescence, whereas shorter stimulation did not result in the same phenotype.
451 *Cdkn2a* (encoding for p16) has been described as a WNT/β-catenin target gene,
452 consistent with the induction we observed after 24h in primary ATII cells [48], in
453 contrast to this, *Cdkn1a* is not upregulated at 24h but only at a later timepoint.
454 Induction of *Cdkn2a* is not sufficient to establish a full senescence phenotype as
455 shown by the fact that SA-ß-Galactosidase is increased only after 7d but not after
456 24h of WNT stimulation. These findings underline that timing, concentration and
457 duration of WNT/β-catenin can lead to different cellular and functional outcomes.

458 While WNT/β-catenin signaling increases with aging; in IPF, this is enhanced by
459 continuous injuries and reprogramming of the lung epithelium, likely further promoting
460 prolonged and chronic WNT/β-catenin activity. Accordingly, inhibition of β-catenin
461 signaling attenuates bleomycin-induced lung fibrosis [26, 49]. Whether these
462 treatments decrease the burden of senescent cells has not been investigated yet.

463 Notably, only canonical, but not non-canonical WNT signaling was able to
464 induce cellular senescence. In addition, the co-activation of both WNT pathways
465 prevented canonical WNT-driven senescence induction, further underlining the
466 importance of proper crosstalk between these pathways. Dysregulation of both,
467 canonical and non-canonical WNT signaling likely contributes to cellular senescence
468 and aging. Studies exploring the expression of WNT ligands in the aging lung are
469 sparse, however, the non-canonical ligand WNT5A was found increased in the aging
470 lung in several reports [5, 34, 50, 51]. Whether WNT5A is upregulated as a feedback
471 mechanism in response to increased senescence or potentially contributes to other
472 aging mechanisms needs to be further investigated.

473 Under homeostatic conditions, WNT/β-catenin signaling is essential for stem
474 cell function, including the progenitor cell potential of ATII cells [35, 41]. We have
475 recently demonstrated that modulation of WNT/β-catenin signaling alters ATII cell-
476 based organoid growth and numbers and further pro-fibrotic activation of the
477 supporting mesenchyme skewed WNT/β-catenin signaling and led to impaired
478 organoid formation [41, 52]. Here, however, we observed that aged ATII cells were
479 characterized by increased WNT/β-catenin signaling but displayed a reduced
480 capacity to form organoids, suggesting a defective progenitor cell function. Similarly,
481 fibrotic ATII cells or ATII cells with shortened telomeres have a decreased organoid
482 forming capacity, further suggesting alveolar progenitor cell dysfunction as a

483 contributor to IPF [53, 54]. Senescence of progenitor cells can lead to cell exhaustion
484 and further senescent niche cells might affect neighboring cells in a cell-autonomous
485 manner by secretion of SASP components that negatively affect progenitor cell
486 function [13]. Along these lines, recent reports describe senescent cells in a latent,
487 stem-like condition and highlight WNT/β-catenin as a major signaling factor in the
488 establishment of this stemness associated senescence (SAS). Altogether, these
489 findings highlight the intricate and overlapping role of WNT signaling as a
490 simultaneous stem-cell factor and senescence inducer [55, 56].

491 Our analysis of a single cell suspension of the whole lung revealed a relative
492 increase in inflammatory cells accompanied by a relative decrease of epithelial cells,
493 consistent with a recent report analyzing single cell sequencing from the aged lung
494 [6]. Analyzing ATII cells by flow cytometry, however, is limited in determining total cell
495 numbers, resulting often in an underestimation of cells, probably due to cell loss
496 during tissue processing [57, 58]. The gold standard to determine total cells numbers
497 in situ remains stereology. Indeed, a recent study found no change in total ATII cell
498 numbers between young and old mice [59]. As such, it is important to note, that while
499 we observe reduced relative numbers of ATII cells by FACS analysis we can not
500 exclude that this finding is in part due to a relative increase of inflammatory cells.

501 The following considerations have to be taken into account given our
502 experimental setup: Throughout the paper, we used primary ATII cells isolated using
503 a well-documented and established isolation protocol [35]. These cells are
504 characterized by high enrichment of EpCAM$^+$ and proSP-C$^+$ cells [58]. While it is
505 well-known that this leads to a high enrichment of ATII cells, we cannot fully exclude
506 that other rare cell populations are present. Moreover, we used different strains
507 (C57BL6/J or /N) as well as the broad range of ages, which might lead to increased

508 variability, however, in our study we observed consistent changes across different
509 strains. We further found that the impairment of progenitor cell function is already
510 apparent in mice aged 16-18 months and not significantly different from even older
511 animals.

512 While senescent cells most likely accumulate in everybody´s lungs, not
513 everybody develops a disease, such as IPF. Thus, other environmental as well as
514 autonomous factors are likely required to develop disease. Notably, telomere attrition
515 is a driving force in IPF and mutations in telomerase genes have been found in
516 familial and sporadic cases of IPF [60]. Telomere attrition is well known to induce
517 cellular senescence [8, 12]. Interestingly, telomere dysfunction in ATII cells, but not
518 mesenchymal cells, led to increased cellular senescence, stem cell failure and lung
519 fibrosis [53, 61]. Moreover, other hallmarks of aging such as mitochondrial
520 dysfunction contribute to IPF [2 , 62] and thus convey increased disease
521 susceptibility in addition to increased cellular senescence.

522 Targeting senescence as a potential therapeutic target is of high interest.
523 Recent advances in the field have led to the development and testing of drugs that
524 target cellular senescence, including senolytics. Senolytics have been effectively
525 shown to attenuate disease in mouse models of various diseases, including
526 pulmonary fibrosis [7, 8, 14, 15] with first in human studies currently being performed
527 [63]. However, current senolytics are rather broad and concerns with regards to their
528 suitability have been raised. While the senolytics target predominantly senescent
529 cells, not every cell type and every type of senescence seems to be affected by
530 these drugs [63]. In IPF, several cell types have been reported to become senescent,
531 including fibroblasts [15, 47] and epithelial cells. It is still a matter of ongoing research,
532 how cell type-specific senescence contributes to IPF pathogenesis. A recent study

533 demonstrated ATII cell-specific induction of cellular senescence was able to drive the
534 development of pulmonary fibrosis in mice [64]. Interestingly, myofibroblast
535 senescence restricts fibrosis in organs other than the lung [8, 12] and recent studies
536 suggest a similar mechanism in the lung [61]. It is intriguing to envision the potential
537 of future therapeutics to address the induction and cell-type specificity of cellular
538 senescence that determines not only the susceptibility to chronic lung disease but
539 also the potential that interference with this process could be developed for novel
540 treatment options for IPF.

541

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550 **Author contributions**

551 ML, MK: conception and design of research. ML, QH, YH, RC planned experiments.
552 ML, QH, YH, RC, KH, AvdB, performed experiments and analyzed the data. ML, QH,
553 MK wrote the manuscript. All authors approved the final version of the manuscript.

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756

757 **Figure legends**

758 **Figure 1: Phenotype of ATII cells in aged mice. (A-H)** Lungs from young (6-21
759 weeks) or old (16-24 months) mice were harvested and ATII cells were isolated. (**A)**
760 Total number of isolated ATII cells per mouse, n=10. **(B)** Freshly isolated ATII cells
761 were analyzed for SA-ß-galactosidase activity by FACS n=4 or **(C)** by conventional
762 SA-ß-galactosidase staining 2 days after plating, image representative of n=4, arrows
763 depict positive cells, Size bar represents 50µm. **(D)** Freshly isolated ATII cells were
764 analyzed for alveolar epithelial cell and senescence markers by qPCR. Values were
765 normalized to *Hprt* and corresponding young controls. n=5-10. **(E-F)** Cytospins of
766 freshly isolated ATII cells were stained for **(E)** P21 or **(F)** proSP-C protein. Image
767 representative of a n=3, Size bar represents 50µm. **(G-H)** Freshly isolated ATII cells
768 were combined with Matrigel and CCL206 fibroblasts and used for an organoid assay
769 as outlined in **(G)** and representative pictures are shown at day 14 of organoid
770 differentiation in **(H)** as well as quantification of numbers of organoids per well, n=5.
771 Data are presented as mean ± s.d. Circles represent C57BL6/J mice, triangles
772 represent C57BL6/N mice. Significance was assessed with one sample t-test
773 compared to a hypothetical value of 0 (D) or Mann Whitney-test (A, B, H)
774 Significance: *P<0.05, **P<0.01.

775

776 **Figure 2: WNT activity is increased in ATII cells from aged mice. (A-B)** Lungs
777 from young (3 months) or old (18 months) WNT GFP mice were harvested and ATII
778 cells were isolated and analyzed for WNT activity (GFP^+) by FACS. Data are
779 presented as mean ± s.d. n=6 old, n=14 young. Significance was assessed with a
780 two-way Anova followed by Sidak´s multiple comparison test. Significance: **P<0.01.

781

782 **Figure 3: Chronic WNT stimulation induces cellular senescence in MLE12 cells.**
783 **(A-D)** MLE12 lung epithelial cells were treated with 1µM CHIR or 2µM CHIR for 7d **(A)**
784 qPCR analysis for WNT target gene *Axin2* normalized to *Hprt* levels were perfomed.
785 n=4. **(B)** SA-β-galactosidase activity was measured by conventional staining after 7d**,**
786 representative of n=4. Size bar represents 50µm. **(C)** SA-β-galactosidase activity was
787 measured by FACS-based staining after 7d, n=5. **(D)** Western Blot for P21 was
788 performed, β-actin was used as a loading control. Blot is representative of n=4. Data
789 are presented as mean ± s.d. Significance was assessed with a one-way Anova
790 followed by Tukey´s multiple comparison test. Significance: *P<0.05, **P<0.01,
791 ***P<0.001.
792
793 **Figure 4: Chronic WNT stimulation induces cellular senescence in pmATII cells.**
794 **(A)** ATII cells were stained by Immunofluorescence for pro Surfactant Protein-C
795 (proSP-C), E-Cadherin (Ecad) or Cytokeratin. **(B)** ATII cells were treated with 1µM
796 CHIR for 7d, n=6**,** **(C)** conditioned medium from WNT3A-overexpressing L-cells
797 (WNT3A CM; 1:1) for 24h (n=6) or 7 days (n=10) **(D)** recombinant mWNT3A for 24h
798 (n=5) and 7d (n=8). **(B-D)** qPCR analysis for WNT target gene *Axin2* and
799 senescence markers *Cdkn1a* and *Cdkn2a* normalized to *Hprt* levels were performed.
800 **(E-F)** ATII cells were treated with 1µM CHIR or WNT3A CM and SA-β-galactosidase
801 activity was measured by **(E)** conventional staining after 24h and 7d, n=6 or by **(F)**
802 FACS-based staining after 7d, n=3. Significance was assessed by Wilcoxon
803 matched-pair signed rank test (B-D) and Student´s t-test (E-F). Significance: *p<0.05,
804 **P<0.01.
805
806 **Fig. 5: Chronic WNT stimulation induces and cellular senescence in epithelial**
807 **cells.**

808 Preciscion cut lung slices (PCLS) were prepared from young mice and treated with
809 **(A)** 2µM CHIR (CHIR) for 7 days, n=5, or **(B)** conditioned medium from WNT3A-
810 overexpressing L-cells (WNT3A CM; 1:1) for 7 days. n=3**.** **(A-B)** qPCR analysis for
811 WNT target gene *Axin2* and senescence markers *Cdkn2a* and *Cdkn1a* was
812 performed and normalized to *Hprt* levels. **(C)** Representative images of
813 immunofluorescence staining for P21 and CDH1 (E-CAD) in PCLS prepared from
814 young mice and treated with WNT3A CM for 7 days**.** Fluorescent images represent a
815 400× magnification. The scale bar represents 50µm. Representative of n=3.
816 Significance was assessed by paired Student´s t-test (A-B). Significance: *p<0.05,
817 **P<0.01.

818

819 **Fig. 6: Chronic WNT stimulation induces Keratin8 and cellular senescence in**
820 **epithelial cells.**

821 **(A-B)** Gene set enrichment analysis was performed on a published RNA sequencing
822 dataset from hATII cells from Donor/IPF patients GSE94555 (Xu et al, 2016, JCI
823 Insight). The dataset was tested for the enrichment of **(A)** WNT/β-catenin (GO:
824 0060070 and https://web. stanford. edu/group/nusselab/cgi - bin/wnt / target_genes)
825 or **(B)** for aging (GO:0007568), senescence (Fridman et al., Oncogene, 2008 [40]) or
826 senescence associated secretory phenotype (SASP; Coppé, Annu Rev Pathol) [9])
827 lists. **(C-D)** pmATII cells were treated with WNT3A CM for 24h (n=5) or 7 days (n=7).
828 **(D)** qPCR analysis of the fibrotic epithelial marker *Krt8* normalized to *Hprt* **(D)** Two
829 representative western blots of Krt8. Quantification of Krt8 signal over β-actin
830 normalized to CTRL CM is shown on the right. n=4. Data are presented as mean ±
831 s.d. Significance was assessed by Wilcoxon matched-pair signed rank test (C) and
832 one sample t-test compared to a hypothetical value of 1 (D). Significance: *p<0.05,
833 **P<0.01.

834

835 **Supplementary Figures**

836 **Figure S1: (A)** Lungs from mice were harvested and a single cell suspension was

837 prepared and analyzed for $CD45^+$ and $EpCAM^+$ cells by FACS and normalized to

838 young controls. n=(5). **(B)** Young (6-21 weeks) or old (16-24 months) mice were

839 weighed and their bodyweight is depicted in [g]. n=5. Significance was assessed with

840 one sample t-test compared to a hypothetical value of 100 (A) or Mann Whitney-test

841 (B) Significance: *P<0.05, **P<0.01.

842

843 **Figure S2: Chronic WNT stimulation induces cellular senescence.**

844 **(A)** MLE12 lung epithelial cells were treated with 2µM CHIR for 7d and LDH was

845 measured. Values are depicted as % of positive control, n=3. No significant increase

846 in LDH was detected. **(B)** MLE12 lung epithelial cells were treated with 1µM CHIR or

847 2µM CHIR for 7d. qPCR analysis for *Cdkn1a* and *Cdkn2a* was performed and

848 normalized to *Hprt* levels were perfomed, n=4.**(C)** ATII cells were treated with 1µM

849 CHIR (CHIR) for 7d, n=6**,** qPCR analysis for *Sftpc* and normalized to *Hprt* levels were

850 performed. Data are presented as mean ± s.d. Significance was assessed with a

851 one-way Anova followed by by Wilcoxon matched-pair signed rank test (A,C)

852 orTukey´s multiple comparison test (B).

853

854 **Figure S3: Non-canonical WNT stimulation does not induce cellular**

855 **senescence in pmATII cells.**

856 **(A-B)** ATII cells were treated with conditioned medium from WNT5A-overexpressing

857 L-cells (WNT5A CM; 1:1) for 7 days (n=6) and **(A)** qPCR analysis for WNT target

858 gene *Axin2* and senescence markers *Cdkn1a* and *Cdkn2a* normalized to *Hprt* levels

859 were performed. **(B)** SA-β-galactosidase activity was measured by conventional

860 staining after 7d, quantification and representative image of n=4 is shown. **(C)** ATII
861 cells were treated with conditioned medium from WNT3A-overexpressing L-cells
862 (WNT3A CM; 1:1) combined with medium from WNT5A-overexpressing L-cells
863 (WNT5A CM; 1:1) for 7 days (n=7). qPCR analysis for WNT target gene *Axin2* and
864 senescence markers *Cdkn1a* and *Cdkn2a* normalized to *Hprt* levels were performed.
865 Values were normalized to control conditioned medium. **(D)** SA-β-galactosidase
866 activity was measured by conventional staining after 7d, quantification and
867 representative image of n=4 is shown. The scale bar represents 50μm. Significance
868 was assessed by Wilcoxon matched-pair signed rank test (A,C) and one sample t-
869 test compared to a hypothetical value of 0 (C). Significance: *P<0.05, **P<0.01. (ctrl
870 to WNT5A; WNT3A to WNT3A+WNT5A), #p<0.05, ##P<0.01 (ctrl to WNT3A).
871