

Hydroxypropyl cyclodextrin improves amiodarone-induced aberrant lipid homeostasis of alveolar cells

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37 **Abstract**

38 Alveolar epithelial type II (AT2) cells secrete pulmonary surfactant via lamellar bodies (LBs).
39 Abnormalities in LBs are associated with pulmonary disorders, including fibrosis. However,
40 high-content screening (HCS) for LB abnormalities is limited by the lack of understanding of AT2 cell
41 functions. In the present study, we have developed LB cells harboring LB-like organelles that secrete
42 surfactant proteins. These cells were more similar to AT2 cells than to parental A549 cells. LB cells
43 recapitulated amiodarone (AMD)-induced LB enlargement, similar to AT2 cells of patients exposed to
44 AMD. To reverse AMD-induced LB abnormalities, we performed HCS of approved drugs and
45 identified 2-hydroxypropyl- β -cyclodextrin (HP β CD), a cyclic oligosaccharide, as a potential
46 therapeutic agent. A transcriptome analysis revealed that HP β CD modulates lipid homeostasis. In
47 addition, HP β CD inhibited AMD-induced LB abnormalities in human induced pluripotent stem
48 cell-derived AT2 cells. Our results demonstrate that LB cells are useful for HCS, and suggest that
49 HP β CD is a candidate therapeutic agent for AMD-induced interstitial pneumonia.

50
51 **Keywords:** alveolar epithelial type 2 cells; amiodarone; lamellar bodies;
52 2-hydroxypropyl- β -cyclodextrin; high content screening

53

54

55 **Introduction**

56 Alveolar epithelial type II (AT2) cells have various functions, including the synthesis and
57 secretion of pulmonary surfactant to maintain the alveolar structure and serving as tissue stem cells
58 with the capacity for self-renewal and differentiation into alveolar epithelial type I (AT1) cells, which
59 contribute to gas exchange in pulmonary homeostasis (1, 2). AT2 cell dysfunction induced by
60 endoplasmic reticulum stress, senescence, and aberrant surfactant homeostasis has been known to occur
61 in pulmonary fibrosis (3–7), emphasizing the importance of understanding the pathological and
62 physiological roles of human AT2 cells (8, 9). Pulmonary surfactants are composed of phospholipids,
63 mainly dipalmitoylphosphatidylcholine (DPCC), and surfactant proteins (10). Surfactant protein A
64 (SFTPA) and surfactant protein D (SFTPD) are hydrophilic proteins that contribute to host defense by
65 binding to and killing respiratory viral, bacterial, and fungal pathogens via alveolar macrophages (11).
66 Surfactant protein B (SFTPB) and surfactant protein C (SFTPC), hydrophobic proteins, are synthesized
67 by the proteolytic cleavage of a precursor molecule and post-translational modification; mature SFTPC
68 and SFTPB accelerate the adsorption of mixed surfactant phospholipids (12). Surfactant proteins
69 (SFTPA, SFTPB, SFTPC, and SFTPD) and phospholipids are assembled into highly packed
70 membranous structures, called lamellar bodies (LBs), which are acidic secretory organelles, and are
71 secreted to maintain the alveolar structure (12).

72 AT2 cells maintain LBs, express surfactant-related genes, and secrete pulmonary surfactant
73 (12). When cultured in a conventional two-dimensional (2D) culture, most AT2 cells differentiate into
74 AT1-like cells (13). In a three-dimensional (3D) culture, however, human AT2 cells can be cultured
75 long-term and their functions can be maintained (14–16). In particular, human induced pluripotent stem
76 cells (iPSCs) differentiate into AT2 cells harboring LBs; these cells express and secrete pulmonary
77 surfactant, the key features of AT2 cells (17–20). However, high-throughput analyses are limited by the

difficulties associated with maintaining AT2 cells in 2D culture and the cost of mass production. A549 cells expressing ABCA3, a transporter of phospholipids to LBs, bear LB-like structures (21, 22), but they differ from LBs *in vivo* in terms of the density of lamellar structures. Additionally, whereas A549 cells in specific culture conditions contain LB-like structures, these cells do not express surfactant proteins (23). Thus, there are no reports of cells containing *bona fide* LBs that express and secrete surfactant proteins, despite the potential applications for high-throughput analyses.

We generated cells harboring LB-like organelles that express and secrete surfactant proteins to establish a model of LB formation and function in AT2 cells. We then used this model to screen candidate drugs for the treatment of aberrant LBs by high-content screening. We generated stable cells, tentatively named LB cells, characterized by ABCA3, SFTPC, SFTPB, SFTPD expression, SFTPB, SFTPC, SFTPD secretion, and LB-like organelles. Using high-content screening, we found that 2-hydroxypropyl- β -cyclodextrin (HP β CD) reduces amiodarone (AMD)-induced LB abnormalities. Our findings demonstrated the value of LB cells for studies of LB abnormalities and identified HP β CD as a candidate drug for AMD-induced interstitial pneumonia.

Methods

Additional details regarding the methods used in this study are provided in the data supplement.

Generation of LB cells

A549 cells stably expressing ABCA3, SFTPB, SFTPC, SFTPD, and GFP were established by a protocol that is detailed in the data supplement.

101 **LB cell culture for subsequent assay**

102 A549 and LB cells were passaged 2:5 twice a week upon reaching 80% confluency on a 10 cm-dish
103 (Greiner). After incubation under specific assay conditions, the cells were harvested for further
104 analysis.

105

106 **Immunofluorescence analysis**

107 Cells were fixed with 4% paraformaldehyde/PBS (Nacalai Tesque) or ice-cold methanol, permeabilized
108 and immersed in the blocking solution. All subsequent antibody reactions were performed as described
109 in the data supplement. Additional details concerning the antibodies used are provided in Table E5.

110

111 **Measurement of phosphatidylcholine (PC) using TopFluor TMR PC (TMR-PC)**

112 PC was visualized using TMR-PC (Avanti Polar Lipids) incorporated into the cells and subsequently
113 quantified. The detailed methods are described in the data supplement.

114

115 **Electron microscopy**

116 Each sample was incubated in the fixative solution, stained with 1% uranyl acetate, dehydrated and
117 embedded in Epon 812 (Nacalai Tesque). The methods are described in the data supplement.

118

119 **Measurement of SFTPb, SFTPC, and SFTPD in the culture supernatant**

120 Culture supernatants of LB or A549 cells were collected and the concentrations of SFTPb, SFTPC, and
121 SFTPD in the culture supernatant were evaluated following the methods given in the data supplement.

122

123 **RNA-sequencing (RNA-seq) and quantitative real-time PCR (qRT-PCR) analysis**

124 Total RNA was extracted using an RNeasy Mini Kit (Qiagen). RNA-seq and qRT-PCR were
125 subsequently performed using the extracted total RNA. All data were deposited in the GEO database
126 (GSE146612). The methods and the list of primers used are detailed in the data supplement and Table
127 E6, respectively.

128

129 **High-content screening**

130 After LB cells were exposed to each compound, the cells were treated with TMR-PC plus DMSO or
131 AMD and analyzed using an Opera Phenix High-Content Imaging System (Perkin Elmer), as described
132 in the data supplement.

133

134 **Drug treatment of iPSC-derived AT2 cells in organoids**

135 SFTPC-GFP reporter iPSCs (B2-3) were first differentiated into SFTPC⁺ AT2 cells. The iPSC-derived
136 AT2 cells were then passaged two to five times (P2–P5), as described previously (18). The alveolar
137 organoids containing iPSC-derived AT2 cells (P2–P5) and human fetal lung fibroblasts were treated
138 with drugs and evaluated. The detailed culture methods are provided in the data supplement.

139

140 **Measurement of LysoTracker and PC in iPSC-derived organoids**

141 Drug-treated alveolar organoids, including iPSC-derived AT2 cells (P2–P5), were dissociated and
142 lysosome-related organelles (LROs) were visualized and quantified using LysoTracker Red DND-99
143 (Thermo Fisher Scientific). The amount of PC in iPSC-derived epithelial cells was evaluated as
144 described in the data supplement.

145

146 **Statistical analyses**

147 Graphs were generated and statistical analyses were performed using GraphPad Prism 8 (GraphPad).
148 The data were analyzed using a two-tailed unpaired Student's *t*-test or ANOVA (one-way) and results
149 are expressed as means $\pm$ S.D.. *P* < 0.05 was considered significant.

150

151 **Results**

152 **A549 cells with exogenous surfactant protein expression harbor LB-like organelles and secrete** 153 **SFTPB, SFTPC, and SFTPD in 3D culture**

154 We hypothesized that surfactant proteins effectively recapitulate the LBs of AT2 cells. Two
155 vectors for the overexpression of ABCA3, SFTPB, SFTPC, SFTPD, and GFP were transfected into
156 A549 cells (Figure 1A). After selection by geneticin and zeocin, we obtained stable A549 cell lines
157 overexpressing ABCA3, SFTPB, SFTPC, SFTPD, and GFP, referred to as LB cells.
158 Immunofluorescence staining revealed that SFTPB and SFTPD were colocalized in cells in 3D culture
159 and that ABCA3 and SFTPC exhibited a scattered, small, patchy distribution, suggesting that they are
160 localized in LB-like structures in cells in 3D culture (Figure 1B). Similarly, ABCA3, SFTPB, SFTPC,
161 and SFTPD were detected in cells in 2D culture (Figure E1A). Because the main component of LBs is
162 dipalmitoylphosphatidylcholine, a PC, we examined whether LB cells increased PC in 2D culture using
163 TMR-PC. FACS analysis indicated that the intracellular TMR-PC intensity was higher in LB cells than
164 in A549 cells (Figure 1C and 1D). Moreover, consistent with a previous report indicating that A549
165 cells overexpressing ABCA3 show LB-like organelles (22), the LB cells in both 2D and 3D cultures
166 showed an LB-like structure in the cells, as determined by transmission electron microscopy (Figure 1E
167 and E2B). Moreover, the SFTPB, SFTPC, and SFTPD concentrations in the culture supernatant were
168 detected in 3D cultures of LB cells, but not in those of A549 cells (Table 1). To assess the
169 transcriptomic profiles of LB cells, RNA-seq was performed. AT2 markers (*ABCA3*, *SFTPB*, *SFTPC*,

170 *SFTPD*, *PCDH9*, *SCD*, *MAOA*, *NPC2*, *AQP3*, *BEX2*, *CMTM8*, *DHCR7*, *ETV5*, and *FASN*) were
171 upregulated in LB cells (Figure 1F, E1B, and Table E1) (18, 24–28). Comparative transcriptome
172 analysis revealed that levels of *ABCA3*, *SFTPB*, and *SFTPD* were similar in LB cells and primary AT2
173 cells (GSE66627), whereas levels of *SFTPC* were lower in LB cells than in primary AT2 cells (Figure
174 E1C) (29). Notably, GO and KEGG pathway analyses using Metascape (30) showed that genes
175 upregulated in LB cells were significantly enriched for “lysosome” and “metabolism of lipids”
176 corresponding to our previous results ($P = 7.53 \times 10^{-22}$ and $P = 9.23 \times 10^{-11}$, respectively) (Figure E1D)
177 (18). A hierarchical clustering analysis of the top 300 differentially expressed genes (DEGs) indicated
178 that expression patterns in LB cells were more similar to those in primary AT2 cells than in A549 cells
179 (Figure 1G and Table E1). Furthermore, mRNA expression of *ABCA3*, *SFTPB*, *SFTPC*, and *SFTPD* in
180 high passaged LB cells (P26–P33) was similar to that in low-passaged LB cells (P12–P20) (Figure
181 E2A). In addition, LB cells harbored LBs in both 2D and 3D cultures, suggesting that LB cells
182 maintained surfactant proteins (Figure E2B). Taken together, LB cells resemble primary AT2 cells at
183 the phenotypic and transcriptome levels.

184

185 **AMD induced LB abnormalities in LB cells**

186 To determine whether LB cells can be used as disease models, they were treated with AMD,
187 which induces LB abnormalities in human AT2 cells (Figure 2A) (31). Fluorescence microscopy
188 indicated that the intracellular intensity of TMR-PC in A549 cells and LB cells treated with AMD
189 increased in a concentration-dependent manner, with a half-maximal effective concentration (EC_{50}) of
190 6 μ M and 2.7 μ M, respectively, suggesting that LB cells were more sensitive to AMD-induced PC
191 increase than A549 cells (Figure 2B). *ABCA3* is expressed predominantly in the limiting membrane of
192 LBs in AT2 cells (32) and can be used as a marker of LBs. We found that the number and size of

193 ABCA3⁺ LBs were higher in LB cells treated with AMD than in the vehicle control (Figure 2C).
194 Furthermore, transmission electron microscopy revealed that LB-like organelles in LB cells treated
195 with AMD in 3D culture were enlarged and more abundant (Figure. 2D), indicating that LB cells are
196 useful for the detection of AMD-induced LB abnormalities.

197 For *in vitro* drug screening to identify chemicals capable of reversing AMD-induced LB
198 abnormalities, we first developed a high-content screening assay using LB cells. Using a PerkinElmer
199 Opera Phenix high-content screening system and Harmony, the number of TMR-PC dots per cell area
200 was assessed (Figure 2E). AMD significantly increased the number of TMR-PC dots per cell area, and
201 the robustness of the assay was validated based on the Z'-factor (0.61 in three independent plates, with
202 six replicates of 0.1% DMSO and 10 μ M AMD in each plate) (Figure 2F). Overall, we developed an *in*
203 *vitro* screening method for LB abnormalities.

204

205 **Identification of HP β CD by high-content screening**

206 We further applied our screening method to evaluate the effect of GW9662, which reduces
207 AMD-induced phospholipid accumulation (33), on LB cells. Concentrations of up to 10 μ M GW9662
208 did not reduce AMD-induced intracellular accumulation of TMR-PC dots (Figure E3A and E3B). The
209 lack of a significant effect of GW9662 on TMR-PC might be attributable to the experimental
210 conditions. We screened 2,345 compounds from the Kyoto University Chemistry library to identify
211 other compounds that can reverse the AMD-induced intracellular accumulation of TMR-PC, obtaining
212 an average Z'-factor of 0.525. We developed a schematic model for the prioritization of compounds and
213 the identification of compounds of high interest (Figure 3A). Compounds that attenuated the
214 AMD-induced intracellular accumulation of TMR-PC dots (with a threshold of 80% inhibition in
215 comparison with the AMD controls) were considered hits (Figure 3B and Table E2). We obtained 32

hits and selected HP β CD with more than 80% cell number (Figure 3C and Table E2). HP β CD inhibited AMD-induced intracellular accumulation of TMR-PC dots in LB cells in a concentration-dependent manner, with a half-maximal inhibitory concentration (IC₅₀) of 0.8 mM (Figure 3D and 3E). Transmission electron microscopy showed that the AMD-induced enlargement of LB-like organelles in LB cells in 3D culture was improved by HP β CD supplementation (Figure 3F). HP γ CD, a series of 2-hydroxypropyl-cyclodextrins, reduces the accumulation of cholesterol and is safer than HP β CD in hepatocyte-like cells derived from Nieman-Pick disease type C (NPC) patient-specific iPSCs (34). Accordingly, HP α CD and HP γ CD were also examined. Both HP α CD and HP γ CD reduced the AMD-induced intracellular accumulation of TMR-PC dots in a concentration-dependent manner, with IC₅₀ values of 1.8 mM and 2 mM, respectively (Figure E3A, E3C, and E3D). These results suggest that HPCDs improved the AMD-induced enlargement of LB-like organelles without reducing the LB cell count.

HP β CD corrects lipid homeostasis in AMD-treated LB cells

To determine the mechanism by which HP β CD improved AMD-induced LB enlargement, RNA-seq was performed using AMD-treated LB cells with HP β CD. Because LB is an LRO containing phospholipids, we hypothesized that AMD induces the abnormal accumulation of phospholipids in lysosomes of LB cells. A GO analysis of upregulated genes in AMD-treated LB cells identified enrichment for the terms “metabolism of lipids”, “lysosome”, and “phospholipid metabolic process” ($P = 3.08 \times 10^{-26}$, 3.23×10^{-22} , and 1.32×10^{-10} , respectively) (Figure 4A, 4B, E4A, and Table E3). Strikingly, an unbiased principal component analysis (PCA) showed that the transcriptomes of AMD-treated LB cells with HP β CD were similar to those of DMSO-treated LB cells (Figure 4C). Hierarchical clustering and GO analyses based on the expression of genes associated with PC2 showed

239 that HP β CD improved lipid biosynthesis and fatty acid metabolism ($P = 4.65 \times 10^{-11}$ and 2.70×10^{-10} ,
240 respectively) in AMD-treated LB cells, whereas HP β CD did not improve AMD-induced alterations in
241 genes associated with PC1 (Figure 4D, 4E, E4B–E4D, and Table E4). Indeed, genes involved in PC
242 degradation were upregulated in AMD-treated LB cells after HP β CD supplementation (Figure E5).
243 These data suggest that HP β CD improved AMD-induced LB abnormalities via alterations in lipid
244 homeostasis.

245

246 **HP β CD reverses AMD-induced LB abnormalities in LB cells and human iPSC-derived AT2 cells**

247 We previously found that AMD increases LROs, as visualized by the fluorescent signal
248 intensity of LysoTracker in iPSC-derived alveolar organoids, including AT2 cells and human fetal lung
249 fibroblasts (18). The efficacy of HP β CD was assessed in AT2 cells derived from human iPSCs to
250 examine the power of the newly developed screening system using LB cells. We treated iPSC-derived
251 alveolar organoids with AMD for 9 days, followed by treatment with HP β CD for 3 days (Figure 5A).
252 We found that HP β CD reduced the AMD-induced fluorescence intensity of LysoTracker, similar to
253 HP α CD and HP γ CD, in SFTPC⁺EpCAM⁺, SFTPC⁻EpCAM⁺, and EpCAM⁻ cells, whereas GW9662
254 had no effect (Figure 5B, 5C, E6A, and E6B). AMD increases PC in the lungs of humans and mice (35,
255 36); further evaluation indicated that HP β CD reduced PC in human iPSC-derived EpCAM⁺ cells
256 (Figure 5D). Transmission electron microscopy revealed that the AMD-induced enlargement of LB-like
257 organelles in human iPSC-derived AT2 cells was attenuated by HP β CD (Figure. 5E). Collectively, our
258 screening system using LB cells was useful for the identification of compounds able to reverse LB
259 abnormalities.

260

261 **Discussion**

262 We developed LB cells expressing ABCA3 SFTPB, SFTPC, and SFTPD derived from A549
263 cells. LB cells harbored LB-like organelles and secreted SFTPB, SFTPC and SFTPD. The
264 transcriptomes of LB cells were more similar to those of primary AT2 cells than to A549 cells.
265 AMD-induced LB abnormalities were recapitulated in LB cells. We used high-content screening to
266 show that HPβCD improved AMD-induced LB abnormalities involving the accumulation of LROs and
267 PC in iPSC-derived alveolar organoids. Thus, we successfully established a tool for the identification
268 of small-molecule inhibitors for reversing human LB abnormalities and specifically identified HPβCD
269 as a candidate therapeutic agent for AMD-induced interstitial pneumonia. LB cells did not differentiate
270 into AT1-like cells as primary AT2 cells do so, but morphologically and physiologically resembled AT2
271 cells. Thus, LB cells may be a good resource for high-throughput screening. Interestingly, SFTPB and
272 SFTPC were not detected in the supernatant of 2D cultures of LB cells (data not shown), although
273 SFTPD was detected (11.2 ± 3.2 ng/mL, $n = 3$). On the other hand, all of these surfactant proteins were
274 secreted in 3D culture of LB cells (Table 1). Thus, future studies will need to optimize culture
275 conditions for surfactant protein secretion. AMD is an iodinated benzofuran derivative and is classified
276 as a class III antiarrhythmic drug; however, it causes severe adverse reactions, including pulmonary
277 toxicity and lung fibrosis (37). Although the mechanism underlying the pulmonary toxicity of AMD is
278 not clear, excessive phospholipidosis, cytotoxicity, and/or oxidative stress may contribute to
279 AMD-induced fibrosis. Moreover, LB abnormalities have been detected in AT2 cells of patients and
280 mice exposed to AMD (31, 35). We previously reported that LB abnormalities are induced by AMD in
281 iPSC-derived AT2 cells (18). Therefore, the AMD-induced LB abnormalities in LB cells observed in
282 this study were consistent with previous results. LB cells could recapitulate AMD-induced AT2 cell
283 injury, especially LB abnormalities.

284 Although cell-type specific mechanisms might be involved in LB abnormalities, many studies

285 have used cell lines that do not have LBs (12, 33). To overcome the limited availability of AT2 cells for
286 high-content screening, we developed LB cells and used these cells for high-content screening to
287 identify compounds able to reverse AMD-induced LB abnormalities. We identified HP β CD as a
288 compound that reverses AMD-induced accumulation of TMR-PC dots in LB cells. To our knowledge,
289 this is the first report of high-content screening using cells containing LB-like organelles. HPCDs are a
290 hydroxyalkyl derivative of cyclodextrin, a widely used carrier for drug delivery (38). HP α CD, HP β CD,
291 and HP γ CD consist of six, seven, and eight D-glucopyranose units, respectively, linked to
292 2-hydroxypropyl groups by α -1, 4 glycosidic bonds to form a macrocycle. Our results indicated that
293 HP β CD could improve AMD-induced LB abnormalities via changes in lipid homeostasis. Furthermore,
294 HP β CD reduced the amount of PC and the intensity of LysoTracker-labeled LROs in iPSC-derived
295 alveolar organoids. Our findings are supported by those of a previous study in which HP β CD was
296 identified as a candidate therapeutic agent for reducing cholesterol accumulation in NPC1-defective
297 cells (39). Although HP β CD and HP γ CD but not HP α CD reduce the accumulation of cholesterol in
298 NPC patient-specific iPSC-derived hepatocyte-like cells (34), all of these HPCDs effectively reversed
299 the AMD-induced accumulation of TMR-PC in LB cells and LysoTracker-labeled LROs in
300 iPSC-derived organoids. This discrepancy may be explained by differences in the structures of each
301 cyclodextrin, which remove phospholipids (especially phosphatidylcholine and sphingomyelin) from
302 the outer half of the membrane bilayer (40) depending on the cell-type specific organelle. Although
303 further *in vivo* studies for investigating the efficacy of each cyclodextrin in AMD-induced pulmonary
304 fibrosis are needed, it would be helpful to know that a single subcutaneous administration of HP β CD
305 was reportedly improved lipid homeostasis and prolonged lifespan in NPC1 knockout mouse (41) and
306 intrathecal HP β CD was also effective in patients with NPC1 disease (42), suggesting that HP β CD is a
307 candidate therapeutic agent for AMD-induced interstitial pneumonia.

308 In conclusion, we developed a cell line harboring LB-like organelles and expressing surfactant
309 proteins. Through high-content screening using LB cells, we identified HPβCD as a candidate
310 therapeutic agent for reversing AMD-induced LB abnormalities. Our findings provide a basis for the
311 identification of therapeutic agents for diseases associated with LB abnormalities.

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319

320 **References**

- 321 1. Whitsett JA, Weaver TE. Alveolar development and disease. *Am J Respir Cell Mol Biol*
322 2015;53:1–7.
- 323 2. Schiller HB, Montoro DT, Simon LM, Rawlins EL, Meyer KB, Strunz M, Vieira Braga FA,
324 Timens W, Koppelman GH, Budinger GRS, Burgess JK, Waghay A, Van Den Berge M, Theis
325 FJ, Regev A, Kaminski N, Rajagopal J, Teichmann SA, Misharin A V., Nawijn MC. The human
326 lung cell atlas: A high-resolution reference map of the human lung in health and disease. *Am J*
327 *Respir Cell Mol Biol* 2019;
- 328 3. Jiang D, Cui H, Xie N, Banerjee S, Liu R-M, Dai H, Thannickal VJ, Liu G. ATF4 Mediates

- 329 Mitochondrial Unfolded Protein Response in Alveolar Epithelial Cells. *Am J Respir Cell Mol*
330 *Biol* 2020;63:478–489.
- 331 4. Hamsanathan S, Alder JK, Sellares J, Rojas M, Gurkar AU, Mora AL. Cellular senescence: The
332 Trojan horse in chronic lung diseases. *Am J Respir Cell Mol Biol* 2019;
- 333 5. Ruwisch J, Sehlmeier K, Roldan N, Garcia-Alvarez B, Perez-Gil J, Weaver TE, Ochs M,
334 Knudsen L, Lopez-Rodriguez E. Air space distension precedes spontaneous fibrotic remodeling
335 and impaired cholesterol metabolism in the absence of surfactant protein C. *Am J Respir Cell*
336 *Mol Biol* 2020;62:466–478.
- 337 6. Kadota T, Yoshioka Y, Fujita Y, Araya J, Minagawa S, Hara H, Miyamoto A, Suzuki S,
338 Fujimori S, Kohno T, Fujii T, Kishi K, Kuwano K, Ochiya T. Extracellular Vesicles from
339 Fibroblasts Induce Epithelial-Cell Senescence in Pulmonary Fibrosis. *Am J Respir Cell Mol Biol*
340 2020;63:623–636.
- 341 7. Rana T, Jiang C, Liu G, Miyata T, Antony V, Thannickal VJ, Liu RM. PAI-1 regulation of
342 TGF- β 1-induced alveolar Type II cell senescence, SASP secretion, and SASP-mediated
343 activation of alveolar macrophages. *Am J Respir Cell Mol Biol* 2020;62:319–330.
- 344 8. Atabai K, Yang CD, Podolsky MJ. You Say You Want a Resolution (of Fibrosis). *Am J Respir*
345 *Cell Mol Biol* 2020;63:424–435.
- 346 9. Ruwanpura SM, Thomas BJ, Bardin PG. Pirfenidone: Molecular mechanisms and potential

347 clinical applications in lung disease. *Am J Respir Cell Mol Biol* 2020;

348 10. Trapnell BC, Nakata K, Bonella F, Campo I, Griesse M, Hamilton J, Wang T, Morgan C, Cottin
349 V, McCarthy C. Pulmonary alveolar proteinosis. *Nat Rev Dis Prim* 2019;

350 11. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity.
351 *Nat Immunol* 2015;

352 12. Beers MF, Moodley Y. When Is an alveolar type 2 cell an alveolar type 2 cell?: A conundrum
353 for lung stem cell biology and regenerative medicine. *Am J Respir Cell Mol Biol* 2017;

354 13. Borok Z, Danto SI, Lubman RL, Cao Y, Williams MC, Crandall ED. Modulation of T1 α
355 expression with alveolar epithelial cell phenotype in vitro. *Am J Physiol - Lung Cell Mol Physiol*
356 1998;275:.

357 14. Shiraishi K, Nakajima T, Shichino S, Deshimaru S, Matsushima K, Ueha S. In vitro expansion
358 of endogenous human alveolar epithelial type II cells in fibroblast-free spheroid culture.
359 *Biochem Biophys Res Commun* 2019;515:579–585.

360 15. Katsura H, Sontake V, Tata A, Kobayashi Y, Edwards CE, Heaton BE, Konkimalla A, Asakura
361 T, Mikami Y, Fritch EJ, Lee PJ, Heaton NS, Boucher RC, Randell SH, Baric RS, Tata PR.
362 Human Lung Stem Cell-Based Alveolospheres Provide Insights into SARS-CoV-2-Mediated
363 Interferon Responses and Pneumocyte Dysfunction. *Cell Stem Cell* 2020;27:890-904.e8.

364 16. Youk J, Kim T, Evans K V., Jeong Y Il, Hur Y, Hong SP, Kim JH, Yi K, Kim SY, Na KJ,

Bleazard T, Kim HM, Fellows M, Mahbubani KT, Saeb-Parsy K, Kim SY, Kim YT, Koh GY, Choi BS, Ju YS, Lee JH. Three-Dimensional Human Alveolar Stem Cell Culture Models Reveal Infection Response to SARS-CoV-2. *Cell Stem Cell* 2020;27:905-919.e10.

17. Gotoh S, Ito I, Nagasaki T, Yamamoto Y, Konishi S, Korogi Y, Matsumoto H, Muro S, Hirai T, Funato M, Mae SI, Toyoda T, Sato-Otsubo A, Ogawa S, Osafune K, Mishima M. Generation of alveolar epithelial spheroids via isolated progenitor cells from human pluripotent stem cells. *Stem Cell Reports* 2014;3:394–403.

18. Yamamoto Y, Gotoh S, Korogi Y, Seki M, Konishi S, Ikeo S, Sone N, Nagasaki T, Matsumoto H, Muro S, Ito I, Hirai T, Kohno T, Suzuki Y, Mishima M. Long-term expansion of alveolar stem cells derived from human iPS cells in organoids. *Nat Methods* 2017;14:1097–1106.

19. Jacob A, Morley M, Hawkins F, McCauley KB, Jean JC, Heins H, Na CL, Weaver TE, Vedaie M, Hurley K, Hinds A, Russo SJ, Kook S, Zacharias W, Ochs M, Traber K, Quinton LJ, Crane A, Davis BR, White F V., Wambach J, Whitsett JA, Cole FS, Morrissey EE, Guttentag SH, Beers MF, Kotton DN. Differentiation of Human Pluripotent Stem Cells into Functional Lung Alveolar Epithelial Cells. *Cell Stem Cell* 2017;21:472-488.e10.

20. Korogi Y, Gotoh S, Ikeo S, Yamamoto Y, Sone N, Tamai K, Konishi S, Nagasaki T, Matsumoto H, Ito I, Chen-Yoshikawa TF, Date H, Hagiwara M, Asaka I, Hotta A, Mishima M, Hirai T. In Vitro Disease Modeling of Hermansky-Pudlak Syndrome Type 2 Using Human Induced

383 Pluripotent Stem Cell-Derived Alveolar Organoids. *Stem Cell Reports* 2019;12:431–440.

384 21. Wambach JA, Yang P, Wegner DJ, Heins HB, Luke C, Li F, White F V., Cole FS. Functional
385 Genomics of ABCA3 Variants . *Am J Respir Cell Mol Biol* 2020;63:436–443.

386 22. Matsumura Y, Sakai H, Sasaki M, Ban N, Inagaki N. ABCA3-mediated choline-phospholipids
387 uptake into intracellular vesicles in A549 cells. *FEBS Lett* 2007;581:3139–3144.

388 23. Cooper JR, Abdullatif MB, Burnett EC, Kempself KE, Conforti F, Tolley H, Collins JE, Davies
389 DE. Long term culture of the A549 cancer cell line promotes multilamellar body formation and
390 differentiation towards an alveolar type II Pneumocyte phenotype. *PLoS One* 2016;11:.

391 24. Plataki M, Fan LC, Sanchez E, Huang Z, Torres LK, Imamura M, Zhu Y, Cohen DE, Cloonan
392 SM, Choi AMK. Fatty acid synthase downregulation contributes to acute lung injury in murine
393 diet-induced obesity. *JCI Insight* 2019;4:.

394 25. Wade KC, Guttentag SH, Gonzales LW, Maschhoff KL, Gonzales J, Kolla V, Singhal S, Ballard
395 PL. Gene induction during differentiation of human pulmonary type II cells in vitro. *Am J Respir
396 Cell Mol Biol* 2006;34:727–737.

397 26. Roszell BR, Tao JQ, Yu KJ, Gao L, Huang S, Ning Y, Feinstein SI, Vite CH, Bates SR.
398 Pulmonary Abnormalities in Animal Models Due to Niemann-Pick Type C1 (NPC1) or C2
399 (NPC2) Disease. *PLoS One* 2013;8:.

400 27. Treutlein B, Brownfield DG, Wu AR, Neff NF, Mantalas GL, Espinoza FH, Desai TJ, Krasnow

- 401 MA, Quake SR. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell
402 RNA-seq. *Nature* 2014;509:371–375.
- 403 28. Liu YL, Matsuzaki T, Nakazawa T, Murata S ichi, Nakamura N, Kondo T, Iwashina M,
404 Mochizuki K, Yamane T, Takata K, Katoh R. Expression of aquaporin 3 (AQP3) in normal and
405 neoplastic lung tissues. *Hum Pathol* 2007;38:171–178.
- 406 29. Marconett CN, Zhou B, Sunohara M, Pouldar TM, Wang H, Liu Y, Rieger ME, Tran E, Flodby
407 P, Siegmund KD, Crandall ED, Laird-Offringa IA, Borok Z. Cross-species transcriptome
408 profiling identifies new alveolar epithelial Type i Cell-specific genes. *Am J Respir Cell Mol Biol*
409 2017;56:310–321.
- 410 30. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK.
411 Metascope provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat*
412 *Commun* 2019;10:.
- 413 31. Bedrossian CWM. Amiodarone pulmonary toxicity: Cytopathology, ultrastructure, and
414 immunocytochemistry. *Ann Diagn Pathol* 1997;1:47–56.
- 415 32. Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y, Morohoshi T, Ogawa J,
416 Shioda S, Inagaki N. ABCA3 is a lamellar body membrane protein in human lung alveolar type
417 II cells. *FEBS Lett* 2001;508:221–5.
- 418 33. Song M, Kim Y-J, Ryu J-C. Phospholipidosis Induced by PPAR γ Signaling in Human Bronchial

- 419 Epithelial (BEAS-2B) Cells Exposed to Amiodarone. *Toxicol Sci* 2011;120:98–108.
- 420 34. Soga M, Ishitsuka Y, Hamasaki M, Yoneda K, Furuya H, Matsuo M, Ihn H, Fusaki N,
421 Nakamura K, Nakagata N, Endo F, Irie T, Era T. HPGCD Outperforms HPBCD as a Potential
422 Treatment for Niemann-Pick Disease Type C During Disease Modeling with iPS Cells. *Stem*
423 *Cells* 2015;33:1075–1088.
- 424 35. Mahavadi P, Henneke I, Ruppert C, Knudsen L, Venkatesan S, Liebisch G, Chambers RC, Ochs
425 M, Schmitz G, Vancheri C, Seeger W, Korfei M, Guenther A. Altered surfactant homeostasis
426 and alveolar epithelial cell stress in amiodarone-induced lung fibrosis. *Toxicol Sci*
427 2014;142:285–297.
- 428 36. Heath MF, Costa-Jussa FR, Jacobs JM, Jacobson W. The induction of pulmonary
429 phospholipidosis and the inhibition of lysosomal phospholipases by amiodarone. *Br J Exp*
430 *Pathol* 1985;66:391–397.
- 431 37. Ernawati DK, Stafford L, Hughes JD. Amiodarone-induced pulmonary toxicity. *Br J Clin*
432 *Pharmacol* 2008;66:82–87.
- 433 38. Gould S, Scott RC. 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD): A toxicology review. *Food*
434 *Chem Toxicol* 2005;
- 435 39. Camargo F, Erickson RP, Garver WS, Hossain GS, Carbone PN, Heidenreich RA, Blanchard J.
436 Cyclodextrins in the treatment of a mouse model of Niemann-Pick C disease. *Life Sci*

437 2001;70:131–142.

438 40. Irie T, Uekama K. Pharmaceutical Applications of Cyclodextrins. III. Toxicological Issues and
439 Safety Evaluation. *J Pharm Sci* 1997;86:147–162.

440 41. Liu B, Turley SD, Burns DK, Miller AM, Repa JJ, Dietschy JM. Reversal of defective
441 lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the
442 npc1^{-/-} mouse. *Proc Natl Acad Sci U S A* 2009;106:2377–2382.

443 42. Ory DS, Ottinger EA, Farhat NY, King KA, Jiang X, Weissfeld L, Berry-Kravis E, Davidson
444 CD, Bianconi S, Keener LA, Rao R, Soldatos A, Sidhu R, Walters KA, Xu X, Thurm A,
445 Solomon B, Pavan WJ, Machielse BN, Kao M, Silber SA, McKew JC, Brewer CC, Vite CH,
446 Walkley SU, Austin CP, Porter FD. Intrathecal 2-hydroxypropyl- β -cyclodextrin decreases
447 neurological disease progression in Niemann-Pick disease, type C1: a non-randomised,
448 open-label, phase 1–2 trial. *Lancet* 2017;390:1758–1768.

449

450 **Tables**

451 **Table 1. Quantification of SFTPb, SFTPC, and SFTPD secretion in 3D cultured A549 and LB**
452 **cells.**

	SFTPb (ng/mL) (n = 3)	SFTPC (pg/mL) (n = 6)	SFTPD (ng/mL) (n = 6)
A549 cells	nd	nd	nd
LB cells	4.25 ± 0.49	3.82 ± 0.86	9.9 ± 0.46

453 Definition of abbreviations: ‘nd’ not detected.

454

455 **Figure legends**

456 **Figure 1. LB cells harbor LB-like organelles**

457 (A) Schematic representation of the generation of LB cells.

458 (B) Immunofluorescence staining of ABCA3, SFTPB, SFTPC, and SFTPD in 3D culture. Nuclei were
459 stained with Hoechst 33342 in blue. Scale bars, 10 μ m.

460 (C and D) FACS analysis of TMR-PC accumulation in LB cells and their parental A549 cells in 2D
461 culture. A549 cells and LB cells were stained with TMR-PC. Data are presented as means $\pm$ S.D. (n = 4
462 from 4 independent experiments). * P < 0.05 versus A549 (unpaired two-tailed Student's t -test).

463 (E) Transmission electron microscope images of LB-like organelles in 3D culture. Arrows mark LBs.
464 Scale bars, 10 μ m.

465 (F) Volcano plot showing DEGs (P < 0.05 using DEseq2) between A549 and LB cells. Previously
466 reported AT2 markers are shown (n = 3 from 3 independent experiments).

467 (G) Heatmap of RNA-seq results for the top 300 DEGs between A549 and LB cells with primary AT2
468 cells (GSE66627). A549 cells and LB cells in 2D culture were subjected to RNA-seq.

469

470 **Figure 2. AMD induced LB abnormalities in LB cells**

471 (A) Schematic representation of the induction of aberrant LB by AMD in LB cells.

472 (B) Live cell imaging of TMR-PC in 2D culture. A549 and LB cells were treated with 10 μ M TMR-PC
473 and indicated concentration of AMD for 24 h in 2D culture and visualized with TMR-PC.
474 Quantification of the intensity of TMR-PC per cell count (mean $\pm$ S.D.; n = 3 from 3 independent
475 experiments). Scale bars, 10 μ m.

476 (C) Immunofluorescence staining of ABCA3 in LB cells. Cells were treated with DMSO or 10 μ M
477 AMD for 24 h in 2D culture and subjected to immunofluorescence analysis. Quantification of the

478 intensity of ABCA3 per cell count (mean $\pm$ S.D.; n = 3 from 3 independent experiments). * P < 0.05
479 versus DMSO-treated LB cells (unpaired two-tailed Student's t -test). Scale bars, 10 μ m.
480 (D) Transmission electron microscope images of LB-like organelles in 3D culture. Cells were treated
481 with DMSO or 25 μ M AMD for 12 days and DMSO for 3 days in 3D culture and analyzed under a
482 transmission electron microscope. Arrows mark lamellar bodies. Scale bars, 10 μ m.
483 (E) Schematic representation of high-content screening to identify chemicals that reverse
484 AMD-induced LB abnormalities in 2D cultured LB cells. Scale bars, 50 μ m.
485 (F) Validation of high-content screening. LB cells were treated with 10 μ M TMR-PC and DMSO or 10
486 μ M AMD for 24 h in 2D culture, followed by live cell imaging (n = 18 from 3 independent
487 experiments).

488

489 **Figure 3. High-content screening identifies HP β CD**

490 (A) Summary of the identification of high-priority compounds. Criteria for each step are shown in blue.
491 (B and C) Summary of the screening data. We performed high-content screening as described in Fig 2E
492 and identified compounds with >80% inhibition of TMR-PC/cell area in both runs as hits (red) (B).
493 Among the hits, HP β CD resulted in >80% retentions in cell counts in both runs (red) (C). Values are
494 shown as a percentage of AMD-induced increase of TMR-PC dots per cell area without compounds at
495 100% and the DMSO-induced ones at 0% (B) and AMD-treated cell number without compounds at
496 100% (C).
497 (D and E) Live cell imaging of TMR-PC in LB cells. Cells were seeded with the indicated
498 concentrations of HP β CD in 2D culture. After 24 h, cells were incubated with 10 μ M TMR-PC and
499 DMSO or 10 μ M AMD for another 24 h. Then, live cell imaging was performed. TMR-PC dots per cell
500 area are shown as a percentage of AMD-treated samples without compounds at 100% and

501 DMSO-treated ones at 0% (mean $\pm$ S.D.; n = 3 from 3 independent experiments, n = 1, 8 fields of
502 view). Scale bars, 50 μ m.

503 (F) Transmission electron microscope images of LB-like organelles in 3D culture. Cells were treated
504 with DMSO or 25 μ M AMD for 12 days and DMSO or 10 mg/mL HP β CD for 3 days in 3D culture and
505 observed under a transmission electron microscope. Arrows indicate LB-like organelles. Scale bars, 5
506 μ m.

507
508 **Figure 4. HP β CD corrects lipid homeostasis in AMD-treated LB cells.**

509 (A) M-A plot of significantly down- (fold-change < -1.2 and adjusted P < 0.05 using DEseq2) and
510 upregulated (fold-change > 1.2 and adjusted P < 0.05 using DEseq2) genes in blue and red,
511 respectively (n = 3 from 3 independent experiments). LB cells in 2D culture with DMSO or 10 μ M
512 AMD treatment were subjected to RNA-seq.

513 (B) Significantly enriched pathways for genes upregulated by AMD (fold change > 1.2 and adjusted P
514 < 0.05 using DEseq2) compared with levels in the DMSO vehicle control using Metascape.

515 (C) Unbiased PCA of RNA-seq data in DMSO-, AMD-, and AMD plus HP β CD-treated LB cells.

516 (D and E) Hierarchical clustering analysis of genes associated with PC2 (PC2 eigenvalue greater than
517 0.01 or less than -0.01) showing two gene signatures (black and blue) normalized by HP β CD (D).

518 Significantly enriched pathways related to the two gene signatures (black and blue) obtained using
519 Metascape (E). Pathways related to lipid function are indicated in red.

520

521 **Figure 5. HP β CD reverses AMD-induced LB abnormalities in human iPSC-derived AT2 cells**

522 (A) Schematic diagram of the effect of HP β CD on human iPSC-derived alveolar organoids.

523 (B and C) Flow cytometry analysis of human iPSC-derived alveolar organoids stained with

524 LysoTracker. Alveolar organoids including iPSC-derived AT2 cells (P2–P5) were treated with DMSO
525 or 25 μ M AMD from day 5 to day 11 and DMSO or 10 mg/mL HP β CD from day 11 to day 14. The
526 fluorescence intensity of LysoTracker was measured in each fraction (mean $\pm$ S.D.; n = 8 from 8
527 independent experiments). *, $\dagger P < 0.05$ versus DMSO-treated control or AMD-treated cells,
528 respectively (one-way ANOVA with Dunnett’s post hoc test).

529 (D) Quantification of PC in iPSC-derived EpCAM⁺ cells. iPSC-derived alveolar organoids (P2–P5)
530 were used in each condition, as indicated. Organoids were collected, and PC was quantified (mean $\pm$
531 S.D.; n = 3 from 3 independent experiments). *, $\dagger P < 0.05$ versus DMSO-treated control or
532 AMD-treated iPSC-derived EpCAM⁺ cells, respectively (one-way ANOVA with Dunnett’s post hoc
533 test).

534 (E) Transmission electron microscopy images of LB-like organelles. iPSC-derived alveolar organoids
535 were treated as indicated in Figure 5A. Scale bars, 5 μ m.

536

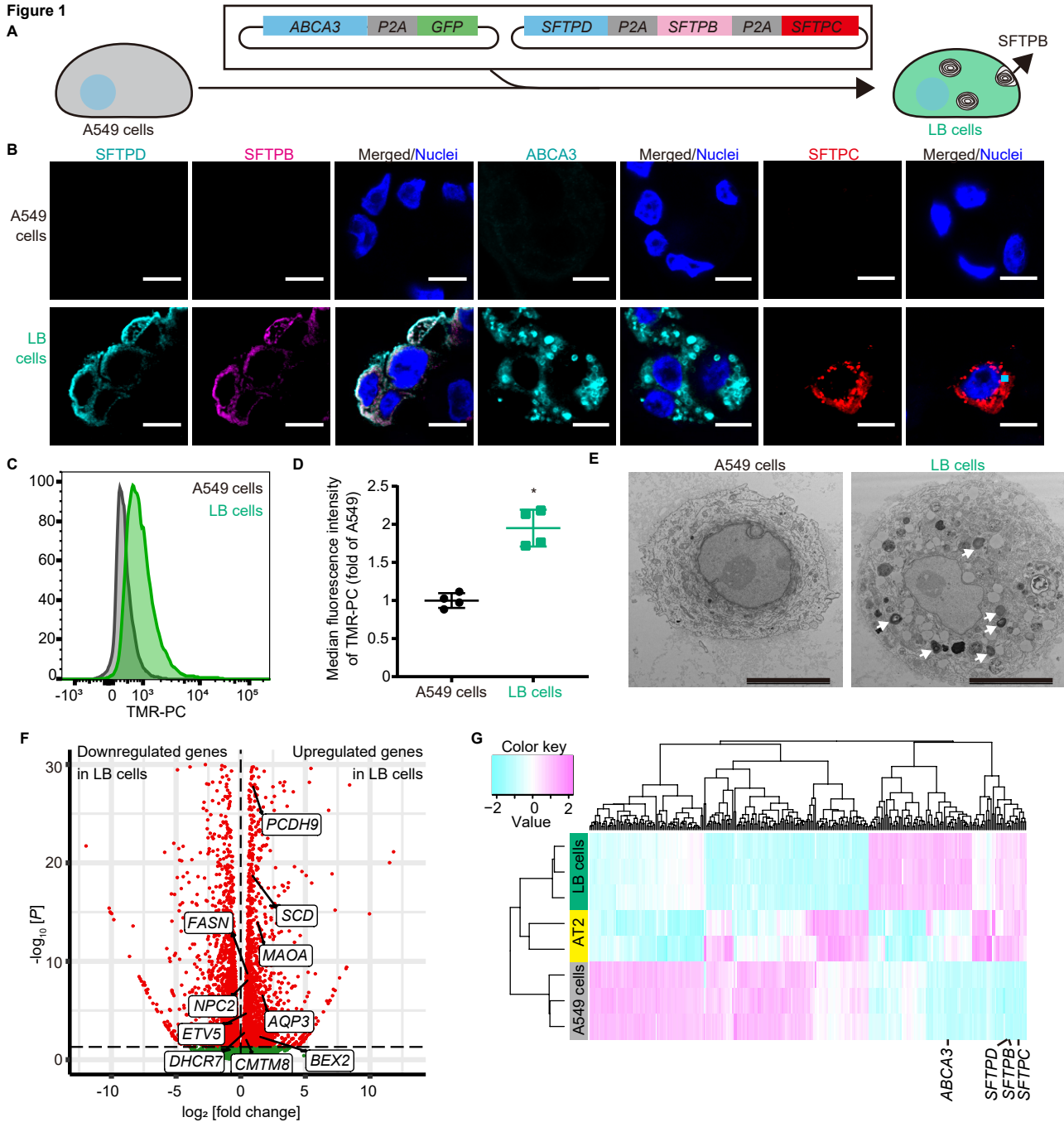


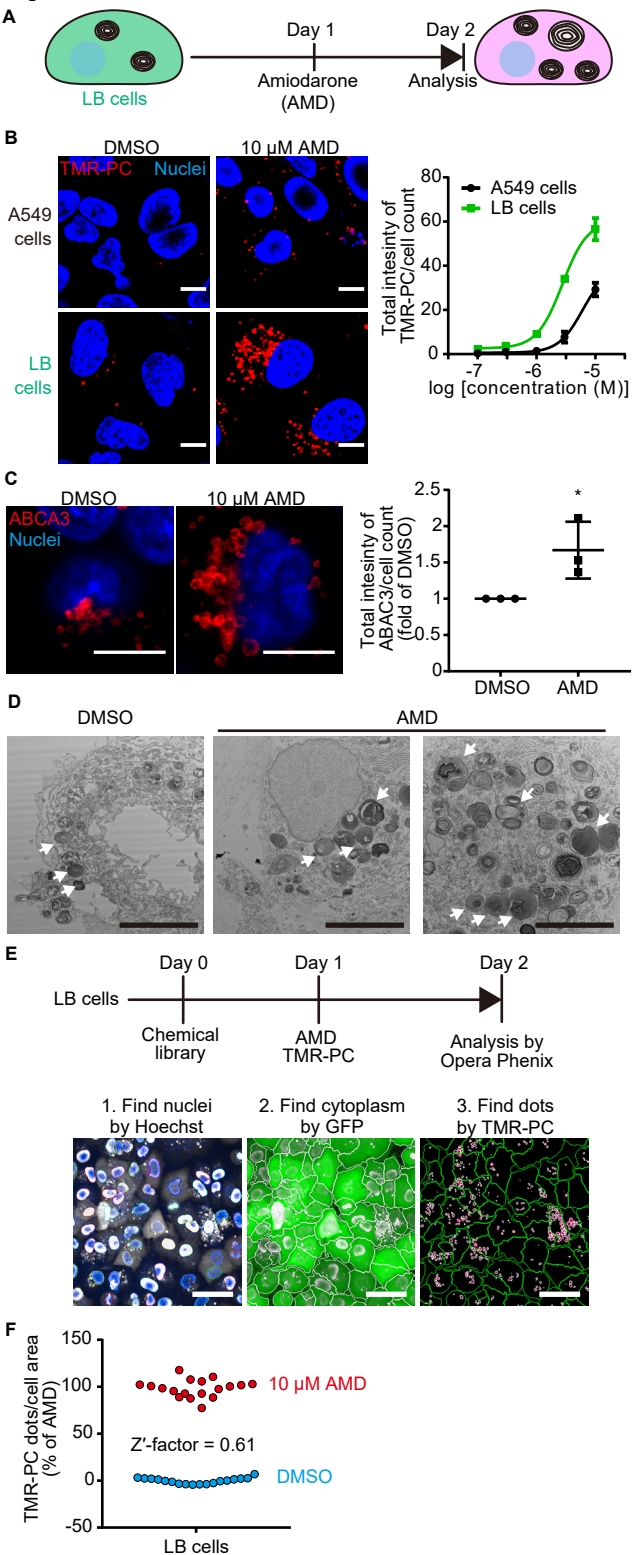
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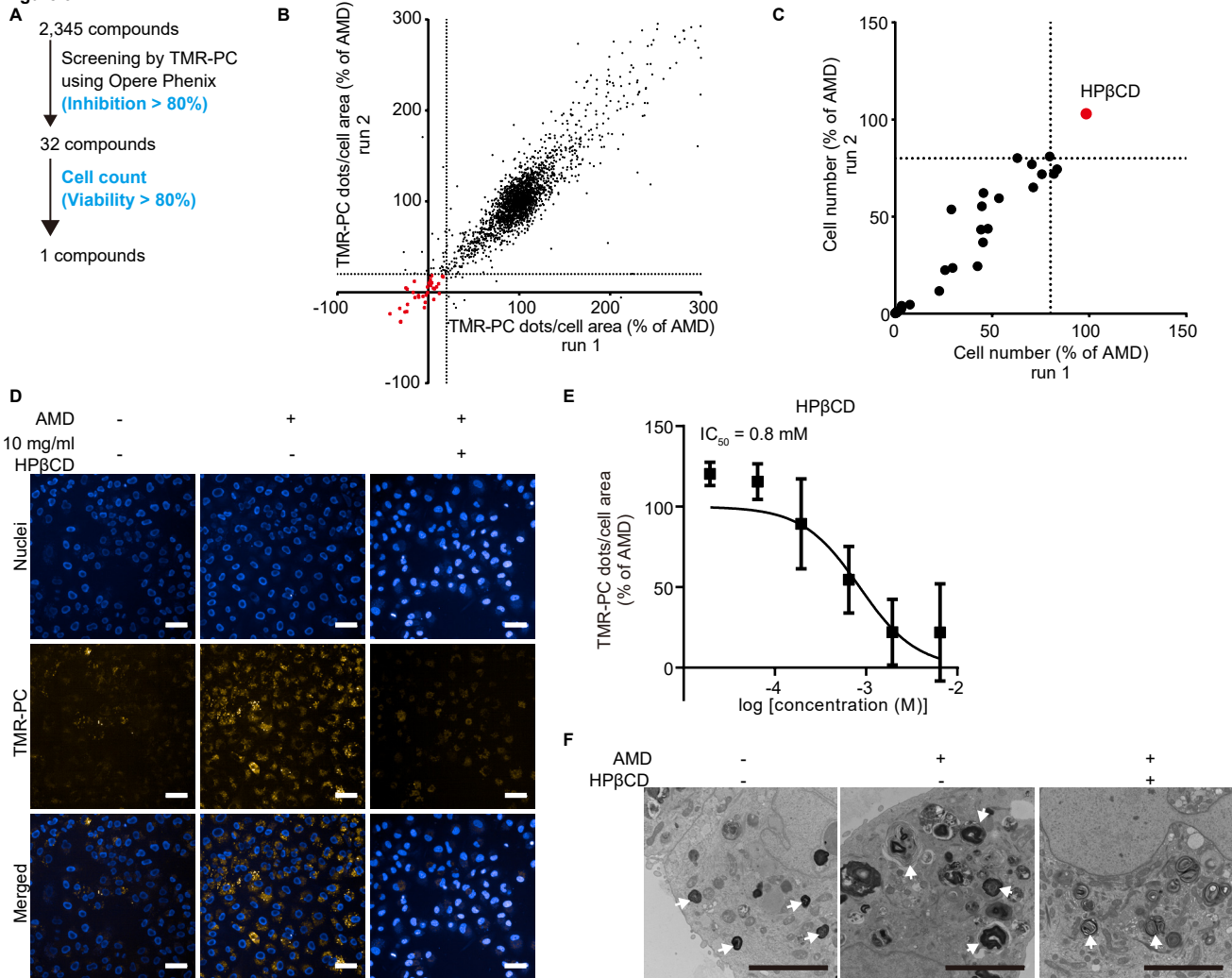
Figure 3

Figure 4

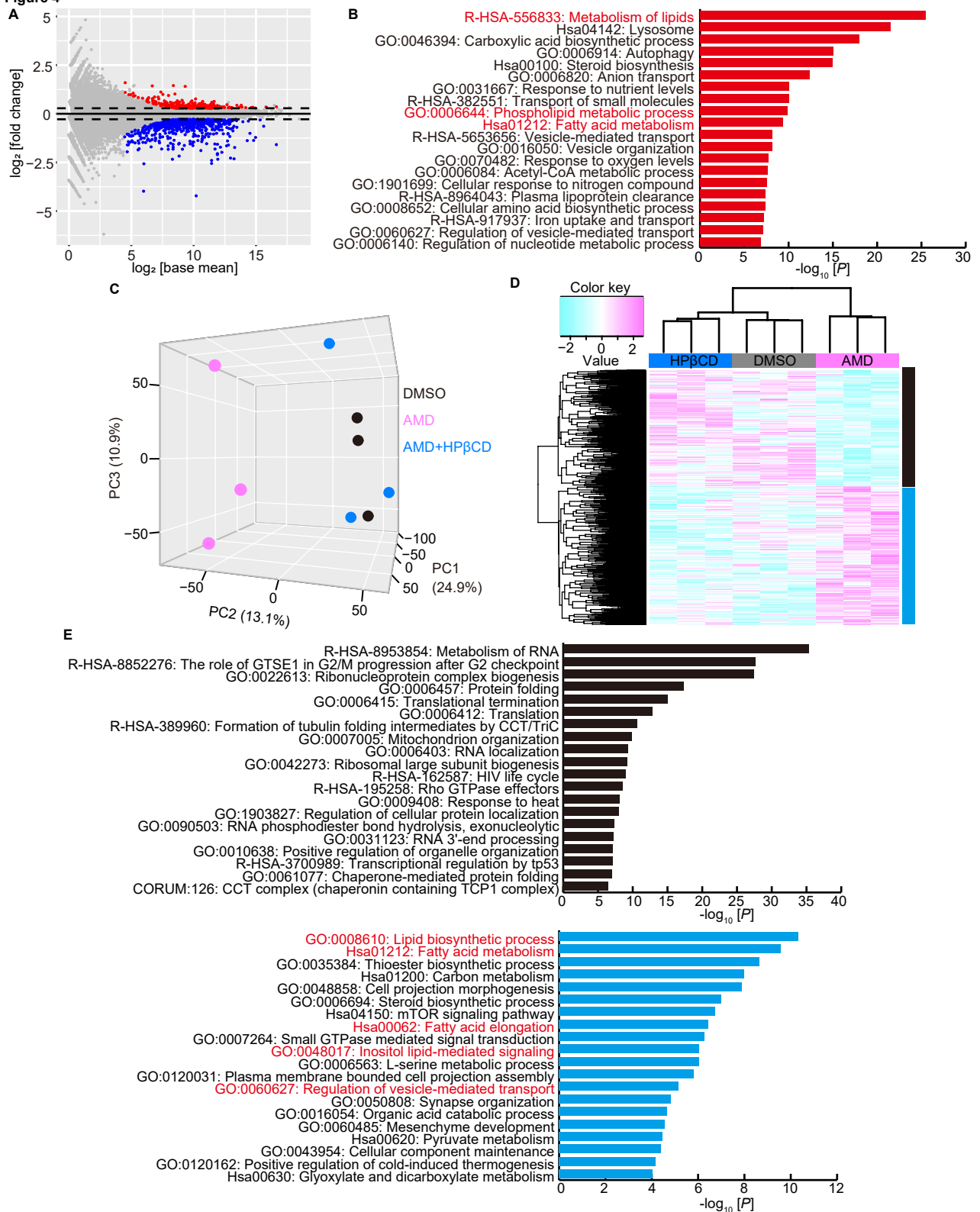


Figure 5