

Tai HR, Wang Z, Gong H, Han XJ, Zhou J, Wang XB, Wei XW, Ding Y, Huang N, Qin JQ, Zhang J, Wang S, Gao F, Chrzanowska-Lightowlers ZM, Xiang R, Xiao HY.

[Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence.](#)

Autophagy 2017, 13(1), 99-113.

Copyright:

This is an Accepted Manuscript of an article published by Taylor & Francis in *Autophagy* on 28/10/2016, available online: <http://www.tandfonline.com/doi/full/10.1080/15548627.2016.1247143>.

DOI link to article:

<https://doi.org/10.1080/15548627.2016.1247143>

Date deposited:

18/07/2017

Embargo release date:

28 October 2017



This work is licensed under a [Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International licence](https://creativecommons.org/licenses/by-nc-nd/4.0/)

1 **Autophagy impairment with lysosomal and mitochondrial**
2 **dysfunction is an important characteristic of oxidative**
3 **stress-induced senescence**

4

5 Haoran Tai^{1*}, Zhe Wang^{1*}, Hui Gong^{1*}, Xiaojuan Han¹, Xiawei Wei¹, Jiao Zhou¹, Xiaobo Wang¹,

6 Yi Ding¹, Ning Huang¹, Jianqiong Qin¹, Jie Zhang¹, Shuang Wang¹, Fei Gao², Zofia M.

7 Chrzanowska-Lightowlers², Rong Xiang³, Henry Xiao^{1#}

8

9 **Author affiliation:**

10 ¹Lab for Aging Research, Center of Gerontology and Geriatrics, State Key Laboratory of

11 Biotherapy & Collaborative Innovation Center of Biotherapy, West China Hospital, Sichuan

12 University, Chengdu 610041, China

13 ²Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle

14 University, Newcastle upon Tyne NE2 4HH, UK

15 ³Department of Clinical medicine, Medical School of Nankai University, Tianjin 300071, China

16 *These authors contributed equally to this work.

17 #To whom correspondence should be addressed.

18

19 **Abstract**

20 Autophagy has profound implications with aging. However, the true features of autophagy in
21 aging development remain to be clarified. In the present study, we explored the status of
22 autophagic flux during the development of cell senescence induced by oxidative stress. In this
23 system, with increased autophagic structures though, the degradation of SQSTM1/p62 protein,
24 the yellow puncta of mRFP-GFP-LC3 fluorescence and the activity of lysosomal proteolytic
25 enzymes all decreased in senescent cells, indicating impaired autophagic flux with lysosomal
26 dysfunction. The influence of autophagy activity on senescence development is confirmed by
27 both positive and negative autophagy modulators and mTOR dependent autophagy activators,
28 rapamycin and PP242, efficiently suppress cellular senescence through a mechanism relevant
29 to restoring autophagic flux. Moreover, by time phased treatment of cells with antioxidant
30 N-acetylcysteine (NAC), mitochondria uncoupler carbonyl cyanide m-chlorophenylhydrazone
31 (CCCP) and ambroxol, a reagent with the effect of enhancing lysosomal enzyme maturation,
32 we found that mitochondrial dysfunction plays a initiative role, while lysosomal dysfunction is
33 more directly responsible for autophagy impairment as well as senescence. Interestingly, the
34 effect of rapamycin on autophagy flux is linked to its role in functional revitalization of both
35 lysosomal and mitochondrial functions. Together, this study demonstrates that autophagy
36 impairment is crucial for oxidative stress-induced cell senescence, thus restoring autophagy
37 activity should be a promising way for senescence retardation.

38 **Key words:** autophagy, senescence, oxidative stress, mitochondria, lysosomes, rapamycin

39

40 **Abbreviations**

- 41 PBS = Phosphate buffered saline
- 42 SA- β -gal = Senescence associated β -galactosidase
- 43 SAHF = Senescence-associated heterochromatin foci
- 44 qRT-PCR = Real-Time Quantitative Reverse Transcription PCR
- 45 MTT = Methyl thiazolyl tetrazolium
- 46 ROS = Reactive oxygen species
- 47 DAPI = 4',6-diamidino-2-phenylindole
- 48 DMEM = Dulbecco's modified eagle medium
- 49 H₂O₂ = Hydrogen peroxide
- 50 SIPS = Stress induced premature senescence
- 51 GFP = Green fluorescent protein
- 52 mRFP = Monomeric red fluorescence protein
- 53 LC3 = Microtubule-associated protein 1 light chain 3
- 54 mTOR = Mammalian target of rapamycin
- 55 TFEB = Transcription factor EB
- 56 HCQ = Hydroxychloroquine
- 57 3-MA = 3-Methyladenine
- 58 VPA = Valproic acid
- 59 COX = Cytochrome c oxidase
- 60 CHX = Cycloheximide
- 61 NAC = N-acetylcysteine
- 62 MMP = Mitochondrial membrane potential

- 63 PtdIns3K = Phosphoinositide 3-kinase
- 64 UCP = Uncoupling protein
- 65 IL = Interleukin
- 66 CCCP = Carbonyl cyanide m-chlorophenylhydrazine
- 67 EBSS = Earle's balanced salt solution
- 68 ULK = Unc-51-like kinase
- 69 UPR^{mt} = Mitochondrial unfold protein response

70

71 **Introduction**

72 Aging is a complicated process with its mechanism remains to be explored. Cellular

73 senescence happens during aging development and is widely used as *in vitro* model for aging

74 research. Stress-induced premature senescence (SIPS) develops faster than replicative

75 senescence and has been established for studying the influence of extracellular or intracellular

76 stress on aging process.¹ Known features of senescent cells include flat and enlarged cellular

77 morphology, increased senescence associated β -galactosidase (SA- β -gal) activity, the

78 appearance of senescence-associated heterochromatin foci (SAHF), cell growth arrest and

79 senescence-associated secretory phenotype (SASP) such as IL-6 and IL-8 secretion.²⁻⁶

80 Autophagy plays cytoprotective roles by turnover long lived proteins and scavenging damaged

81 cellular components. Among different types of autophagy, macroautophagy is the most

82 studied one and is composed of two stages: the early stage acting for the formation of

83 autophagosome with capturing damaged cellular molecules and organelles; the late stage

84 responsible for the digestion of entrapped components within autolysosome formed by the

85 fusion of autophagosome and lysosome.^{7, 8} The updated consensus appeals that the real
86 status of autophagy should be assessed not only by the number of autophagosomes and
87 autolysosomes but also by evaluating the actual autophagic flux, such as monitoring the
88 clearance of cell components in autolysosomes.^{9, 10}

89 The relationship between autophagy and senescence is still inconclusive.¹¹ Some studies
90 reported that autophagy is positively correlated with senescence, with increased autophagy
91 activity in senescent cells.^{12, 13} On the other hand, there are reports showing opposite view.
92 For example, Kang *et al.* reported that autophagy impairment induces premature senescence
93 in primary human fibroblasts.¹⁴ Moreover, it is known that rapamycin, an mTOR inhibitor and
94 an autophagy activator, could extend the lifespan of mice and be a potential anti-aging drug.¹⁵

95 The free radical theory of aging emphasizes the reactive oxygen species (ROS)-induced
96 cellular damages for aging development.¹⁶ Although ROS are physiologic signaling molecules,
97 excessive produced ROS in cells under stressful situations, happened either naturally or
98 artificially, are detrimental to cell components and homeostasis.¹⁷ As mitochondria are primary
99 source of ROS and primary target for ROS damage, their functional status is closely linked
100 with aging development.^{18, 19} In fact, age-related changes in cells are associated with declined
101 mitochondrial function, accompanying with increased ROS and decreased ATP.²⁰

102 To clarify the relationship between autophagy and senescence, the features of oxidative
103 stress-induced senescent fibroblasts are characterized in the present study, with particular
104 focusing on the status of autophagy flux and its role in senescence development. As an
105 attempt for analyzing underlying mechanisms, we also closely concerned the influence of
106 lysosomal and mitochondrial dysfunction on autophagy impairment, together with the effect of

107 autophagy modulators on autophagy flux restoration and cellular senescence attenuation.

108

109 **Results**

110 **H₂O₂ treatment induces cellular senescence with intracellular ROS elevation**

111 First, we utilized a method involving 45 min incubation of NIH3T3 cells in suspension with 400

112 μ M of H₂O₂ treatment to establish SIPS cell model and monitored the progress of cellular

113 senescence up to 7 days after H₂O₂ treatment. As shown in Fig. 1A, H₂O₂-treated cells

114 gradually became enlarged, flattened and most of these cells were SA- β -gal staining positive

115 after 3 days. SAHFs were also evident in H₂O₂-treated cells as large nuclei and irregularly

116 shaped puncta appeared (Fig. 1B). In addition, we observed apparent cell growth arrest (Fig.

117 1C), as well as elevated expressions of p53 protein and *Cdkn1a* (P21), *Il6* mRNAs in

118 H₂O₂-treated cells (Fig. 1D-F). H₂O₂ treatment also triggered subsequent intracellular ROS

119 elevation (Fig. 1G). Besides, in MRC-5, a human lung fibroblast cells, we also successfully

120 induced cellular senescence by using the same method (Fig. S1). These results collectively

121 demonstrate that in fibroblasts, the H₂O₂ treatment protocol we used could reliably induce

122 cellular senescence in 3 to 5 days and the following experiments were mostly done in 3 or 5

123 days after H₂O₂ treatment.

124

125 **Autophagic structures increase but autophagic flux impairs in senescent cells**

126 The biological relevance of autophagy and senescence has been under investigation.^{21, 22} To

127 elucidate the status of autophagy during SIPS, autophagic structures were observed by

128 transmission electronic microscope and an apparent increase in the number of vacuole or

129 vesicular-like structures in the cytoplasm of senescent cells was found (Fig. 2A). To
130 characterize the increased vacuole or vesicular-like structures, LysoTracker Red was used to
131 stain lysosomes or autolysosomes, leading to the observation that lysosomal structures
132 greatly increased in H₂O₂-treated cells (Fig. 2B). Moreover, in a NIH3T3 cell line stably
133 expressing mRFP-LC3, the punctate LC3 distribution was observed from day 1 after H₂O₂
134 exposure, with an apparent increase at day 3 and day 5. These results show the increase of
135 autophagic structures in H₂O₂-induced senescent cells.

136 Given the accumulation of autophagic structures can be resulted from either increased
137 autophagic induction or decreased autophagic degradation,^{9, 10} we measured autophagic flux
138 in our model. First, endogenous SQSTM1/p62 protein was examined as it mainly degraded
139 through autophagic pathway.²³ The SQSTM1 protein in H₂O₂-treated cells increased
140 apparently at day 3 and day 5 compared to control cells (Fig. 2D). Then, to justify the increase
141 of SQSTM1 protein is due to increased synthesis or decreased degradation, a
142 degradation-blocking experiment was conducted using hydroxychloroquine (HCQ), a reagent
143 which elevates lysosomal pH and inhibits lysosomal degradation. As shown in Fig. 2E,
144 different from the apparent increase of SQSTM1 protein in control cells caused by HCQ
145 treatment, the level of SQSTM1 protein kept almost unchanged in H₂O₂-treated cells,
146 suggesting that lysosomal degradation capacity in H₂O₂-treated cells was low enough to
147 compare to that caused by HCQ treatment. Next, we tested the half-life of SQSTM1 protein by
148 inhibiting protein synthesis with cycloheximide (CHX), and found that SQSTM1 protein in
149 H₂O₂-treated cells degraded much slower than that in control cells, with the half-life being
150 around 12 h and 7 h in H₂O₂-treated and control cells, respectively (Fig. 2F). Furthermore,

151 using NIH3T3 stably expressing tandem mRFP-GFP-LC3,²⁴ we found that while EBSS
152 starvation induced puncta like distribution of LC3 protein accompanied with the quenching of
153 green fluorescence, H₂O₂ treatment did not (Fig. 2G). These results collectively indicate that
154 impairment of autophagic flux happened in H₂O₂ induce senescent cells.

155

156 **Chronic blockage of autophagic flux is sufficient to induce cellular senescence**

157 To verify the relationship between autophagy and cellular senescence, we tried to assess
158 whether the impairment of autophagic flux could induce cellular senescence. When normal
159 NIH3T3 cells were treated with sublethal dose of HCQ and leupeptin, two lysosome inhibitors
160 capable of blocking autophagic degradation,¹⁰ the cells exhibited increased intracellular ROS
161 (Fig. 3A). In parallel, SA-β-gal positive cells and *l6* mRNA expression also increased
162 apparently after 7 days (Fig. 3A & 3B). Moreover, we used shRNA-mediated *Atg5* knockdown
163 that inhibited autophagy in NIH3T3 (Fig. 3C & 3D). We found that SA-β-gal positive cells are
164 conspicuous in *Atg5* gene silenced cells rather than in control shRNA cells that both
165 experienced a 15 days culturing without H₂O₂ treatment (Fig. 3E). As another senescence
166 marker, *l6* mRNA expression also increased apparently in *Atg5* knockdown cells (Fig. 3F).
167 These results indicate that chronic autophagy impairment could induce cellular senescence.

168

169 **mTOR dependent autophagy activators attenuate senescence in a manner related to** 170 **autophagy flux restoration**

171 We further investigated how autophagy regulators affect cell senescence. Seven different
172 autophagy regulators, including 4 activators and 3 inhibitors were tested for their influences on

173 SIPS. The concentrations of these chemical compounds were determined by preliminary
174 experiments. As shown in Fig. 4A, rapamycin and PP242, two mTOR-dependent autophagy
175 activators, showed a potent anti-senescent effect as they dramatically reduced the percentage
176 of SA- β -gal positive cells; on the other hand, two mTOR-independent autophagy activators,
177 valproic acid (VPA) and LiCl, showed minor influence on the senescent state of H₂O₂-treated
178 cells (Fig. 4A). As to autophagy inhibitors, bafilomycin A1 and HCQ, two reagents blocking
179 lysosomal function, increased SA- β -gal positive cells in H₂O₂-treated cells (Fig. 4B), while
180 3-methyladenine (3-MA), an autophagy inhibitor targeting to phosphoinositide 3-kinase
181 (PtdIns3K) which influences autophagy at early stage,²⁵ did not (Fig. 4B). Further
182 investigations were performed using two representative reagents, rapamycin and bafilomycin
183 A1. As shown, their regulatory role in senescence tested by the *p16* mRNA level was in
184 accordance with SA- β -gal staining (Fig. 4C). To confirm if the role of these reagents in SIPS
185 development is relevant to autophagy regulation, we tested whether autophagy flux is
186 influenced by these two reagents in our model. Using NIH3T3 cells stably expressing tandem
187 mRFP-GFP-LC3, we found that rapamycin increased while bafilomycin A1 decreased the ratio
188 of red/yellow puncta in H₂O₂-treated cells, showing the restoration and aggravation of
189 autophagic flux, respectively (Fig. 4D). In addition, the abundance of SQSTM1 decreased in
190 rapamycin treated cells and increased in bafilomycin A1 treated cells at day 3 after H₂O₂
191 treatment (Fig. 4E). The effects of rapamycin and bafilomycin A1 on SIPS were similar in
192 human fetal lung fibroblast cell line MRC-5 as in NIH3T3 cells (Fig. S1). These results
193 demonstrate that rapamycin is an efficient anti-senescence reagent that can act through
194 up-regulating autophagic flux in our system.

195

196 **Lysosomal and mitochondrial dysfunction happens during SIPS development**

197 Since the capacity of lysosomal degradation is a rate-limiting factor for autophagic flux,^{26, 27} we
198 assessed the function of lysosomes in cells. We found that the activity of lysosomal acid
199 phosphatase decreased in senescent cells (Fig. 5A) and the size of lysosomes increased in
200 these cells (Fig. 5B).²⁸ Moreover, the abundance of cathepsin B, a representative protease in
201 lysosome, reduced in senescent cells (Fig. 5C), concurrently with a decline of cathepsin B
202 activity measured by Magic Red Cathepsin B kit (Fig. 5D). Taken together, these results reveal
203 that the oxidative stress-induced impairment of autophagic flux in our senescent cells is
204 closely associated with reduced degradation ability of autolysosomes and lysosomes.
205 As damaged or dysfunctional mitochondria have been connected to senescence,^{18, 20} we
206 functionally assayed mitochondria in our system. We found that mitochondrial DNA
207 actually increased after H₂O₂ treatment. This increase also happens to mitochondrial DNA
208 integrity, although H₂O₂ indeed decreased its integrity right after treatment (Fig. S2). These
209 results indicate that mitochondria biogenesis is activated during the development of
210 senescence. However, the function of mitochondria is not activated, since mitochondrial
211 depolarization was detected in H₂O₂-treated cells (Fig. 5E), with a decrease of both cellular
212 ATP content (Fig. 5F) and mitochondrial cytochrome c oxidase (COX) activity (Fig. 5G). To
213 clarify the role of mitochondria in SIPS, a mitochondrial uncoupling reagent carbonyl cyanide
214 m-chlorophenylhydrazine (CCCP) was used in the experiment.¹⁹ As shown, when cells were
215 pretreated with CCCP, the mitochondrial content remains stable (Fig. S3). However, the
216 intracellular ROS generation after H₂O₂ treatment significantly reduced (Fig. 5H), coinciding

217 with attenuated cellular senescence revealed by SA- β -gal staining (Fig. 5I) and *IL6* mRNA
218 expression (Fig. 5J). These results indicate the important role of dysfunctional mitochondria
219 derived ROS in SIPS development.

220 The importance of intracellular ROS on the dysfunction of lysosomes and autophagy in our
221 senescent cells was further proved by another SIPS model established by treatment of
222 pyocyanin, a reagent inducing intracellular ROS production.²⁹ We found that both ROS and
223 cellular senescence were induced by pyocyanin in a dosage dependent manner (Fig. S4).

224 Under this kind of oxidative stress, the decrease of lysosomal acid phosphatase activity and
225 the increase of SQSTM1 protein were also prominent (Fig. S5). Consistently, the treatment of
226 cells with antioxidant NAC attenuated the senescence induced by pyocyanin (Fig. S6).

227

228 **Asynchronous dysfunction of mitochondria and lysosomes during SIPS development**

229 To explore the mechanistic relationship between lysosomal and mitochondrial dysfunction
230 during SIPS development, the time sequenced functional assays for these two organelles
231 were conducted. First, we tested the effect of antioxidant NAC on the development of SIPS.

232 Although SIPS did not be rescued from co-treatment of NAC and H₂O₂, it was weakened when
233 NAC was included in culture medium after H₂O₂ treatment (Fig. S7), indicating that
234 intracellular ROS induction after H₂O₂ treatment might be a crucial event for SIPS
235 development. This possibility was confirmed by time phased detection for cellular ROS and we
236 found an increase in ROS level following H₂O₂ treatment, peaking at 4 to 8 h (Fig. 6A),
237 accompanying with mitochondrial depolarization (Fig. S8). On the other hand, different from
238 this implication of mitochondria dysfunction in the early phase of SIPS development, the sign

239 of lysosomes dysfunction appeared much later, showing as the activity of cathepsin B did not
240 decrease, but slightly increased, within the first 24 h after H₂O₂ treatment (Fig. 6A). Prolonged
241 experiments showed that cathepsin B activity began to decrease from day 3 after H₂O₂
242 treatment, together with a further elevation of ROS production (Fig. 6B). Importantly, NAC
243 application did increase lysosomal cathepsin B activity in our system (Fig. 6C), suggesting a
244 possible causal relationship between dysfunctional mitochondria derived ROS overproduction
245 and lysosomal dysfunction. This temporal sequenced dysfunction of lysosomes and
246 mitochondria in our system was further revealed by using NAC, CCCP and ambroxol, the last
247 one acts as an enhancer of lysosome enzyme maturation.^{30,31} After the suppressive effect of
248 CCCP on ROS overproduction and the promoting effect of ambroxol on lysosomal cathepsin B
249 activity were confirmed (Fig 6D and 6E), we applied these reagents at 24 h duration after H₂O₂
250 treatment. As the results, NAC showed an obvious anti-senescence effect when applied in the
251 first 48 h after H₂O₂ treatment (Fig. 6F), while the effect of CCCP was only seen when applied
252 within the first 24 h (Fig. 6G). Interestingly, the anti-senescence time window of ambroxol was
253 the longest, prolonging up to 48-72 h (Fig. 6H). These results suggest that, mitochondria
254 dysfunction precedes lysosomes dysfunction during SIPS development.

255

256 **Autophagy modulators regulate the function of mitochondria and lysosomes in** 257 **senescent cells**

258 Then, we asked if the influence of autophagy modulators on cellular senescence is relevant to
259 the function of mitochondria and lysosomes, which might be helpful to provide the ground for
260 developing new interventions against aging. With respect to mitochondrial function, we found

261 that mitochondrial membrane potential (MMP) was elevated by rapamycin and lowered by
262 bafilomycin A1 (Fig. 7A). Accordingly, rapamycin substantially decreased intracellular ROS
263 and increased intracellular ATP while bafilomycin A1 did the opposite (Fig. 7B & 7C). As to
264 lysosomal function, we found that rapamycin apparently increased the expression of cathepsin
265 B (Fig. 7D), as well as the activity of this enzyme in senescent cells (Fig. 7E). Collectively, our
266 results provide novel evidence for the influence of autophagy modulators on senescence,
267 specifically revealing the effect of autophagy activator rapamycin on protecting mitochondria
268 and on activating lysosome related degradation.

269

270 **Discussion**

271 In this study, we addressed the functional role of autophagic flux in the development of
272 oxidative stress-induced cellular senescence. The main findings are: 1. autophagic flux
273 impaires in our SIPS cells; 2. mitochondrial dysfunction and related ROS production, are
274 initiative while subsequently lysosome dysfunction is directly responsible for autophagy
275 impairment and senescence development; 3. the effect of mTOR-dependent autophagy
276 activator rapamycin on senescence prevention associates with its role in the restoration of
277 autophagic flux as well as mitochondrial and lysosomal function. Our findings provide novel
278 evidence to demonstrate that autophagy activation is a promising strategy to counteract
279 cellular senescence or even aging, and are supportive for the idea that the interplay between
280 mitochondria and lysosomes plays an important role in the maintenance of cell health, herein
281 should be a reasonable intervention point for anti-aging intervention.

282 In recent years, the relationship between autophagy and senescence is a hot topic with

283 contradictory conclusions existed in previous reports.¹¹ Although many factors, such as cell
284 type, the kind of inducer, stress intensity and measuring time point, can impact the conclusion
285 about autophagy status, we consider the methods and indexes used for evaluating autophagy
286 activity should be carefully considered. For this reason, instead of emphasizing the increase in
287 the quantity and size of autophagic structures, which can be caused by the alterations either in
288 autophagic structure formation or in autophagic degradation,¹⁰ we particularly evaluated the
289 status of autophagic flux during SIPS development. By utilizing functional measurements,
290 particularly those working on the assessment of autolysosomal protein degradation, we get
291 chance to ensure that autophagy impairment is positively correlated with senescence
292 development. This result is in accordance with some other reports.^{14, 32, 33} We also noticed that
293 opposite correlation is demonstrated in some researches. For example, it is reported that
294 inhibition of autophagy by knocking down Atg5 or Atg7 in a tumor cell line suppressed the
295 induction of senescence.¹² We consider that the reasonable interpretation for these
296 inconsistent might lie on the distinct metabolic characteristics of cells and senescence
297 induction approaches used by different studies.

298 The restoration of autophagic flux is an attractive concept for anti-aging therapeutics.
299 Consistent with previous studies,^{34, 35} our study confirmed the effectiveness of autophagy
300 activation on senescence prevention. Interestingly, we found that different autophagy
301 activators and inhibitors have different influences on SIPS. Two mTOR-dependent autophagy
302 activators, rapamycin and PP242, exhibited obvious anti-senescence effect, while
303 mTOR-independent/PtdIns3K-dependent ones, VPA and LiCl, showed minor influence. These
304 results not only confirmed the crucial role of autophagic flux in SIPS development but also

305 revealed the effectiveness of mTOR inhibition on SIPS repression.

306 The connection between mTOR inhibition and autophagy activation has not been established

307 completely. Previous interpretation emphasizes the role of mTOR inhibition in the restoration of

308 ULK activity which influences the early stage of autophagy.³⁶ Recent evidence show that

309 mTOR also regulates autophagic flux by influencing the late stage of autophagy.^{28, 37} However,

310 the role of mTOR in autophagic flux regulation has not yet been studied in senescent cells. In

311 the present study, the effect of rapamycin-mediated mTOR inhibition on autolysosomal activity

312 is proved as rapamycin increased the expression and revived the activity of cathepsin B during

313 SIPS (Fig. 7D & 7E). This is inconsistent with a previous report which states that mTOR

314 suppression by PP242/Torin induced lysosomes activation but not rapamycin.³⁸ In our

315 consideration, this inconsistency might result from the different concentrations of rapamycin

316 used in two experiments: that they used is 10 folds higher (1 μ M) than we used (100 nM). It

317 has been reported that high concentration of rapamycin could elevate the pH of lysosomes,

318 thus covering up its role in lysosomal activation through mTOR suppression.³⁹ The situation

319 about mTOR downstream protein TFEB, an essential transcription factor for

320 autophagy-related genes, is under pursuing in our system. We surprisingly found that, TFEB

321 localized in nucleus of H₂O₂ treatment-induced senescent cells, either rapamycin was added

322 or not; however, the transcription of TFEB-targeted lysosomal genes, such as *Gns* and *Lamp1*,

323 was not activated.⁵¹ These data provide two open questions: If mTOR could regulate the

324 function of autolysosomes through a way independent of TFEB? If TFEB could be functionally

325 regulated in a manner independent of mTOR? Intensive investigations are needed to clear

326 these questions.

327 Aspect of the importance of mitochondria dysfunction on SIPS induction, evidence obtained in
328 the present study, showing as elevated ROS generation, the protective role of NAC and
329 pyocyanin-promoted SIPS development. Importantly, the pretreatment of cells with CCCP
330 alleviated ROS production along with attenuated development of SIPS. In fact, the imperative
331 role of mitochondria in cellular senescence was i evidenced by Clara Correia-Melo *et al.*
332 recently when this manuscript in preparing, They found that mitochondria depletion reduced a
333 spectrum of senescence effectors and phenotypes while preserving ATP production and cell
334 survival via enhanced glycolysis.⁴⁰ Their work is inspiring because it affirmed the concept that
335 mitochondria should be a major putative therapeutic target for interventions impacting on the
336 senescent phenotypes because of their undesired role in cellular senescence. Our time course
337 experiments are supportive for this concept, by having the data about enhanced ROS
338 generation with the increased mtDNA content during SIPS development (supplementary data),
339 The increase in mtDNA indicates that although experienced an injury and became
340 dysfunctional within hours after H₂O₂ treatment, mitochondria content in cells actually
341 increased afterwards. For the mechanism about the biogenesis of mitochondria upon stress
342 loading, a recent published article concerned the role of mitochondrial unfolded protein
343 response (UPR^{mt}).⁵² Whether UPR^{mt} is responsible for the increase of mitochondrial
344 biogenesis during SIPS needs to be investigated in future studies. It is worthy of noting here
345 that using CCCP weakened ROS generation and SIPS development in our system. This result
346 matches the protective role of UCP protein under stress.⁵³ Our results are also consistent with
347 the “uncoupling to survive” theory,⁴¹ which suggests that partially uncoupling of mitochondria
348 under stressed condition may be beneficial for SIPS prevention.

349 The interplay between mitochondria and lysosomes in cellular senescence is attractive but yet
350 proved. As the attempt to clarify this issue, time-dependent alterations of the function of these
351 two organelles were investigated in this study. What we understand upon this investigation are:
352 1. The biogenesis of both mitochondria and lysosomes is activated during the development of
353 SIPS, but the function of newly generated organelles is compromised comparing to that in
354 proliferating cells; 2. the dysfunction of mitochondria happens within hours after H₂O₂
355 treatment, whereas that of lysosomes happens days later, so that the former seems to be an
356 initiative event for senescence development and to play as a trigger of autophagy impairment;
357 3. although the dysfunction of lysosomes happening subsequently after the dysfunction of
358 mitochondria, the consistency of its dysfunction with autophagy impairment indicates its
359 importance role for SIPS development; 4. the influence of lysosomes on mitochondria should
360 not be neglected, as the inhibitors of lysosomes induced intracellular ROS generation (Fig. 3A),
361 and rapamycin alleviated the dysfunction of mitochondria (Fig. 7A-C). Our results are
362 supportive for the mitochondrial-lysosomal axis theory of aging proposed by Brunk and
363 Terman,⁴³ as the drugs ameliorate the function of each of these organelles exhibit potent
364 anti-senescence effect. Upon this finding, we believe that a combined treatment targeting both
365 mitochondrial and lysosomes should be beneficial for aging-related diseases, such as
366 neurodegenerative and cardiovascular diseases.

367 In conclusion, this study provided functional evidence showing the occurrence of autophagy
368 impairment in H₂O₂-induced senescent cells and emphasized the characteristics of the
369 dysfunction of autolysosomes/lysosomes for SIPS development. By exploring the initiative role
370 of intracellular ROS and mitochondria malfunction during SIPS development, interplay

371 between mitochondria and autophagic flux was discovered. Furthermore, this study revealed
372 the efficiency of mTOR inhibition-mediated autophagy restoration on SIPS prevention,
373 expanded our understanding on the relationship between autophagy and senescence as well
374 as the possible mechanisms about the role of rapamycin in aging prevention. Further study is
375 needed to understand the specific underlying mechanism that how impaired autophagy relates
376 to senescence and to open new way for developing safe and effective strategy against aging.

377

378 **Materials and Methods**

379 **Reagents and antibodies**

380 Rapamycin (R0395), VPA (P4543), LiCl (203637), bafilomycin A1 (B1793), HCQ (H0915),
381 3-MA (M9281), NAC (A7250) CHX (C7698) and pyocyanin (P0046) are from Sigma. PP242 is
382 from Cayman Chemical Company (13643). Leupeptin is from Amresco (J580). 2',7'-dichlor-
383 fluorescein-diacetate (DCFH-DA) is from Applygen (C1300). JC-1 and CCCP are from
384 Beyotime (C2006). Ambroxol hydrochloride is from Boehringer Ingelheim (Mucosolvan®, 2 ml :
385 15 mg). Anti-ACTB (sc-47778), anti-SQSTM1 (sc-28359) and anti-CSTB antibodies (sc-6493)
386 are from Santa Cruz Biotechnologies. Anti-TP53 antibody is from Cell Signaling Technology
387 (#9282).

388

389 **Cell culture and treatments**

390 The murine fibroblast NIH3T3 and human fetal lung fibroblast MRC-5 cell line were obtained
391 from Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences and

392 cultured in a complete medium (Dulbecco's Modified Eagle Medium supplemented with 10%
393 fetal bovine serum) in a humidified incubator at 37 °C and 5% CO₂.

394 For monitoring cell proliferation status, normal, H₂O₂-treated and starved cells were inoculated
395 in 6-well plates in 3 replicates for each time point and harvested by trypsinization at 24 h
396 intervals for 7 days. The numbers of cells were counted under microscope.

397 For senescence induction, growing cells at about 80% confluence were collected into an
398 Eppendorf tube after trypsinization, suspended in PBS with 1×10⁶ cells/ml density and
399 exposed to 400 μM H₂O₂ at 37 °C. During H₂O₂ exposure, the tube was turned upside down
400 gently 5-10 times with a 5 minutes interval. After 45 min, cells were washed with PBS once
401 and resuspended with complete medium. The control cells are treated with PBS at the same
402 time. Then both control cells and H₂O₂-treated cells were split into individual wells in culture
403 plate with a density about 2×10⁴ cells/cm² and cultured with complete medium for various
404 durations as indicated in individual experiments. After treatment, the media of both control and
405 H₂O₂-treated cells were changed every 3 days.

406 To evaluate the effect of activators and inhibitors of autophagy activity on SIPS processing,
407 different chemical reagents were added into culture medium after H₂O₂ treatment, with their
408 concentrations indicated as in the figure legends. These concentrations were determined by
409 preliminary experiments. The stock solutions of rapamycin and bafilomycin A1 were dissolved
410 in DMSO, those of PP242, VPA, LiCl, HCQ and 3-MA in PBS, respectively.

411

412 **SA-β-gal staining**

413 SA-β-gal activity was determined using SA-β-gal staining kit (Beyotime, C0602) according to

414 standard protocol.³ Senescent cells were identified as bluish green stained cells by
415 microscopy. More than 500 cells in 6 random fields were counted to determine the percentage
416 of SA- β -gal-positive cells in total cells.

417

418 **SAHF detection**

419 Cells were fixed *in situ* with 4% paraformaldehyde and washed by PBS. DAPI (Beyotime,
420 C1006) at 300 nM concentration in PBS was added for 5 min incubation. The cells were then
421 washed 3 times by PBS, drained and mounted. DAPI-stained nuclei with blue fluorescence
422 were viewed by fluorescence microscope.

423

424 **Western blot analysis**

425 Whole cell lysates were prepared by directly denaturing cell pellets in 2 \times SDS loading buffer,
426 and then boiling for 10 min. Western blotting assays were done as described previously.⁴⁴
427 Anti-TP53, anti-SQSTM1, anti-CSTB antibodies were used as primary antibodies. Every
428 experiment was repeated 3 times, and representative data were shown.

429

430 **Real-time quantitative reverse transcription PCR (qRT-PCR)**

431 Total RNA extraction, reverse transcription and real-time PCR amplification were performed
432 as described previously.⁴⁵ PCR primers for *Cdkn1a* gene are 5'-GTGGCCTTGTCGCTGTC
433 TT-3' (forward) and 5'-GCGCTTGGAGTGA-3' (reverse), for *Il6* gene are 5'-ACTCACCTCT
434 TCAGAACGAATTG-3' (forward) and 5'-CCATCTTTGGAAGGTTTCAGGTTG-3' (reverse), for
435 *18s* rRNA are 5'-TTGACGGAAGGGCACCACCAG-3' (forward) and 5'-GCACCACCACCCAC

436 GGAATCG-3' (reverse). The experiments were triplicated, and the data about the *Cdkn1a* and
437 *Irf6* genes were adjusted by the values for *18s* gene and shown as relative fold changes
438 against control.

439

440 **Electron microscopy**

441 PBS washed cells were collected to Eppendorf tubes by cell scrapers. After centrifuging, 4%
442 paraformaldehyde was loaded carefully on cell pellets for an overnight fixation at 4 °C. Fixed
443 cells were further treated and sliced, and then the images of their ultrastructure were recorded
444 under a transmission electron microscope.

445

446 **Lysosome labeling with LysoTracker Red DND-99 and mitochondrion labeling with** 447 **MitoTracker Green**

448 Lysosomes were labeled by LysoTracker Red (Life technologies, L7528) according to
449 manufacturer's protocol. Briefly, LysoTracker Red was added into cultural cells with the
450 concentration of 1:20000 dilution from stock solution and incubated at 37 °C for 30 min.

451 Images were taken by fluorescent microscope and the size of lysosomes were measured by
452 using Image J software to analyze the pixels a single lysosome occupying. We counted at
453 least 50 cells of random area in each group and the size in control cells was normalized to 1.

454 Mitochondria were labeled by MitoTracker Green FM (Life technologies, M7514) according to
455 manufacturer's protocol. Briefly, MitoTracker Green FM was added to the cultured cells with
456 the concentration of 1:10000 dilution from stock solution and incubated at 37 °C for 30 min.

457 Images were taken by fluorescent microscope

458

459 **Stable transfected Cells expressing fluorescent LC3 protein**

460 mRFP-LC3 expression construct (pmRFP-LC3) and mRFP-GFP-LC3 expression construct
461 (ptf-LC3) were provided by Dr. Yoshimori.²⁴ DNA was transfected into NIH3T3 using
462 Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's protocol. 500
463 µg/ml G418 (Invitrogen, 11811) was used for selecting stable expression cell clones. More
464 than 50 clones were pooled, expanded and used for experiments. These two pooled stable
465 cell populations were named as mRFP-LC3 cells and mRFP-GFP-LC3 cells, respectively.

466

467 **Intracellular ROS detection**

468 Reactive oxygen species (ROS) production was detected by ROS detecting probe DCFH-DA
469 (Applygen, C1300) combined with flow cytometry detection.⁴⁶ DCFH-DA turns to green
470 fluorescent molecule called DCF when oxidized by ROS, so that intracellular ROS level can be
471 reflected by fluorescent intensity produced by DCF. Three independent experiments were
472 conducted.

473

474 **Knockdown of Atg5 by shRNA**

475 ShRNA plasmid targeting mouse *Atg5* (AGAACCATACTATTTGCTT) were synthesized by
476 Genechem (25978). NIH3T3 cells were transfected with control or *Atg5* shRNA and a
477 polyclonal pool of NIH3T3 cells were selected by adding 2 µg/ml puromycin (Sigma, P8833).

478

479 **Lysosomal acid phosphatase assay**

480 Lysosomal acid phosphatase activity was assayed by a commercially available kit (Beyotime,
481 P0326) according to manufacturer's instructions. The activity of lysosomal acid phosphatase
482 was normalized by average lysosome content which was measured via a process including
483 Lyso-tracker staining of living cells, PBS washing, cell splitting with 1% Triton X-100 and
484 fluorescence intensity reading by a fluorescence microplate reader.

485

486 **Cathepsin B activity assay**

487 Cathepsin B activity was measured by Magic Red cathepsin B detection kit (ImmunoChemistry
488 Technologies, #937). Control or H₂O₂ treated NIH3T3 cells were cultured in 24-well plates with
489 different treatment for indicated time. Then cells were loaded with Magic Red Cathepsin B
490 reagent for 1 h and washed with PBS twice. More than 10 fluorescent images were taken and
491 representative images were shown. For quantification of cathepsin B activity, the fluorescence
492 of Magic Red cathepsin B probe was measured by fluorescent microplate reader.

493

494 **Mitochondrial membrane potential assay (JC-1)**

495 Mitochondrial membrane potential was monitored by a mitochondrial-specific dual
496 fluorescence probe, JC-1.⁴⁷ Briefly, JC-1 (Beyotime, C2006) was added to reach a final
497 concentration of 5 µg/ml and incubated for 20 min, and then the cells were washed twice with
498 medium and imaged under a fluorescent microscope.

499

500 **Measurement of mitochondrial DNA content and integrity using qPCR**

501 Mitochondrial DNA integrity was measured according to a qPCR method described

502 previously.⁵⁰ The primers used for qPCR are TGCCCCTCTTCTCGCTCCGG (forward),
503 GGCGATAACGCATTTGATGGCC (reverse) for amplifying short fragment in D-loop and
504 TGGGGGCCAACCAGTAGAACA (forward), TCGTCTAGACTGTGTGCTGTCC (reverse) for
505 amplifying semi long fragment in D-loop. The mitochondrial DNA content was calculated
506 according to the ratio of short D-loop/genomic fragment. The mitochondrial DNA integrity was
507 calculated according to the ratio of semi long/short fragment.

508

509 **Intracellular ATP level**

510 Intracellular ATP level was measured by a commercially available intracellular ATP
511 measurement kit (Nanjing Jiancheng, A095) according to manufacturer's instructions.

512

513 **Cytochrome c oxidase (COX) activity**

514 Determination of COX activity by spectrophotometry in protein extracts from the cells was
515 performed using a commercially available COX activity kit (GenMed Scientifics,
516 GMS10014.3.1) according to manufacturer's instructions.

517

518 **Statistical analysis**

519 Data are expressed as means \pm SD from at least three biological replicates. The difference
520 between control and treated was examined by Student's t-test. The difference between
521 multiple groups was examined by one-way ANOVA with Bonferroni post-hoc. $p < 0.05$ is
522 considered to be significant and $p < 0.01$ was considered highly significant.

523

524 **Acknowledgments**

525 This work was supported by National Natural Science Foundation of China (Grant Number
526 81273224), National 973 Basic Research Programs of China (Grant Number 2013CB911300
527 and Grant Number 2013CB967204). The authors thank Dr. Canhua Huang and Yuquan Wei
528 for continuous supports and Dr. Ping Lin, Xiujie Wang and Yi Chen for all around convenience.

529

530 **Conflict of Interest**

531 The authors declare no conflict of interest.

532

533 **References**

- 534 1. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of
535 stress-induced premature senescence (SIPS) of human diploid fibroblasts and
536 melanocytes. *Exp Gerontol.* 2000; 35:927-945.
- 537 2. Chen Q, Amos BN. Senescence-like growth arrest induced by hydrogen peroxide in
538 human diploid fibroblast F65 cells. *Proc Natl Acad Sci USA.* 1994. 91:4130-4134.
- 539 3. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M,
540 Rubelj I, Pereira-Smith O, *et al.* A biomarker that identifies senescent human cells in
541 culture and in aging skin in vivo. *Proc Natl Acad Sci USA.* 1995; 92:9363-9367.
- 542 4. Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe
543 SW. Rb-mediated heterochromatin formation and silencing of E2F target genes during
544 cellular senescence. *Cell.* 2003; 113:703-716.

- 545 5. Blagosklonny MV. Cell senescence and hypermitogenic arrest. *EMBO Rep.* 2003;
546 4:358-362.
- 547 6. Kojima H, Inoue T, Kunimoto H, Nakajima K. IL-6-STAT3 signaling and premature
548 senescence. *JAKSTAT*.2013; 2(4):e25763.
- 549 7. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. *Nat Cell Biol.* 2010;
550 12:814-22.
- 551 8. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW,
552 Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, *et al.* Regulation of mammalian
553 autophagy in physiology and pathophysiology. *Physiol Rev.* 2010; 90:1383-1435.
- 554 9. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell.*
555 2010; 140:313-326.
- 556 10. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K,
557 Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, *et al.* Guidelines for the use and
558 interpretation of assays for monitoring autophagy. *Autophagy.* 2012; 8(4):445-544.
- 559 11. Gewirtz D. Autophagy and senescence: A partnership in search of definition. *Autophagy.*
560 2013; 9(5):808-812.
- 561 12. Young A, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavares S, Arakawa S,
562 Shimizu S, Watt FM, *et al.* Autophagy mediates the mitotic senescence transition. *Genes*
563 *Dev.* 2009; 23(7):798-803.
- 564 13. Goehe RW, Di X, Sharma K, Bristol ML, Henderson SC, Valerie K, Rodier F, Davalos AR,
565 Gewirtz DA. The autophagy-senescence connection in chemotherapy: must tumor cells
566 (self) eat before they sleep? *J Pharmacol Exp Ther.* 2012; 343(3):763-78.

- 567 14. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature
568 senescence in primary human fibroblasts. *PLoS One*. 2011; 6:e23367.
- 569 15. Kaeberlein M. Rapamycin and aging: When, for how long, and how much? *J Genet*
570 *Genomics*. 2014; 41(9):459-463.
- 571 16. Hekimi S, Lapointe J, Wen Y. Taking a "good" look at free radicals in the aging process.
572 *Trends Cell Biol*. 2011; 21(10):569-76.
- 573 17. Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. *Cell*.
574 2007; 17(9):422-427.
- 575 18. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species
576 (ROS)-induced ROS release: a new phenomenon accompanying induction of the
577 mitochondrial permeability transition in cardiac myocytes. *J Exp Med*. 2000;
578 192:1001-1014.
- 579 19. Mailloux RJ, Harper ME. Uncoupling proteins and the control of mitochondrial reactive
580 oxygen species production. *Free Radic Biol Med*. 2011; 51(6):1106-1115.
- 581 20. Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. *Cell*.
582 2006; 125:1241-1252.
- 583 21. Young AR, Narita M. Connecting autophagy to senescence in pathophysiology. *Curr Opin*
584 *Cell Biol*. 2010; 22:234-40.
- 585 22. Rubinsztein DC, Mariño G, Kroemer G. Autophagy and aging. *Cell*. 2011; 46:682-695.
- 586 23. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H,
587 Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a
588 protective effect on huntingtin-induced cell death. *J Cell Biol*. 2005; 171:603-614.

- 589 24. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by
590 a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy*. 2007; 3(5):452-60.
- 591 25. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, Ong CN, Codogno P, Shen HM.
592 Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of
593 inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem*. 2010;
594 285(14):10850-61.
- 595 26. Bampton ET, Goemans CG, Niranjana D, Mizushima N, Tolkovsky AM. The dynamics of
596 autophagy visualized in live cells: from autophagosome formation to fusion with
597 endo/lysosomes. *Autophagy* 2005; 1:23-36.
- 598 27. Zhang XD, Qi L, Wu JC, Qin ZH. DRAM1 regulates autophagy flux through lysosomes.
599 *PLoS One*. 2013; 8(5):e63245.
- 600 28. Yu L, McPhee C, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, *et*
601 *al.* Autophagy termination and lysosome reformation regulated by mTOR. *Nature*. 2010;
602 465(7300):942-946.
- 603 29. Muller M. Pyocyanin induces oxidative stress in human endothelial cells and modulates the
604 glutathione redox cycle. *Free Radic Biol Med*. 2002; 33(11):1527-1533.
- 605 30. Bendikov-Bar I, Maor G, Filocamo M, Horowitz M. Ambroxol as a pharmacological
606 chaperone for mutant glucocerebrosidase. *Blood Cells Mol Dis*. 2013; 50(2):141-5.
- 607 31. McNeill A, Magalhaes J, Shen C, Chau KY, Hughes D, Mehta A, Foltynie T, Cooper JM,
608 Abramov AY, Gegg M, *et al.* Ambroxol improves lysosomal biochemistry in
609 glucocerebrosidase mutation-linked Parkinson disease cells. *Brain*. 2014; 137:1481-1495.

- 610 32. Bitto A, Lerner C, Torres C, Roell M, Malaguti M, Perez V, Lorenzini A, Hrelia S, Ikeno Y,
611 Matzko ME, *et al.* Long-term IGF-I exposure decreases autophagy and cell viability. *PLoS*
612 *One.* 2010; 5(9):e12592.
- 613 33. Fujii S, Hara H, Araya J, Takasaka N, Kojima J, Ito S, Minagawa S, Yumino Y, Ishikawa T,
614 Numata T, *et al.* Insufficient autophagy promotes bronchial epithelial cell senescence in
615 chronic obstructive pulmonary disease. *Oncoimmunology.* 2012; 1(5):630-641.
- 616 34. Simonsen A, Cumming RC, Brech A, Isakson P, Schubert DR, Finley KD. Promoting basal
617 levels of autophagy in the nervous system enhances longevity and oxidant resistance in
618 adult *Drosophila*. *Autophagy.* 2008; 4:176-184.
- 619 35. Demidenko ZN, Zubova SG, Bukreeva EI, Pospelov VA, Pospelova TV, Blagosklonny MV.
620 Rapamycin decelerates cellular senescence. *Cell Cycle.* 2009; 8(12):1888-1895.
- 621 36. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct
622 phosphorylation of Ulk1. *Nat Cell Biol.* 2011; 13:132-141.
- 623 37. Settembre C, DiMalta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU,
624 Huynh T, Medina D, Colella P, *et al.* TFEB links autophagy to lysosomal biogenesis.
625 *Science.* 2011; 332:1429-1433.
- 626 38. Zhou J, Tan SH, Nicolas V, Bauvy C, Yang ND, Zhang J, Xue Y, Codogno P, Shen HM.
627 Activation of lysosomal function in the course of autophagy via mTORC1 suppression and
628 autophagosome-lysosome fusion. *Cell Res.* 2013; 23:508-523.
- 629 39. Ni HM, Bockus A, Wozniak AL, Jones K, Weinman S, Yin XM, Ding WX. Dissecting the
630 dynamic turnover of GFP-LC3 in the autolysosome. *Autophagy.* 2011; 7(2):188-204.

- 631 40. Correia-Melo C, Marques FD, Anderson R, Hewitt G, Hewitt R, Cole J, Carroll BM, Miwa S,
632 Birch J, Merz, A, *et al.* Mitochondria are required for pro-ageing features of the senescent
633 phenotype. *EMBO J.* 2016; e201592862.
- 634 41. Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp*
635 *Gerontol.* 2000; 35(6-7):811-20.
- 636 42. Heid ME, Keyel PA, Kamga C, Shiva S, Watkins SC, Salter RD. Mitochondrial reactive
637 oxygen species induces NLRP3-dependent lysosomal damage and inflammasome
638 activation. *J Immunol.* 2013; 191(10):5230-8.
- 639 43. Brunk UT, Terman A. The mitochondrial-lysosomal axis theory of aging: accumulation of
640 damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem.* 2002;
641 269(8):1996-2002.
- 642 44. Hu J-P, Nishishita K, Sakai E, Yoshida H, Kato Y, Tsukuba T, Okamoto K. Berberine
643 inhibits RANKL-induced osteoclast formation and survival through suppressing the NF- κ B
644 and Akt pathways. *Eur J Pharmacol.* 2008; 580:70-79.
- 645 45. Mo C, Wang L, Zhang J, Numazawa S, Tang H, Tang X, Han X, Li J, Yang M, Wang Z, *et*
646 *al.* The Crosstalk Between Nrf2 and AMPK Signal Pathways Is Important for the
647 Anti-Inflammatory Effect of Berberine in LPS-Stimulated Macrophages and
648 Endotoxin-Shocked Mice. *Antioxid Redox Signal.* 2014; 20(4):574-88.
- 649 46. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and
650 flow-cytometry. *Methods Mol Biol.* 2010; 594:57-72.

- 651 47. Perelman A, Wachtel C, Cohen M, Haupt S, Shapiro H, Tzur A. JC-1: alternative excitation
652 wavelengths facilitate mitochondrial membrane potential cytometry. *Cell Death Dis.* 2012;
653 3:e430.
- 654 48. Tewari S, Santos JM, Kowluru RA. Damaged mitochondrial DNA replication system and
655 the development of diabetic retinopathy. *Antioxid Redox Signal.* 2012; 17(3):492-504.
- 656 49. Mijaljica D, Prescott M, Devennish RJ. V-ATPase engagement in autophagic processes.
657 *Autophagy.* 2011; 7(6):666-8.
- 658 50. Tewari S, Santos JM, Kowluru RA. Damaged mitochondrial DNA replication system and
659 the development of diabetic retinopathy. *Antioxid Redox Signal.* 2012; 17(3):492-504.
- 660 51. Han X, Tai H, Wang X, Wang Z, Zhou J, Wei X, *et al.* AMPK activation protects cells from
661 oxidative stress-induced senescence via autophagic flux restoration and intracellular
662 NAD⁺ elevation. *Aging Cell.* 2016; 15(3):416-427.
- 663 52. Lin YF, Schulz AM, Pellegrino MW, Lu Y, Shaham S, Haynes CM. Maintenance and
664 propagation of a deleterious mitochondrial genome by the mitochondrial unfolded protein
665 response. *Nature.* 2016; 533:416-419.
- 666 53. Mailloux RJ, Harper ME. Uncoupling proteins and the control of mitochondrial reactive
667 oxygen species production. *Free Radic Biol Med.* 2011; 51(6):1106-1115.

668

669 **Figure legends**

670 **Figure 1. Short term of H₂O₂ treatment is sufficient to induce cellular senescence.**

671 NIH3T3 cells were treated with PBS (Ctrl) or with 400 μM H₂O₂ in PBS as described in

672 Materials and Methods, and shifted to culture in a complete medium for indicated days.

673 Representative control cells were cultured to the longest time point in the experiment. (A)
674 Images showed the cellular morphology and SA- β -gal staining of control and H₂O₂-treated
675 NIH3T3 cells. (B) H₂O₂-treated cells were stained with DAPI at indicated days to show SAHFs.
676 Circles indicate typical SAHFs. Apoptosis was induced by 1.5 mM H₂O₂ for comparison. (C)
677 Cell counting was conducted for 7 days and growth curves of control, H₂O₂-treated and serum
678 starved NIH3T3 cells are shown. (D) TP53 protein was measured by Western blot. (E) (F)
679 Relative mRNA levels of *Cdkn1a* and *Il6* genes were analyzed by qRT-PCR. (G) ROS
680 generation in NIH3T3 cells was labeled by DCFH-DA probe and quantified by flow cytometry.
681 Data are presented as the means \pm SD from 3 independent experiments. *p <0.05 and **p
682 <0.01 compared to control.

683

684 **Figure 2. Autophagic structures increase but autophagic flux impairs in senescent cells.**

685 NIH3T3 cells were treated with or without H₂O₂ as described in Fig. 1. (A) Transmission
686 electronic microscopy of control or H₂O₂-treated NIH3T3 cells at day 5. Images on the right of
687 each group are enlarged areas in rectangles. Arrows show the vesicle-like structures. (B) The
688 lysosome content of control or H₂O₂-treated NIH3T3 cells was probed by Lyso-tracker Red
689 DND-99 and images were taken under a fluorescent microscope by using the same exposure
690 parameters. (C) Fluorescent images of mRFP-LC3 NIH3T3 cells treated with or without H₂O₂
691 at indicated time points. (D) Samples of control or H₂O₂-treated NIH3T3 were collected at
692 indicated time points and Western blots were performed by using SQSTM1 antibody. (E)
693 SQSTM1 protein expression at day 3 in control or H₂O₂-treated NIH3T3 cells with or without 5
694 μ g/ml HCQ for 12 h. (F) Control or H₂O₂-treated NIH3T3 cells were cultured for 3 days before

695 adding CHX (100 μ g/ml). Samples were collected at indicated time points after the addition of
696 CHX and SQSTM1 protein was determined by Western blot. Statistics show the time phased
697 degradation of SQSTM1 protein from 3 independent experiments. (G) Fluorescent images of
698 EBSS starved or H₂O₂-treated NIH3T3 cells stably expressing mRFP-GFP-LC3.

699

700 **Figure 3. Blockage of autophagy flux is sufficient to induce cellular senescence.**

701 (A) NIH3T3 cells were treated with HCQ (3 μ g/ml) or leupeptin (5 μ g/ml) for 7 days. DCFH-DA
702 fluorescence and SA- β -gal staining was used to detect intracellular ROS and the senescent
703 state of these cells respectively. Statistics show the percentages of SA- β -gal positive cells. (B)
704 Relative *Il6* mRNA expressions of the cells in (A) were quantified by qRT-PCR. (C) NIH3T3
705 cells were transfected with control shRNA or Atg5 shRNA and positive cells were selected by
706 2 μ g/ml puromycin for 7 days. Western blots of ATG5 in control and ATG5 knockdown cells. (D)
707 Western blot of SQSTM1 in control and ATG5 knockdown cells. (E) The same cells in (C) were
708 cultured for another 5 days before treated with or without H₂O₂ and incubated for 3 days.
709 Images show SA- β -gal staining of control and ATG5 knockdown cells. Statistics showed the
710 percentages of SA- β -gal positive cells. (F) Relative *Il6* mRNA expression of control and Atg5
711 knockdown cells cultured for 15 days. The data are presented as means \pm SD from 3
712 independent experiments and **p <0.01.

713

714 **Figure 4. Autophagy restoration attenuates SIPS development.**

715 (A) (B) Different autophagy regulators including rapamycin (100 nM), PP242 (500 nM), VPA (1
716 mM), LiCl (10 mM), bafilomycin A1 (50 nM), HCQ (3 μ g/ml) and 3-MA (500 μ M) were added to

717 H₂O₂-treated NIH3T3 cells and incubated for 3 or 5 days. Images of SA-β-gal staining are
718 shown. Statistics show the percentages of SA-β-gal positive cells at day 5. (C) *β6* mRNA level
719 in H₂O₂-treated cells cultured with rapamycin or bafilomycin A1. (D) Confocal images of
720 H₂O₂-treated tandem mRFP-GFP-LC3 cells with rapamycin or bafilomycin A1. (E) SQSTM1
721 proteins in H₂O₂-treated NIH3T3 cells with rapamycin or bafilomycin A1 were examined by
722 Western blots. The data are presented as means ± SD from 3 independent experiments. *p
723 <0.05 and **p <0.01 when compared to DMSO.

724

725 **Figure 5. Autophagy impairment couples with lysosomal and mitochondrial dysfunction.**

726 NIH3T3 cells treated with or without H₂O₂ as described in Fig. 1. (A) *In vitro* acid phosphatase
727 activity assay was performed at day 3 after H₂O₂ treatment. (B) Lysosomes of control or
728 H₂O₂-treated NIH3T3 cells were loaded with LysoTracker Red DND-99 at day 3 and images
729 were taken by confocal microscope. The images in rectangles are 1.5 fold enlarged. Statistics
730 show the relative lysosome size in control and H₂O₂-treated cells. (C) Endogenous cathepsin
731 B protein in control and H₂O₂-treated cells were detected by Western blot. (D) Cellular
732 cathepsin B activity was visualized by using Magic Red Cathepsin B detection kit at indicated
733 days. Cells treated with bafilomycin A1 (50 nM) for 12 h were shown as negative control. (E)
734 Mitochondrial membrane potential in cells was measured by JC-1 staining. Fluorescent
735 images were taken at indicated days. (F) Cellular ATP level of control (5d) or H₂O₂-treated (1d,
736 3d, 5d) NIH3T3 was measured. (G) Mitochondrial COX activity of control (5d) or H₂O₂-treated
737 (1d, 3d, 5d) NIH3T3 was assayed. (H) NIH3T3 cells pretreated with DMSO or 10 μM CCCP for
738 6 h accepted H₂O₂ treatment as described in Fig. 1. Images of DCFH-DA fluorescence was

739 taken at indicated days. (I) SA- β -gal staining was performed at day 5 of the cells in (H). (J)
740 Relative *U6* mRNA expression of the cells in (I). The data are presented as means \pm SD from 3
741 independent experiments. *p <0.05 and **p <0.01 when compared to ctrl or DMSO.

742

743 **Figure 6. Mitochondria dysfunction precedes lysosomes dysfunction during SIPS**

744 **development.**

745 NIH3T3 cells were treated with or without H₂O₂ as described in Fig. 1. (A) Intracellular ROS
746 and lysosomal cathepsin B activity within 24 h after H₂O₂ treatment. Intracellular ROS was
747 measured by flow cytometry using DCFH-DA probe. Lysosomal cathepsin B activity was
748 measured by the fluorescence intensity of Magic Red Cathepsin B probe. 0 h means the end
749 time point of H₂O₂ treatment. The data of untreated cells (time point -1 h) was normalized to 1.
750 (B) Intracellular ROS and lysosomal cathepsin B activity at 0, 1, 3, 5 day after H₂O₂ treatment.
751 (C) H₂O₂-treated NIH3T3 cells were cultured in medium with or without 2 μ M NAC for 3 days.
752 Cathepsin B activity was visualized by Magic Red Cathepsin B kit. (D) Images of DCFH-DA
753 fluorescence of control or H₂O₂-treated NIH3T3 cells at day 3. 10 μ M CCCP was added to the
754 culture medium after H₂O₂ treatment. (E) Images of Magic Red Cathepsin B fluorescence of
755 control or H₂O₂-treated NIH3T3 cells at day 3. 50 μ M ambroxol was added to the culture
756 medium after H₂O₂ treatment. (F-H) H₂O₂-treated NIH3T3 cells were incubated with NAC (2
757 μ M), CCCP (10 μ M) or ambroxol (50 μ M) at different periods of time. Cells were stained with
758 SA- β -gal at day 5. Arrow indicates the proliferating cells in NAC group. Statistics show the
759 percentages of SA- β -gal positive cells in each group. The data are presented as means \pm SD
760 from 3 independent experiments. *p <0.05 and **p <0.01 when compared to control or

761 samples not treated with NAC/CCCP/ambroxol.

762

763 **Figure 7. Rapamycin restores mitochondrial and lysosomal function in H₂O₂-treated cells.**

764 NIH3T3 cells were treated with H₂O₂ and incubated with DMSO, rapamycin or bafilomycin A1

765 for 3 or 5 days. (A) Images of JC-1 fluorescence from H₂O₂-treated cells with DMSO,

766 rapamycin or bafilomycin A1 for 3 days. (B) DCFH-DA probed intracellular ROS at day 3. (C)

767 Intracellular ATP levels were measured at day 3. (D) Cathepsin B protein in cells incubated

768 with rapamycin or bafilomycin A1 was examined by Western blots. (E) Intracellular cathepsin

769 B activity at day 3 was visualized by using Magic Red Cathepsin B kit. The data are presented

770 as means ± SD from 3 independent experiments. *p <0.05 and **p <0.01 when compared to

771 DMSO.