FASN activity is important for the initial stages of the induction of senescence

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Abstract
Senescent cells accumulate in several tissues during ageing and contribute to several pathological processes such as ageing and cancer. Senescence induction is a complex process not well defined yet and is characterized by a series of molecular changes acquired after an initial growth arrest. We found that fatty acid synthase (FASN) levels increase during the induction of senescence in mouse hepatic stellate cells and human primary fibroblasts. Importantly, we also observed a significant increase in FASN levels during ageing in mouse liver tissues. To probe the central role of FASN in senescence induction, we used a small-molecule inhibitor of FASN activity, C75. We found that C75 treatment prevented the induction of senescence in mouse and human senescent cells. Importantly, C75 also reduced the expression of the signature SASP factors interleukin 1α (IL-1α), IL-1β and IL-6, and suppressed the secretion of small extracellular vesicles. These findings were confirmed using a shRNA targeting FASN. In addition, we find that FASN inhibition induces metabolic changes in senescent cells. Our work underscores the importance of C75 as a pharmacological inhibitor for reducing the impact of senescent cell accumulation.

Introduction
Accumulation of senescent cells in different tissues during ageing has been extensively reported, especially in cardiovascular and other age-related diseases1. Senescence is a process primarily characterized by a stable cell proliferation arrest and development of a secretory state known as senescence-associated secretory phenotype (SASP)2. SASP factors are mainly composed by cytokines, chemokines and growth factors. Senescent cells are able to affect adjacent cells through SASP factor activation of various cell-surface receptors and a resulting intracellular signal transduction3,4. As part of the SASP, senescent cells also secrete extracellular vesicles (EVs)5,6. Importantly, it was shown that the protein p53 is responsible for the regulation of EVs secretion7.

A key characteristic of senescent cells is their high metabolic activity8. Increased glycolysis and redox homoeostasis has been frequently detected in vitro in senescent cells, with decreased fatty acid oxidation9. Several signals and stressors can trigger senescence via accumulation of the protein p53, which consequently activates the cell cycle inhibitor p21CIP10. p53 belongs to a small family of tumour suppressor proteins known as the p53 family11,12. This family regulates several pathways in cells, the most well-known being apoptosis, cell cycle and senescence2,11.

p53 regulates glycolysis, oxidative phosphorylation and amino acid metabolism in cancer cells13–15, and also modulates metabolic adaptation in senescence cells16. Glycolysis and oxidative phosphorylation are the two major metabolic pathways involved in regulating most
cellular activities. Although glycolysis and its metabolites have been widely studied in the context of senescence and cancer, fewer studies have been conducted on the role of lipid metabolism in inducing replicative senescence. It is of note that p53 activation has been linked to alterations in fatty acid metabolism but the role of lipid synthesis in regulating senescence induction has been understudied.

Lipid synthesis is an important metabolic process in cells. Lipids are essential for the production of fatty acids, phospholipids, sterols and sphingolipids. Fatty acids can be derived from two sources: exogenous sources or de novo fatty acid synthesis (FAS). Normal cells rely on exogenous fatty acids whereas tumour cells and senescent cells use de novo synthesis of fatty acids. The FAS pathway produces long-chain fatty acids, using the enzyme fatty acid synthase (FASN) to combine acetyl-CoA produced from glycolysis with malonyl-CoA. FASN plays a major role in metabolism and has been shown to be upregulated in different cancer cells. However, limited evidence has implicated FASN pathway in cellular senescence.

To understand the link between FAS and cellular senescence, we decided to study the role of FASN activity at the initial phases of senescence activation in both mouse and human cells. We focused on the initial phase of senescence activation in order to identify future therapies that would prevent the activation of a full senescent programme. For this, we took advantage of a well-documented FASN inhibitor, C75. C75 has been largely used as a tool for studying FAS role in metabolic disorders, senescence and cancer. In addition, we confirmed our findings using a previously characterized short hairpin RNA (shRNA) targeting FASN (shFASN). We next assessed the role of FASN during the initial stages of activation of the senescence programme. We found increased levels of FASN mRNA during the induction of senescence. Treatment of senescent cells with C75 and shFASN prevented the induction of senescence. C75 and shFASN also reduced the secretion of SASP factors interleukin 1α (IL-1α), IL-1β and IL-6, and suppressed the secretion of EVs. We found that FASN inhibition also induces metabolic changes in senescent cells.

Senescent cells accumulate during ageing in degenerative tissues, so drugs that can control SASP activity have the potential of improving several age-related diseases. The results from this study support the use of C75 as a novel therapeutic agent for reducing the effect of the activation of senescence on different age-associated diseases.

Results

p53 endogenous expression progressively induces senescence in mouse hepatic stellate cells

In order to investigate the metabolic adaptation during the activation of senescence, we took advantage of hepatic stellate cells (HSCs) derived from adult mouse liver, previously described by Lujambio et al. HSCs were cultured in the presence of doxycycline (Dox) inducing the expression of a green fluorescent protein (GFP)-tagged shRNA targeting TP53 (GFP-shp53) in a reverse tetracycline-controlled transactivator transgene vector (Supplementary Fig. S1A). HSCs were withdrawn of Dox and collected at different time points, allowing the endogenous expression of p53 (Fig. 1a). The endogenous expression of TP53 and other senescence markers (CDKN2A, CDKN1A, IL6, IL1A and IL1B) were monitored by reverse transcription-PCR (RT-PCR) (Fig. 1b). We found a time-dependent increase in TP53 mRNA level expression upon Dox removal. We also found increased expression of CDKN2A (encoding p16INK4A) and CDKN1A (encoding p21CIP), genes important in inducing cell cycle arrest and senescence, after Dox was withdrawn (Fig. 1b). Notably, we observed increased transcription of IL6, IL1A and IL1B, key mediators of the proinflammatory elements of SASP (Fig. 1b). In the presence of Dox HSCs express high levels of GFP and after Dox withdrawal GFP expression was significantly reduced (Fig. 1c, d). Indeed, HSCs after Dox removal showed increased SA-β-gal (senescence-associated β-galactosidase) activity (Fig. 1c, e) with reduced cell proliferation, measured by cell number by 4′,6-diamidino-2-phenylindole (DAPI) and quantifying cells positive for Ki67 staining (Fig. 1c, f, g). Overall, these results show that after Dox removal HSCs undergo a cell cycle arrest and express different markers of senescence.

FASN activity is essential for mitochondria energy in senescent cells

p53 regulates the expression of several target genes involved in different metabolic pathways. We explored whether senescence induction in HSCs was affecting lipogenesis. To this end, we measured changes in the FASN pathway by detecting mRNA levels of FASN and Acetyl-CoA carboxylase enzymes (ACACA1 and ACACA2) (Fig. 2a). After removing Dox to induce senescence, we observed significant FASN mRNA upregulation with a significant reduction of ACACA2. ACACA1 mRNA levels reached a peak at day 2 without Dox, after which it drops to basal levels.

To determine whether FASN increased mRNA levels in senescence correlated during ageing in vivo, we extracted RNA from C57BL/6J mice liver of 4 (young) and 25 (old) months. RT-PCR analysis of the livers from aged mice revealed elevated levels of FASN mRNA as compared with young mice (Fig. 2b). Importantly, we also observed increased levels of several well-established biomarkers of senescence such as CDKN2A, CDKN1A, IL1A and IL1B in livers from aged mice, suggesting increased senescence in vivo (Fig. 2b). Together, these
Fig. 1 (See legend on next page.)
data indicate that FASN mRNA levels increase during ageing in vivo.

As the FASN pathway plays a central role in mitochondrial energy metabolism\(^{27}\) (Fig. 2c), we next measured the oxygen consumption rate (OCR) in both control and senescent HSCs after 8 days of Dox withdrawal. As expected, senescent HSCs showed a greater increase in OCR than the control (Fig. 2d). These data are in agreement with previous reports showing that senescent cells have a marked increase in mitochondrial activity\(^{6,28}\). To assess whether this increase in OCR during senescence was due to FASN activity, we used increasing concentrations of C75. An 80% OCR reduction was observed in senescent HSCs after C75 treatment (Fig. 2d, e). Importantly, only a 20% OCR reduction was observed in control after C75 treatment. Next, we inhibited fatty acid oxidation in HSCs with increasing concentrations of etomoxir (Eto), an irreversible inactivator of the carnitine palmitoyltransferase-1 (CPT-1) transporter\(^{30}\). Eto reduced senescent cell OCR by almost 80% but only reduced control OCR by 35% (Fig. 2e). A combination of 2 \(\mu\)M rotenone and 1 \(\mu\)M antimycin A (mitochondria complex I and III inhibitors, respectively) was added to the cells, to shut down mitochondrial respiration as a positive control for OCR reduction (Fig. 2d, e). Collectively these data suggest that FASN activity is important for the mitochondrial energy metabolism during senescence.

It is well documented that senescent cells have an increase in mitochondrial number and mitochondria hyperfunction\(^{28}\). Therefore, we measured mitochondria membrane potential and reactive oxygen species (ROS) in control and senescent HSCs with or without C75 (Supplementary Fig. S1B, C). As expected, we observed significant mitochondrial hyperpolarization in senescent cells with increased ROS, measured by quantifying hydrogen peroxide production. Importantly, C75 treatment was able to inhibit mitochondria hyperpolarization and ROS production (Supplementary Fig. S1B, C). To determine whether the mitochondria hyperpolarization and ROS production could be due to an increase in the number of mitochondria in our model, we measured COXIV, a mitochondrial marker, by western blotting (Supplementary Fig. S1D). In fact, we detect a clear increase in COXIV in senescent cells as compared with control as previously described\(^{28}\). Importantly, C75 was able to reduce the mitochondria mass at similar levels to control (Supplementary Fig. S1D). As ATP levels in the cells mainly depend on mitochondrial activity\(^{31}\), we next measured ATP levels in senescent cells after 6 days of Dox withdrawal vs. control. We found that ATP levels in senescent cells increased significantly in comparison with control (Fig. 2f). Importantly, strong reduction in ATP levels was observed after C75 treatment in senescent cells (Fig. 2f). These data are in agreement with our previous findings that mitochondria in senescent cells exhibited a marked increase in basal oxygen consumption, which was greatly reduced following treatment with C75 (Fig. 2d). In addition, C75 is able to prevent mitochondria dysfunctional during senescence with enhanced levels of ROS. Next, to validate these data in an in vivo system, we quantified the levels of mRNA of peroxisome proliferator-activated receptor-\(\gamma\) coactivator 1-\(\alpha\) (PGC1A) as a marker of mitochondria biogenesis in ageing mice. We detect a significant increase of PGC1A transcript in the livers of old mice as compared with that of young mice (Supplementary Fig. S1E), suggesting that old mice have an increased mitochondria number and biogenesis. In agreement with these results, a significant increase in mitochondria basal respiration was observed in mitochondria isolated from the liver of old mice as compared with that of young mice (Supplementary Fig. S1F). Collectively, these data suggest that FASN inhibition prevents mitochondria transformation arising during senescence.

The FASN inhibitor C75 affects mTOR activity and reverses growth arrest in senescent cells

The mammalian target of rapamycin (mTOR) senses signals from various growth factors and nutrients, and regulates several pathways involved in protein and lipid synthesis\(^{32}\). mTOR is activated in cellular senescence, promoting protein synthesis\(^{33}\). We found that senescent HSCs displayed a significant increase in the amount of puromycin-labelled peptides compared with control, indicating that global protein synthesis\(^{34}\) is increased in senescent cells and suggesting an increase in mTOR activity (Fig. 3a). Importantly, a significant reduction of
Fig. 2 (See legend on next page.)
protein synthesis of 90% was found in senescent cells treated with C75 (Fig. 3a). We used 1 μM rapamycin, a mTOR inhibitor, as a positive control for mTOR activity and could observe a 50% reduction in protein synthesis in senescent cells treated with rapamycin. Our results suggest that senescent cells upregulate global rate of protein synthesis, and that FASN inhibition is able to restore the normal ratio of protein synthesis in the cells.

To confirm the implication of mTOR, we analysed mTOR signalling after 6 days of senescence induction and we compared this with control. To our surprise, mTOR activity was repressed during senescence as shown by a decrease in the phosphorylation levels of some of its downstream targets: S6, 4EBP1 and AKT (Fig. 3b, c). However, it was recently reported that mTOR is quickly activated in senescence but after 72 h this signal is inhibited by a negative feedback loop. Remarking, senescent cells treated with C75 were able to restore mTOR signalling at levels similar to control (Fig. 3b, c).

To validate the role of mTOR during ageing in an in vivo system, we measured in aged mouse liver EEF2 and MAPKAPK2 transcripts, which are two canonical targets of mTOR. We observed a significant increase in EEF2 and MAPKAPK2 mRNA levels in the livers of old mice (Supplementary Fig. S1G), suggesting an upregulation of mTOR during ageing as previously described.

mTOR inhibition has been shown to inhibit the SASP, but there is controversy whether it reverses the proliferation arrest characteristic of senescence. Therefore, we measured cell number after 6 and 8 days of senescence induction (Fig. 3d). We observed a significant cell number reduction after the induction of senescence and treatment with C75 rescued the growth arrest of senescent HSCs (Fig. 3d). However, mTOR inhibition with 1 μM rapamycin did not rescue the cell cycle arrest induced in senescence (Fig. 3d).

FASN inhibition prevents the initiation of senescence induction in HSCs

To assess the relevance of FASN activity in the establishment of the senescent programme, we treated HSCs with C75 before the establishment of senescence after 4 days of Dox withdrawal (Fig. 4a). Next, we evaluated whether senescence was established at day 6 of Dox removal by measuring several senescence markers. As shown in Fig. 4b, after 6 days of Dox withdrawal, HSCs displayed a senescent phenotype. Indeed, HSCs show a significant increase in SA-β-gal activity and in the percentage of cells expressing the tumour suppressor p53-binding protein 1 (33BP1), an indication of DNA damage and significant reduction in GFP expression (Fig. 4b, c). Thus, bromodeoxyuridine (BrdU) immunostaining revealed cell proliferation arrest after Dox withdrawal compared with control (Fig. 4b, c). Importantly, C75 treatment of HSCs showed a marked ability to prevent the activation of the senescent phenotype indicated by higher levels of BrdU staining and low SA-β-gal activity (Fig. 4b, c). In agreement with these data, p53, p16 and p21 RNA and protein levels were also upregulated after senescence induction and significantly reduced after C75 treatment (Supplementary Fig. S2A, B, C). Importantly, C75 treatment did not induce apoptosis in HSCs senescent cells (Supplementary Fig. S2D).

Senescent cells secrete large amounts of molecules as part of the SASP, which induce changes in the microenvironment. We next evaluated whether C75 could reduce the secretion of the SASP factors by RT-PCR measuring the mRNA levels of IL1A, IL1B and IL6 in senescent cells. Figure 4d shows that senescent cells treated with C75 significantly reduced IL1A, IL1B and IL6 mRNA levels in HSCs.

It was reported that cancer cells upregulate FAO and the β-oxidation pathway to achieve the extra energy demand, and it has been suggested that these two
processes are linked together. Therefore, we used the β-oxidation pathway inhibitor Eto and we observed a marked senescence-induction inhibition indicated by a significant reduction in SA-β-Gal staining when senescent HSCs were treated with Eto (Supplementary Fig. S3A, B). Indeed, RNA levels for the cell cycle
Fig. 4 The FASN inhibitor, C75, prevents the induction of cellular senescence. 

a. Schematic representation of the experimental settings to determine the role of FASN pathway in preventing the induction of senescence in HSCs. After 4 days of Dox withdrawal, 10 μM C75 was added to HSCs. 

b. Representative images of control, senescent and senescent with 10 μM C75 treatment in HSCs. SA-β-Gal staining was assessed by light microscopy. GFP (green), DAPI (blue), 53BPI and BrdU (red) staining was assessed and quantified using the automated fluorescent microscope InCell 2200. Scale bar: 100 μm. 

c. Quantification of the percentage of HSC staining positive for SA-β-gal, GFP, 53BPI and BrdU incorporation in control and senescent HSCs with or without 10 μM C75 treatment. Data represent the mean ± SEM of three independent experiments. 

d. Relative expression of IL1A, IL1B and IL6 in control, senescent and senescent plus 10 μM C75 HSCs. 

e. Extracellular vesicles (EVs) released in control, senescent and senescent plus 10 μM C75 HSCs were measured using Nanoparticle Tracking Analysis (NTA). 

f. Evaluation of the size distribution of the EVs released by HSCs in control, senescent and senescent plus 10 μM C75 treatment. Data represent the mean ± SEM of two independent experiments. Two-tailed Student’s t-test was used to calculate the significance and it was represented as follows: *P < 0.05; **P < 0.001; ***P < 0.0001.
inhibitors TP53, CDKN2A and CDKNA1 were significantly downregulated upon Eto treatment (Supplementary Fig. S3C). These results further support the idea that fatty acid metabolism (synthesis and oxidation) play a central role in the induction of senescence. We therefore conclude that blocking lipid metabolism with two independent inhibitors prevents the activation of senescence in HSCs.

A key and novel aspect of p53 function is the regulation of EVs secretion, which is an important process involved in cell–cell communication. Our analysis revealed that FASN activity regulates p53 expression and, due to this, we wondered whether FASN activity could regulate EVs secretion. To this end, we induced senescence in HSCs and then we treated the cells with and without C75. We observed that senescent cells secrete high numbers of EVs as described before, but after treatment with C75 we observed a statistically significant decreased in the secretion of EV compared with senescent cells (Fig. 4e). Moreover, no differences in EVs size distribution between control, senescent and senescence plus C75 was observed (Fig. 4f).

To determine the specificity of C75 in inhibiting FASN, we silenced FASN expression in HSCs using a previously validated lentiviral vector encoding a FASN-specific shRNAs (shFASN), which reduced FASN levels (Fig. 5a, b). Importantly, FASN knockdown in senescent HSCs prevented the senescent-induced cell cycle arrest (Fig. 5c–e), in addition to preventing the upregulation of different markers of senescence such as SA-β-gal activity, p53, p16 and p21 (Fig. 5d–g). Notably, FASN knockdown also inhibit the increase in the expression of mRNA levels of SASP factors IL1A, IL1B and IL6 in HSCs (Fig. 5h). These data show that FASN activity is important for the induction of senescence in HSCs.

C75 is able to prevent the induction of senescence in human primary fibroblasts

In order to understand whether the mechanism underlying C75 prevention of cellular senescence initiation was conserved in human cells, we induced senescence in human primary foreskin fibroblasts (HFFF2) by a stable infection with a construct expressing the oncogene H-RASG12V (RAS) inducible by tamoxifen (Fig. 6a). First, we confirmed that FASN mRNA levels were indeed upregulated during senescence induced by RAS in human cells (Fig. 6b). Furthermore, RAS-induced senescent cells exhibited high RNA and protein levels of the senescent markers p53, p16 and p21 (Fig. 6c–e). Compared with control, RAS-induced senescence resulted in a strong increase in the levels of the secreted cytokines IL6 and IL8 (Fig. 6c). Significant inhibition of cell cycle inhibitors and SASP genes in RAS-induced senescent cells were observed after treatment with C75 (Fig. 6c–e).

Together, our data show that FASN activity is essential for the induction of senescence in human cells. In summary, our results support the idea that FASN has a novel role as a metabolic-controlling protein.

Discussion

Cellular senescence is a well-defined phenotype characterized by a stable cell cycle arrest in response to different stress signals such as oncogene activation and DNA damage, and accompanied by a characteristic secretory phenotype of EVs and SASP. Here we focused on elucidating the biological role of FASN in controlling the induction of cellular senescence.

FASN is the key enzyme involved in the regulation of de novo FAS. Normal human tissues will use exogenous lipids for membrane synthesis, whereas de novo FAS is suppressed, and FASN expression is maintained at very low levels. We show here that FASN endogenous levels are upregulated during senescence in mouse and human cells. Numerous evidence suggest that cancer cells have deregulated lipid metabolism (de novo FAS and β-oxidation pathway), which resemble our findings.

We show here that FASN inhibition in mouse and human cells stops cellular senescence induction, by reducing mitochondrial energy levels, a finding that confirms and further extends the original description that senescent cells display high metabolic activity.

FASN has a well-defined role in lipogenesis, by catalysing de novo biogenesis of fatty acids. New reports have given to FASN a central role in supporting cancer cell growth rather than functioning as an anabolic energy-storage pathway. Importantly, a recent study shows that FASN indirectly controls the mTOR, a central regulator of cellular metabolism. Therefore, we set out to assess whether FASN regulates any aspect of mitochondria oxygen consumption in senescent cells and characterized the relationship between FASN inhibitor and cellular senescence induction. We induced senescence in mouse HSCs and assessed metabolic changes by measuring mitochondria respiration and ATP levels. Mitochondrial respiration is the most important generator of cellular energy under most circumstances. It is a process of energy conversion of substrates into ATP. Importantly, we observed enhanced mitochondrial respiration with a robust increase in ATP levels in senescent cells. These results are in agreement with previous reports showing that RAS-induced senescent cells have increased OCR than control. Overall, our study shows that FASN activity is important for mitochondrial bioenergetic generation in senescent cells.

This study unveils a role for FASN in controlling senescence establishment that contributes to explain the effect of FASN inhibitor, C75. Importantly, C75 was reported to inhibit senescence in human diploid
Fig. 5 (See legend on next page.)
fibroblasts, although it was unclear which senescent pathway was affected, whereas other reports show that C75 induce senescence in fibroblasts. Here we report that cellular senescence is inhibited in HSCs not only by treatment with C75 but also by FASN knockdown using a specific shRNA. Indeed, mouse and human cells treated with C75 inhibit cellular senescence induction and start to divide; they show reduced β-galactosidase staining and reduced p53 levels.

Rise of senescent cells in organs is considered to be the one of the hallmarks of ageing and therefore partially responsible for different age-associated diseases. Senescent cells can play a central role in this phenomenon, by secreting different SASP and EVs factors. We show that C75 was able to inhibit the key mediators of the proinflammatory elements of the SASP and EVs secretion, possibly because we observed a dramatic reduction in p53 levels known to be the regulator of SASP and EVs. Also, we observed increase expression of FASN and it was associated with increased levels in several biomarkers of senescence in liver tissue from aged mice. As senescent cells accumulate during ageing, causing cancer and several age-related pathologies, C75 could be a potential therapeutic route to increase the lifespan and delay age-related pathologies.

In summary, we show that FASN activity was vital for mitochondria respiration in senescent cells. Our results support the use of C75, as a drug for reducing the effect of different age-associated diseases.

Materials and methods

Cell culture

Mouse HSCs were a kind gift from Scott Lowe and were grown in 1 µg/ml of Dox. Human foreskin fibroblasts (HFF/2; 86031405) were purchased from Public Health England (UK). All cells were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% antibiotic–antimycotic solution.

RNA extraction, cDNA synthesis and RT-PCR

Cells grown in six-well plates or 10 cm dishes were washed with phosphate-buffered saline (PBS) and lysed directly into the culture dish using TRIzol Reagent (ThermoFisher). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcriptase Kit (ThermoFisher). RT-PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 Fast System RealTime PCR cycler (Applied Biosystems). Primer sequences are listed in Supplementary Fig. S4.

Stable gene expression

Stable retroviral and lentiviral expressions were performed as in previous studies.

β-Galactosidase staining

Cells were washed with PBS and fixed with 0.05% (w/v) glutaraldehyde (in PBS) for 15 min at room temperature (RT). Cells were washed a second time with PBS and incubated with 5-bromo 4-chloro-3-indolyl-beta-d-galacto-pyranoside solution (Promega) for 1 h at 37 °C. Cells were imaged using a light microscope (Nikon) at ×20 magnification and single representative images of each well were taken.

Immunofluorescence staining and analyses

Cells grown in 96-well plates were washed with PBS and fixed in 4% paraformaldehyde for 15 min at RT. Cell were then washed in PBS twice before being permeabilized and blocked for 40 min with 0.2% Triton X-100 together with 1% bovine serum albumin and 0.2% gelatin fish (Sigma). For immunofluorescence (IF) staining, cells were incubated overnight with the primary antibody (Supplementary Fig. S5); in the case of BrdU, cells were treated with DNsase and MgCl2. Cells were then washed in PBS and incubated 1 h with secondary antibody, DAPI (Sigma-Aldrich). IF images were acquired using IN Cell 2200 automated 991 microscope (GE) and the IN Cell 2200 Developer software version 1.8 (GE).
Protein analysis by western blotting

Proteins from cells were lysed using the RIPA Buffer Lysis (ThermoFisher), separated in an SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore) and probed with different antibodies (Supplementary Fig. S5).

Fig. 6 The C75 inhibitor blocks senescence in human primary fibroblasts. a Schematic representation of the experimental settings to determine the role of FASN pathway in human primary fibroblast. Human primary foreskin fibroblasts (HFFF2) were transduced with retroviruses expressing H-RAS<sup>G12V</sup> or with empty vector control. After 4 days of senescent induction using 200 nM tamoxifen (4OHT), the cells were treated or not with 10 μM C75. b RT-PCR determining the mRNA levels of FASN in control and senescent human fibroblasts. c Relative mRNA levels of two cell cycle inhibitors, CDKN2A and CDKN1A, and two genes associated with SASP, IL6 and IL8, were analysed in control, senescent and senescent plus 10 μM C75 treatment HFFF2 cells. Data represent the mean ± SEM of three independent experiments. d Western blottings of cellular lysates corresponding to cells transduced with control or with H-RAS<sup>G12V</sup>. Senescent cells were treated with DMSO or 10 μM C75. Expression of p53, p16 and p21 protein levels were analysed. β-Actin and vinculin (Vcl) were used as a loading control. e Densitometry quantification for p53, p16 and p21 expression normalized with respect to vinculin or β-actin in HFFF2 expressing a control vector or H-RAS<sup>G12V</sup>. Senescent cells were treated with 10 μM C75 or DMSO. Two-tailed Student’s t-test was used to calculate the significance and it was represented as follows: *P < 0.05, ***P < 0.0001.
ATP levels
HSCs were depleted of Dox for 6 days. Cells were trypsinized and centrifuged at 1000 r.p.m. Collected cells were suspended in 50 µL CellTiter-Glo Luminescent Cell Viability Assay solution (Promega, Madison, WI, USA) and incubated for 10 min. The luminescence intensity was measured using a Synergy HT Multi-Mode Microplate Reader. All the readings were expressed as rlu (relative luminescence unit)/cell number.51

Measurement of cellular OCR
XF-24 cell culture microplates were coated with 3.4 mg/mL BD Cell-Tak tissue adhesive solution (BD Bioscience 354240) according to the manufacturer’s instruction. Forty-three microlitres of the Cell-Tak solution was added to each well of a XF-24 cell culture plate and incubated for 20 min at RT. Mouse HSCs were plated at densities of 30,000 cells per well in a Seahorse XF-24 plates pre-coated with Cell-TAK on the same day of the analysis. Microplates containing the cell suspension were centrifuged at 700 × g for 5 min and then incubated at 37 °C for at least 1 h, to allow attachment. Before measurements, the growth medium was replaced with 600 µl assay medium (Seahorse Bioscience), a low buffered DMEM containing no bicarbonate, and incubated for 45 min in a 37 °C non-CO2 incubator. Basal OCR was determined using XF-24 Extracellular Flux analyser. The different drugs were added automatically during measurement, after establishing the baseline of OCRs. We measured the OCR in response to sequential treatment with the irreversible inhibitor of CPT-1, Eto at 50 µM and 100 µM, an inhibitor of FASN, C75 at 10 µM and 20 µM, and with a mix of the electron transport chain inhibitors rotenone (2 µM) and antimycin A (1 µM).51

Nanoparticle tracking analysis
The NanoSight LM10 (Malvern Instruments) was calibrated using Silica Microspheres beads (Polyscience). Samples were diluted in PBS in order to obtain a particle number between 10^8–10^9 particles. At least three repeated measurements of 60 s were taken per each individual sample and the mean value was used to determine particle number. The movement of each particle in the field of view was measured to generate the average displacement of each particle per unit time, which was calculated using the NTA 3.0 software.52

Newly synthesized proteins
HSCs cells were plated and treated as indicated. After 6 days, control, senescent, senescent + C75 and senescent + 1 µM rapamycin cells were treated for 1 h with 2.5 µg/ml puromycin. Cells were collect with RIPA buffer. The incorporation of puromycin in the newly synthesized proteins was assessed by western blotting with an antipuromycin antibody.34

Liver mice tissue
Livers were taken from female C57BL/6J mice. All mice (4- and 25-months old) were maintained in the same housing with identical environmental conditions. Tissues were provided by the Tissue Bank provider ShARMUK.

Isolation of liver mitochondrial
Livers were taken from female C57BL/6J mice. All mice (4- and 25-months old) were maintained in the same housing with identical environmental conditions. Mitochondria were isolated as previously described.53

Apoptosis measurement
Apoptosis was quantified using the Annexin V, Alexa Fluor® 647 conjugate (ThermoFisher Scientific, Catalogue number A23204). Briefly, cell medium and cell pellets were collected in a fluorescence-activated cell sorting (FACS) tube. Annexin V-647 (25 µg/ml) was added to the cells. The cells were incubated for 10 min in the darkness at RT. DAPI staining solution (1 µg/ml) was then added and FACS was carried out using a BD FACS CANTO II flow cytometer.

Mitochondrial membrane potential
3,3′-dihexyloxacarbocyanine iodide (DiOC6) (Invitrogen, Catalogue number D273) staining was carried out as previously described.51 Briefly, cells were washed once in FACS buffer and then resuspended in 300 µL of the same buffer containing 4 nM DiOC6. Cells were then incubated for 30 min at RT, followed by one wash with PBS. Cells were resuspended in PBS containing 200 ng/mL DAPI staining.

Hydrogen peroxide assay (ROS)
Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher Scientific) was used to determine the amount of hydrogen peroxide (H2O2) present in cell samples as a marker for oxidative stress. Absorbance at 562 nm was measured using a Synergy HT Multi-Mode Microplate Reader.

Statistical analyses
All results are expressed as mean values ± SD or ±SEM of at least three independent experiments, except when otherwise indicated. The unpaired Student’s t-test and one-way analysis of variance were used to compare and identify statistically significant differences.

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