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Senolytic treatment preserves biliary regenerative capacity lost through cellular senescence during cold storage

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Overline: LIVER TRANSPLANTATION

One Sentence Summary. Damage caused to livers by pre-transplantation cold storage can be ameliorated by ablation of cellular senescence.

Abstract
Liver transplantation is the only curative option for patients with end-stage liver disease. Despite improvements in surgical techniques, non-anastomotic strictures (characterised by the progressive loss of biliary tract architecture) continue to occur after liver transplantation, negatively impacting liver function and frequently leading to graft loss and re-transplantation. To study the biological effects of organ preservation before liver transplantation, we generated murine models that recapitulate liver procurement and static cold storage.
In these models, we explored the response of cholangiocytes and hepatocytes to cold storage, focusing on responses that affect liver regeneration, including DNA damage, apoptosis and cellular senescence.

We show that biliary senescence is induced during organ retrieval and exacerbated during static cold storage, resulting in impaired biliary regeneration. We identified Decoy Receptor 2 (DCR2)-dependent responses in cholangiocytes and hepatocytes, which differentially affect the outcome of those populations during cold storage. Moreover, in vitro knockdown of DCR2 via CRISPR, increased cholangiocyte proliferation and decreased cellular senescence whilst having the opposite effect in hepatocytes.

Using the p21KO model to inhibit senescence onset, we showed that biliary tract architecture was better preserved during cold storage. Similar results were achieved by administering senolytic ABT737 to mice before procurement. Finally, we perfused senolytics into discarded human donor livers and showed that biliary architecture and regenerative capacities are better preserved.

Our results indicate that cholangiocytes are susceptible to senescence and identify the use of senolytics and the combination of senotherapies and machine-perfusion preservation to prevent this phenotype and reduce the incidence of biliary injury post-transplantation.
Introduction

Liver transplantation is the only curative option for patients with end-stage liver disease. Annually, over 6000 patients receive a liver transplant in Europe (1), 900 in the UK alone (2) and the accumulated incidence of chronic liver disease indicates that the need for donor livers will continue to rise (3).

After liver transplantation, patient survival depends on many different factors including graft function. In the initial phases the successful function of the graft depends on hepatocyte function and vasculature recovery, whereas in the long term, complications such as non-anastomotic biliary strictures (NAS) can have a profound impact on graft and patient survival (1, 4, 5). NAS are characterised by the progressive loss of biliary tract architecture (6), frequently resulting in graft loss (45.8% requiring re-transplantation) or death (25%) (7).

During the retrieval process, donor livers are perfused with preservation solutions, quickly removed, and cold stored for transport. After cold perfusion, extrahepatic bile ducts typically do not demonstrate signs of damage. However, after cold storage, a series of histological studies have shown that a high percentage of livers exhibited severe biliary epithelial loss, mural necrosis, and peribiliary vascular damage (8-10), indicating that the cold storage period may contribute to the deterioration of the biliary tract. Despite these histological observations, little is known about the underlying molecular mechanisms that lead to biliary damage during cold storage.

Recently developed preservation techniques, such as normo- or hypothermic ex situ perfusion technologies, have shown a 50% lower occurrence of graft injury, as measured by hepatocellular enzyme release. However, these studies have shown no significant difference in the incidence of NAS after perfusion (11, 12).

Cellular senescence is an irreversible cell cycle arrest implemented by the cell as a result of stressful insults (13). Characterised by phenotypic alterations, including secretome changes and genomic instability, senescent cells upregulate Decoy Receptor 2 (DCR2) (14, 15), a transmembrane receptor that belongs to the tumour necrosis factor receptor (TNFR) superfamily, and that is associated both with pro-apoptotic and anti-apoptotic pathways (16). Unlike other members of the TNFR family, DCR2 lacks the intracellular domain necessary for signal transduction, effectively inhibiting apoptosis (17). Therefore, DCR2 expression confers senescent cells resistance to extrinsic apoptosis (18, 19).

Accumulating experimental evidence indicates that senescence plays a relevant role in the occurrence and development of biliary pathology, exacerbating biliary injury, transmitting the senescent phenotype to surrounding hepatocytes, and impairing the regenerative response of the liver (20). Senescent cholangiocytes have been identified in liver allograft rejection and obstructive cholangiopathies (21, 22). Senescent cells have also been associated with detrimental roles in organ transplantation, limiting survival of cardiac allografts (23) and reducing the regenerative capacity of transplanted kidneys (24). Therefore, inhibition of senescence (either by targeting biological pathways or by pharmacological abrogation) has recently emerged as a potential approach to improve kidney and cardiac transplant outcomes (23, 24).

Although the detrimental presence of senescent cells in the liver has been thoroughly described (21, 22), the biological mechanisms of senescence in liver transplantation remain largely unknown. We hypothesized that biliary senescence is induced during organ retrieval and exacerbated during cold storage, resulting in impaired regenerative responses of the biliary tract. Senolytics (drugs that selectively clear senescent cells) might therefore be used to prevent this phenotype and reduce the incidence of biliary complications post-transplantation.
Here, we developed a mouse model of liver procurement and static cold storage that reproduces pre-transplantation stages. Using immunohistochemical techniques, we characterised the biliary and hepatocellular compartment. We cultured cholangiocytes extracted from livers exposed to 0 or 10 hours of cold storage to explore their in vitro proliferative capacities.

In these models, we explored the response of cholangiocytes and hepatocytes to cold storage at gene and protein level, focusing on responses that affect liver regeneration, including DNA damage, apoptosis and cellular senescence.

We found out that cholangiocytes express high levels of DCR2 during homeostasis and performed a mass spectrometry analysis to identify factors that interact with DCR2 in the context of liver cold storage. Via CRISPR, we explored the effects of DCR2 ablation in the proliferative and senescent response of cholangiocytes and hepatocytes.

To explore the functional effects of senescence in the biliary tract of donor livers, we exposed p21KO mouse livers to cold storage and administered the senolytic ABT737 to mice before liver procurement. Finally, we perfused senolytics into discarded human donor livers to study the regenerative and senescent response of the biliary tracts.

**Results**

**Increasing liver static cold preservation time alters murine biliary architecture.**

To address the physiological effects of organ preservation by static cold storage we designed a murine ex situ model that is representative of liver pre-transplantation (Fig 1A) (25). In a similar process to DBD (donation following brain death) organ procurement, the liver is perfused in situ with Universal Wisconsin (UW) preservation solution via the portal vein, and removed and submerged in UW in a cold receptacle for 1, 5 or 10 hours post-extraction.

Liver procurement in the setting of human organ donation is done by perfusing UW solution in the aorta, to allow simultaneous perfusion of all abdominal organs. However, access to the aorta in the mouse requires a microsurgical approach and, to ensure that perfusate enters the hepatic artery, ligation of aortic and coeliac artery branches may be required. Therefore, for the mouse procurement model, we perfused UW through the portal vein, with a good degree of perfusion (Fig S1).

Increasing cold storage time did not alter gross anatomy of the liver (Fig 1B), which histologically presented a preserved parenchyma with small areas of dead hepatocytes (Fig S2A) and accumulation of inflammatory cells (Fig S2B). Conversely, increasing time in cold storage altered bile duct morphology as assessed by haematoxylin-eosin (Fig 1C) and Keratin19 (K19) staining (Fig 1D). Alterations included impaired duct continuity and detachment of cholangiocytes into the lumen (Fig 1E,F; p < 0.01), indicating a compromised biliary architecture similar to that reported in human NAS (Fig S2C) (26).

Upon isolation, bile ducts from mouse livers maintained for 10 hours in cold storage showed significant growth delay in comparison with bile ducts directly isolated from fresh livers (0 hours of cold storage) (Fig 1G,H and Fig S2D). Furthermore, the percentage of cholangiocytes undergoing proliferation significantly decreased in bile ducts isolated after 10 hours of cold storage (assessed by Ki67; p < 0.01, and PCNA; p < 0.001; Fig 1I) whereas markers associated with stemness capacity such as LGR5 (27) displayed no significant changes (Fig 1I; p = 0.1607).

Our model of cold storage also displayed a significant increase of α-SMA-positive fibroblasts surrounding K19-positive bile ducts (Fig S3A; p < 0.05,) but no significant changes in collagen deposition
The extent of fibrosis remained unchanged compared to 0 hours of cold storage, as assessed by Picrosirius Red (PiSR) quantification (Fig S3C). The total number of F4/80-positive macrophages significantly increased during the first hour of cold storage ($p<0.05$) and plateaued after the initial increase (Fig S3D,E), whereas the inflammatory infiltrates proximal to the K19-positive cholangiocytes appeared to be actively proliferating as indicated by the presence of Ki67 (Fig S3F).

To provide a comprehensive view of the clinical scenarios of deceased donations, we modelled DBD and DCD (donation after circulatory death) procurements and cold storage (Fig S4A). Haematoxylin & Eosin staining revealed similar degrees of bile duct damage (Fig S4B) and mononuclear cell infiltration (Fig S4C) for both DBD and DCD procurements. However, DCD presented more necrosis in the hepatic parenchyma in comparison with the DBD model (Fig S4C). The time points assessed after DCD procurement (10 min warm ischemic time (WIT), WIT + 5 hours of cold storage and WIT + 10 hours of cold storage) displayed no significant changes in proliferation (assessed by PCNA, Fig S4D), DNA damage response (H2A.X, Fig S4E), or DCR2 abundance (Fig S4F) for either cholangiocytes or hepatocytes.

Comparative histological analysis between DBD and DCD procurement at all time points suggested that DCD livers displayed a higher percentage of proliferating cholangiocytes at all time points in comparison with DBD procurement (Fig S5A), suggesting they may actively proliferate in response to the WIT. However, cholangiocytes in DCD procurements also displayed higher amounts of DNA damage marker H2A.X (Fig S5B), senescence marker DCR2 (Fig S5C), and TUNEL (Fig S5D), suggesting that this proliferative response is accompanied by cellular damage.

Overall, our model of DCD procurement presents more damage than DBD, with an accelerated period of damage during the WIT. Altogether, these data suggest that, independent on the procurement model, the hepatocyte and cholangiocyte compartments respond differently to static cold storage conditions, which severely damaged the cholangiocyte population contributing to the collapse and destruction of the bile ducts.
Mouse cholangiocytes and hepatocytes present different senescent and apoptotic responses during static cold storage.

To test whether the presence of anatomical abnormalities impairs the regenerative response of the biliary tract (10), we first explored the proliferative capacity of cholangiocytes during cold storage. Ki67 expression was significantly increased in cholangiocytes at 5 hours (p<0.05), albeit by a small amount (4.97% ± 0.6850 mean ± SEM of total cholangiocytes), suggesting that this population starts proliferating in response to cold storage (Fig 2A and Fig S6A). Hepatocytes showed no significant change in proliferation (p=0.2058) during cold storage (Fig S6A).

Next, we examined the presence of cellular senescence. In contrast to their low proliferation, a large percentage of cholangiocytes (41.68% ± 3.298) expressed H2A.X (an established marker of DNA damage and senescence (28)) after 5 hours of cold storage (Fig 2B and Fig S6B; p<0.05). Similarly, hepatocytes had increased abundance of H2A.X in comparison with the 0-hour control group (Fig S6B; p<0.05). Bile ducts presented increased abundances of markers associated with cell cycle arrest (p21, Fig S6C; p16, Fig S6D), DNA damage (H2A.X, Fig S6E) and senescence (DCR2, Fig S6F), all suggestive of a cellular senescence response (13).

We further explored this senescent phenotype by assessing gene expression changes in cholangiocytes and hepatocytes isolated from mouse healthy livers and livers subjected to 10 hours of cold storage (Fig S7A). Isolated bile ducts (Fig S7B) express known markers of cholangiocytes (EpCAM and Keratin 19) with minimal contamination by α-SMA myofibroblasts or F4/80 macrophages (Fig S7C). Isolated hepatocytes (Fig S7D) expressed HNF4α and CYP2D6, with minimal contamination from other cell populations (Fig S7E). At the mRNA level, as expected, bile ducts highly expressed Krt19 (p<0.05) and lowly expressed Hnf4a (p<0.05), in contrast to hepatocytes (Fig S7F). qRT-PCR of Itgam suggested low macrophage contamination for both bile ducts and hepatocytes (Fig S7F).

After 10 hours of cold storage bile ducts had significantly increased levels of Cdkn1a (p21; p<0.001), Cdkn2a (p16; p<0.001), Rb1 (p<0.01) (Fig 2C). Bile ducts significantly increased antiapoptotic Bcl2l1 expression (BCL-XL, p<0.05) after 10 hours of cold storage whereas hepatocytes showed increased Casp3 (p<0.01) (Fig 2D). Bile ducts displayed increased Aldh3a1 (p<0.001), Tnf (p<0.001), and Trp53bp1 (p<0.01) whereas hepatocytes showed no changes in senescence inductors (Fig 2E). Senescence-Associated Secretory Phenotype (SASP) markers, including Tgfb1 (p<0.01), Tgfb2 (p<0.001), Tgfb3 (p<0.01), I2b1 (p<0.001), Cxcl1 (p<0.001) and Coll (p<0.05), also increased in the cold-stored bile duct group (Fig 2F).

To test the functional capacities of these cholangiocytes, we isolated bile ducts from control livers (0 hours of cold storage), DBD livers at 10 hours of cold storage, and DCD livers (10 min WIT + 10 hours cold storage), and cultured them as organoids for 1 week. Then we assessed their function by evaluating their ability to efflux Rhodamine 123, a fluorescent substrate that measures the activity of the multidrug resistance protein 1 (MDR1) biliary transporter (29). Organoids grown from bile ducts isolated from control livers presented a highly efficient efflux of Rhodamine 123 into the lumen (Fig S8A). However, this efflux was significantly impaired in DBD-organoids and the effect was even more pronounced in DCD-organoids (Fig S8B). This suggests that both DBD and DCD procurement affect the functional capacities of bile ducts, with bile ducts procure after DCD being the most affected. Supporting the hypothesis of a cellular senescence response, we observed an accumulation of Senescence Associated-β-Gal (SAbGal) staining in DBD- and DCD-organoids in comparison with control organoids (Fig S8C). These organoids also displayed significantly increased DCR2 (p<0.05), and a non-significant increase for other markers of senescence such as p16 and p21 (Fig S8D).

Altogether, these data suggest that although a small proportion of cholangiocytes starts to proliferate during cold storage, the vast majority of these cells shows signs of DNA damage and cellular

[94x714]Mouse cholangiocytes and hepatocytes present different senescent and apoptotic responses during static cold storage.

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Altogether, these data suggest that although a small proportion of cholangiocytes starts to proliferate during cold storage, the vast majority of these cells shows signs of DNA damage and cellular
senescence. Furthermore, cholangiocytes and hepatocytes present a differential response with cholangiocytes prone to establish cellular senescence in response to long periods of cold storage whereas hepatocytes upregulate apoptosis-related factors.
Decoy Receptor 2 (DCR2) expression is characteristic of murine and human cholangiocytes during homeostasis and exacerbated during cold storage.

In a context of cellular stress, cells can either enter apoptosis (a form of programmed cell death (30)) or cellular senescence, an irreversible cell cycle arrest driven by dominant cell-cycle inhibitors and characterised by changes in morphology, DNA damage response (31), and the activation of the SASP (13, 32). To maintain a senescent response, cells must actively block apoptosis by engaging with several regulatory factors to ensure this blockage (17). Of particular interest is Decoy Receptor-2 (DCR2, TRAIL-R4, TRUNND) a member of the TNF-receptor superfamily and encoded by TNFRSF10D. DCR2 is a well-known marker of cellular senescence and a pivotal factor at the crossroad between apoptotic and senescent pathways (33, 34) (Fig S9A).

To study in detail the differential response between senescence and apoptosis in cholangiocytes and hepatocytes, we stained cold-stored livers for Caspase-3, a direct effector of apoptosis. Caspase-3 abundance remained unchanged in cholangiocytes but significantly increased in hepatocytes (p<0.01, Fig 3A,B and Fig S9B,C). Cleaved-Caspase-3, the active form of Caspase-3, was present in hepatocytes at 10 hours of cold storage (Fig 3B), suggesting that apoptotic-related mechanisms are active in hepatocytes but not in cholangiocytes. TUNEL staining also confirmed that hepatocytes significantly increased apoptosis with increasing cold storage time (p<0.05), whereas K19-positive cholangiocytes did not (Fig S9D,E). The loss of FADD (FAS-Associating Death Domain, an apoptosis adaptor required for activation of cell death (35, 36)) in hepatocyte nuclei provided further evidence that this population is prone to apoptosis (Fig S10). In contrast, DCR2 expression was significantly higher in cholangiocytes than hepatocytes during cold storage (Fig 3C-E and Fig S11A,B; p<0.05), suggesting that bile ducts implement senescence rather than apoptosis. Increased expression of DCR2 was also found in bile ducts isolated from livers maintained for 10 hours in cold storage and grown as three-dimensional organoids in culture (p<0.001) (Fig S11C-D).

We found that prior to cold storage, fresh cholangiocytes already expressed high amounts of DCR2 (26.05% ± 6.497 in vivo and 41.36% ± 2.467 in isolated bile ducts (Fig S11B,C)) whereas hepatocytes did not (Fig 3C-E and Fig S11B), and this was also reflected in isolated normal human cholangiocytes (NHC) in culture (37, 38) when compared to human hepatocytes (HepG2) (Fig S11E). A similar response was found in mouse EpCAM-positive FACS-sorted cholangiocytes and subpopulations of cholangiocytes such as Hepatic Progenitor Cells (39) in 2D and 3D cultures, where cholangiocytes had higher constitutive DCR2 expression in comparison with primary hepatocytes (Fig S12A-D).

To test the functional response of DCR2 in cholangiocytes, we exposed human cholangiocytes (NHC) and human hepatocytes (HepG2) to increasing concentrations of Fas-Ligand (FasL, a member of the TNF-family that induces apoptosis upon binding to its receptor, as represented in Fig S9A). As expected (40, 41), hepatocytes undergo cell death when exposed to Fas-ligand (Fig S12E). However, cholangiocytes were unresponsive to FasL-exposure (Fig S12F), suggesting that DCR2 expression in this population confers cholangiocytes protection against stressful/lethal stimuli.

To understand if homeostatic DCR2 expression is restricted to cholangiocytes or is ubiquitous to all epithelial cells indistinctly of the organ, we performed immunostaining on a panel of mouse tissue (Fig S13 and Fig S14A) and found DCR2 was highly expressed in (but not restricted to) epithelial surfaces of the gut, stomach, kidney, bladder, heart, and eye (Fig S14B). Because DCR2 has been shown to protect cells from apoptosis (17, 19), this pattern of epithelial expression suggests that certain tissues have constitutive high DCR2 to protect them from damaging endo/exogenous agents (for example cholangiocytes in bile ducts, which are continuously challenged by toxic factors present in bile). Our results suggest that cholangiocytes have high constitutive DCR2 expression and are inherently prone to enter cellular senescence after stressful insults such as cold storage. In contrast, low DCR2
expression in hepatocytes may trigger apoptotic pathways and death upon cold storage-induced injury.
DCR2 engages apoptotic-related pathways in hepatocytes, but sustains a cellular senescence-related response in cholangiocytes.

To explore the functional effects of DCR2 in cholangiocytes, we subjected mouse livers to 0 or 10 hours of cold storage, isolated cholangiocytes and hepatocytes from those livers, and performed an immunoprecipitation for DCR2. Mass spectrometry analysis of these samples allowed us to identify proteins that interact with DCR2, indicating potential functional effects (data Files S1-3). In hepatocytes, at 0 hours of cold storage, DCR2 potentially interacted with 80 factors that exclusively bind to hepatocytes but not to cholangiocytes (Fig 4A). These factors included known regulators of P53 (RPS26 (42), RPS27A (43)), members of the death receptor signalling pathways (HADHA (44), EHHADH (45)) and regulators of programmed cell death (SEC61b (46)) (fig S15A) (for the complete list of 80 factors, check data Files S1-3). Functional analysis revealed that these factors were significantly associated with biological processes including negative regulation of cell death (FDR=0.0019) and negative regulation of apoptotic process (FDR=0.0058) (fig S15A).

We found 51 cholangiocyte-specific potential interactors, including factors known to suppress apoptosis (HSP90AB1 (47)), regulate necrosis (HSP90AA1 (48)) and increase cell viability (MIF (49,50)) (Fig 4A, fig S15B) (for the complete list of 51 markers, check data Files S1-3). These factors that potentially bind to DCR2 in cholangiocytes are significantly associated with negative regulation of cell cycle arrest (FDR=0.0035), regulation of response to DNA damage stimulus (FDR=0.0429), and response to stress (FDR=0.0108), among others (fig S15B). Further pathway analysis indicated that, in hepatocytes, DCR2 potentially interacts with factors associated with programmed cell death, regulation of p53 activity, and the cell cycle, whereas in cholangiocytes DCR2 binds to suppressors of apoptosis and regulators of necrosis, among others (fig S15C).

After 10 hours of cold storage, 145 factors were found to potentially interact with DCR2 in hepatocytes but not cholangiocytes (Fig 4B). Together, these factors were significantly associated with the regulation of intrinsic apoptotic signalling (FDR=0.0179), response to stress (FDR=0.0204), and the processing of SMDT1 (FDR=0.0038) which has been shown to inhibit cell proliferation and induce apoptosis (51) (Fig 4B and fig S16A). We found 77 cholangiocyte-specific potential DCR2 interactors, significantly associated with biological processes including negative regulation of telomere maintenance (FDR=2.76e-05), negative regulation of oxidative stress-induced apoptosis (such as Nono (52) and Trap1 (53); FDR=0.0064), DNA repair elements (FDR=0.0135), and proteins that regulate cell cycle (FDR=0.0405) (Fig 4B and fig S16B). Overall, at 10 hours of cold storage, our analysis indicated that DCR2 may interact with apoptosis-related factors in hepatocytes and may bind elements of cellular senescence in cholangiocytes (fig S16C).

Because DCR2 is poorly conserved, hindering direct mouse-to-human comparison (54) (fig S17A), we focused on murine DCR2 interactors that have a conserved DCR2-link in Homo sapiens (fig S17B and data file S4). Of particular interest are Tripartite Motif-containing 28 (TRIM28, also known as KAP1) and the macrophage migration inhibitory factor (MIF, also known as GIF). The interaction of these factors with DCR2 appears to be conserved both in mouse and human (Fig 4A,B and fig S17B) and they retain a high degree of conservation between species, both at the domain and sequence level (fig S17C,D).

TRIM28, a known direct regulator of senescence (55), binds to DCR2 specifically in cholangiocytes at 0 hours of cold storage, suggesting that this is a conserved link for cholangiocytes during homeostasis (Fig 4A,C). However, DCR2 and TRIM28 were found to be uncoupled in cholangiocytes after 10 hours of cold storage (Fig 4B,C). TRIM28 abundance did not significantly increase in cholangiocytes during cold storage (Fig 4D,E and fig S18A), suggesting that any potential effects of TRIM28 on the senescent phenotype are dependent on the uncoupling of DCR2 and TRIM28. In contrast, DCR2 and TRIM28 did
not interact in hepatocytes, although overall TRIM28 abundance significantly increased in hepatocytes during cold storage ($p<0.05$; Fig 4E and fig S18A).

MIF is an inflammatory cytokine that has been shown to prevent cellular senescence in mesenchymal cells through the PI3K-AKT signalling pathway (56), to delay senescence as an effector of the hypoxia-inducible factor HIF1α (57), and to mediate hepatocyte protection in models of hepatotoxin-induced liver fibrosis (58). In cholangiocytes, we found DCR2 interacted with MIF at 0 hours but uncoupled after 10 hours of cold storage, whereas in hepatocytes we found the opposite effect: DCR2 uncoupled from MIF at 0 hours and started interacting with MIF after 10 hours (Fig 4F). MIF abundance did not significantly change in cholangiocytes (Fig 4G,H and fig S18B) but significantly increased in hepatocytes over the course of cold storage ($p<0.05$; Fig 4H and fig S18B), suggesting that any hepatoprotective effect might be directly linked to the DCR2-MIF interaction.

Computational protein inference of the DCR2 amino acid sequence led to a predicted structure model (Fig 4I; fig S18C) and associated equipotential map (Fig 4J) that shows the major forces of hydrophobic effect in folding, electrostatic interactions and hot spots for binding (59). Together, these identify potential areas of interaction for DCR2 with TRIM28 and MIF.

Altogether, our results suggest that DCR2 preferentially interacts with proteins associated with apoptotic related pathways in hepatocytes, but with proteins associated with a senescent response in cholangiocytes: DCR2 is potentially coupling and uncoupling from MIF and TRIM28 in a differential manner in cholangiocytes and hepatocytes during cold storage.
**DCR2 loss increases proliferation in cholangiocytes.**

To explore the functional role of DCR2, we next knocked down DCR2 *in vitro* using CRISPR editing. First we knocked down DCR2 in the NHC line (normal human cholangiocytes (Fig 5A)). In comparison with DCR2-WT cells, DCR2-KO cholangiocytes have significantly increased MKI67 mRNA (Ki67; *p*<0.05) and decreased CDKN1A mRNA (p21; *p*<0.01), but no significant difference in BCL2 (BCL-2; *p*=0.1953) or TP53 (p53; *p*=0.1909) (Fig 5B), potentially indicating that DCR2-KO cholangiocytes are prone to proliferate while supressing senescence-associated programmes. DCR2-KO cholangiocytes also showed a higher basal percentage of BrdU (p<0.01; Fig 5C).

For a comparative analysis, we also knocked down DCR2 in human hepatocytes (HepG2 line) (Fig 5D). No morphological changes were observed between DCR2-KO and DCR2-WT, for either cholangiocytes or hepatocytes (Fig 5E). Consistent with the pro-proliferative pattern seen in DCR2-KO NHC, we found decreased DCR2 in cholangiocytes during mouse ductular reaction (Fig 5F,G), a phenomenon in which the cholangiocyte compartment proliferates, expands and contributes to the repair of the liver parenchyma when the proliferative capacity of the hepatocytes is compromised (62). We observed no ductular reaction in healthy, sham-operated controls or 48 hours post-partial hepatectomy (a model of hepatocyte-driven regeneration where little ductular reaction is observed (Fig 5F,G). Ductular reaction is a consistent phenomenon across different types of liver damage including dietary models 3,5-diethoxycarbonyl-1,4-dihydrocolidine (DDC), and choline-deficient diet supplemented with ethionine (CDE), but barely present in healthy or regenerating livers (partial hepatectomy) (Fig 5H). DCR2 levels significantly decrease in the expanding EpCAM-positive cholangiocytes (Fig 5I,J). Conversely, DCR2 in hepatocytes increases with the expansion of EpCAM-positive cholangiocytes (Fig 5I,J). This expression pattern suggests that cholangiocytes downregulate DCR2 for proliferation and expansion, while hepatocytes accumulate DCR2 in response to a damage that impairs their proliferative response.

Upon etoposide exposure, a significantly higher percentage of DCR2-WT cholangiocytes were observed to be SAbGal-positive, whilst DCR2-WT hepatocytes showed no significant change in their response (Fig S19A,B). After DCR2 knockdown, we observed no significant differences in the percentage of SAbGal-positive DCR-KO cholangiocytes or hepatocytes in the senescence response to etoposide exposure (Fig S19B).

As shown previously, DCR2-WT cholangiocytes were unresponsive to Fas-Ligand exposure (see Fig S12F). Conversely, hepatocytes were sensitive to Fas-L exposure, resulting in increased cell death (Fig S19C). DCR2-KO cholangiocytes exposed to FasL consistently decreased mRNA of senescence markers p53, p21, and p16 in comparison with DCR2-WT cholangiocytes (Fig S19D). In contrast, DCR2-KO hepatocytes increased markers of senescence upon FasL exposure (Fig S19D), suggesting that DCR2 plays a pivotal role in the shift between senescence and apoptosis for these two populations.

The percentage of DCR2-KO cholangiocytes undergoing cell death (as measured by DRAQ7) significantly increased upon FasL exposure (*p*<0.001, Fig S19E). Conversely, hepatocytes that previously responded to FasL-induced cell death lost this ability upon DCR2 knockdown (Fig S19E). We noted that despite the increase of cell death in DCR2-KO cholangiocytes upon FasL exposure, CASP3 mRNA decreased (Fig S19D), suggesting engagement with alternative pathways for apoptosis induction. Together, these data suggest that DCR2 protects cholangiocytes from apoptosis by implementing senescence in the presence of stressful stimuli. In hepatocytes, knockdown of DCR2 results in increased apoptosis, suggesting a highly complex, cell-dependent scenario for DCR2.
Inhibition of cellular senescence decreases DCR2 and improves preservation of biliary anatomy during cold storage.

To explore the consequences of sustained cellular senescence and DCR2 expression in bile ducts, we used the tamoxifen-inducible K19-Mdm2\textsuperscript{flox/flox} mouse model (\textbf{fig S20A}) (20). Murine Double Minute 2 (\textit{Mdm2}) is a key negative regulator of p53, that positively regulates p21 expression. Therefore, cholangiocyte-specific (via Keratin19Cre\textsuperscript{ERT2}) deletion of \textit{Mdm2} results in p21 expression in bile ducts and cellular senescence (\textbf{fig S20B}) (20). Induction of the K19-Mdm2\textsuperscript{flox/flox} model resulted in p21-driven cholangiocyte senescence and sustained DCR2 expression in bile ducts and adjacent hepatocytes within 12 weeks (\textbf{fig S20C}). Furthermore, the K19-Mdm2\textsuperscript{flox/flox} model displayed altered biliary architecture resembling non-anastomotic stricture injury (\textbf{fig S20C}) (26).

Non-anastomotic strictures (NAS) are a type of biliary complication found after liver transplantation that severely impair the structure and function of the biliary tract (26,63). NAS account for significant morbidity and mortality, frequently requiring a second transplantation (26). At the histological level, NAS present moderate to severe loss of the luminal epithelial lining of the bile ducts (26) and lack of regeneration (10). Our set of human NAS displayed biliary anatomy disruption (\textbf{Fig 6A}) and NAS-cholangiocytes decreased in proliferation (\textit{p}=0.0501), as assessed by Ki67 staining (\textbf{Fig 6B}). NAS-hepatocytes showed no significant changes in proliferation in comparison with healthy livers (\textbf{Fig 6B}). DCR2 was significantly increased in both cholangiocytes (\textit{p}<0.001) and hepatocytes (\textit{p}<0.05) in NAS in comparison to healthy livers (\textbf{Fig 6B}), potentially indicating a disrupted regenerative response and presence of senescence mechanisms.

To test the regenerative capacities of the biliary tract in absence of a competent senescence response, we applied the cold storage setting to livers obtained from the p21\textsuperscript{KO} murine model (64). p21\textsuperscript{KO} mice presented normal biliary tracts after 10 hours of cold storage (\textbf{Fig 6C}) and significantly increased proliferative marker Ki67 in cholangiocytes (\textit{p}<0.05, \textbf{Fig 6D}). In contrast with the WT model, in the p21KO mouse the percentage of cholangiocytes and hepatocytes expressing DCR2 does not increase during cold storage (\textbf{Fig 6D} and \textbf{fig S21A}) although a significant increase in the extent of DNA damage response (assessed by H2AX immunofluorescence) was observed for both cholangiocytes and hepatocytes (\textbf{fig S21B}). These data suggest that the cold storage-driven damage response is still present in the liver. However, in the absence of senescence (and DCR2 response) the biliary compartment retained its proliferative and regenerative capacities, indicating the importance of cellular senescence in the development of biliary injury. TUNEL and Caspase3 abundance in cholangiocytes and hepatocytes after 10 hours of cold storage showed no significant change (\textbf{fig S21C,D}), suggesting that DCR2 effects are uncoupled from the senescence-apoptosis balance in the p21KO model.

Next, we explored whether pharmacologically disrupting cellular senescence during mouse liver cold storage improved biliary preservation. Senolytic regimes that selectively clear senescent cells, such as ABT737 (65) and the combination of Dasatinib plus Quercetin (D+Q) (66) were administered to the mice, before liver procurement and cold storage (\textbf{fig S22A}). Upon ABT737 administration, livers subjected to cold storage displayed less biliary damage and fewer areas of hepatocellular death (\textbf{Fig 6E}). Bile ducts isolated from livers treated with ABT737 or D+Q prior cold storage and grown as organoids exhibited increased growth rates (\textbf{fig S22B} and \textbf{C}, respectively) and displayed lower amounts of p21 and p16 (\textbf{fig S22D}).

DCR2 abundance significantly decreased \textit{in vivo} after ABT737 treatment (\textit{p}<0.001; \textbf{Fig 6F}), as well as \textit{in vitro}, as seen in bile ducts isolated from cold stored livers after senolytic treatment (\textbf{fig S22E,F}). The
percentage of cholangiocytes undergoing proliferation following senolytic treatment significantly increased in vivo (Fig 6F; \( p < 0.01 \)) as well as in bile ducts isolated after 10 hours of cold storage (Fig S22F; \( p < 0.01 \)), whereas LGR5 (27) displayed no significant changes (Fig 6F and Fig S22F). p21 immunohistochemistry in cold stored livers suggests that senolytic-treated mice have fewer senescent cholangiocytes (Fig S22G). We note that livers of senolytic-treated mice had less H2A.X after 10 hours of cold storage (Fig S22H), suggesting that ABT737 may have a protective effect against stressful stimuli, which may account for the decreased cellular senescence and improved biliary regeneration seen in this model.

To test the functional effects of senolytics upon ischemic insults in vivo, we administered ABT737 to mice for a week and then induced ischemic injury by clamping the hepatic artery, portal vein, and common bile duct of the mouse. After 45 min of hepatic ischemia, the clamp was removed allowing reperfusion of the ischemic tissues (ischemia-reperfusion injury, IRI; (67)) (fig S23A). At 48 hours after the ischemia upon senolytic treatment, we did not observe differences in the morphology of the biliary tract or the extent of parenchymal damage (fig S23B). However, transaminases showed a significant reduction of AST at 24 hours (\( p < 0.001 \)) and cholesterol at 48 hours (\( p < 0.05 \)) (fig S23C), indicating that inhibition of senescence during IRI may be beneficial in restoring liver function. Senolytic treatment before IRI significantly increased proliferation in hepatocytes (\( p < 0.001 \)), although this increase was not significant for cholangiocytes (fig S23D). The increased apoptotic marker Caspase 3 in both cholangiocytes and hepatocytes (\( p < 0.001 \) and \( p < 0.01 \) respectively, fig S23E) may indicate a shift from senescence into apoptosis, as expected from treatment with senolytics. Last, we observed decreased DCR2 abundance in cholangiocytes (\( p < 0.05; \) fig S23F), similar to the reduction observed in cold stored livers procured from ABT737-treated mice.

Administration of senolytics to a potential donor using this regime is not feasible with the current strategies for liver donation after circulatory arrest. An alternative option could be to perfuse the donor livers with senolytics during procurement and keep the organs submerged in senolytic-supplemented solution during cold storage (fig S24A). We have explored this strategy and found that it significantly increased proliferation of bile ducts and hepatocytes (fig S24B). However, we found no significant differences in DCR2 abundance in bile ducts (\( p = 0.0599 \), fig S24C), suggesting that this method may not be sufficient to prevent biliary damage during cold storage.

Current efforts to implement machine perfusion for liver transplantation represent an exciting leap forward for the reconditioning of donor livers (for example, administration of cell therapy for biliary disease (68)). To prove the feasibility of this approach for the administration of senolytics, we tested first the effects of ex vivo machine perfusion on DCR2 using a pre-clinical, proof of concept hypothermic perfusion (without oxygenation) in mouse DCD livers (HMP-DCD, fig S25A). Our experimental setting included two peristaltic pumps (one to cool the chamber where the liver is placed and the second to perfuse the liver) (fig S25B,C) that keep the system below 10°C for the whole perfusion (fig S25D). After perfusion, H&E showed similar degrees of damage in the bile ducts of DCD livers and HMP-DCD livers, with shedding of cholangiocytes into the lumen and destruction of biliary architecture (Fig S25E). The cholangiocytes of HMP-DCD livers had an increased proliferation rate (as assessed by Ki67, \( p < 0.05 \)) but no significant changes in DCR2 were observed (fig S25F), suggesting that damage, once present in cholangiocytes, cannot be reversed by perfusion alone.
We next tested the clinical value of targeting cellular senescence in discarded human livers during organ preservation, by administering D+Q in a proof-of-concept continuous perfusion. Briefly, following Couinaud anatomical classification, segments II and III were dissected and perfused normothermically for 2 hours with D+Q (one segment) or with vehicle solution (the other segment) (fig S26A-B). In the same liver, the D+Q perfused segment showed significantly decreased DCR2 abundance in cholangiocytes in comparison with the vehicle segment (p<0.01; Fig 6G,H) but no significant increase in cholangiocyte proliferation (p=0.0735; Fig 6H). The number of TUNEL-positive cholangiocytes (potentially shifting from senescence to apoptosis) showed a significant increase in D+Q perfused segments, but not in hepatocytes in D+Q perfused segments in comparison with vehicle segments (Fig 6H). Analysis of liver segments prior and after D+Q perfusion (fig S26C) showed that D+Q perfused segments had better preserved biliary tracts (fig S26D). The number of TUNEL-positive cholangiocytes increased (although not significantly, as we present individual values for each liver) in both cholangiocytes (p=0.3615) and hepatocytes (p=1763) (Fig S26E). D+Q livers had two-fold increase in the levels of cholangiocyte proliferation (p=0.0971) and one-fold decrease in the levels of DCR2 (p=0.1083). A similar increase in proliferation and decrease in DCR2 levels was found in hepatocytes of D+Q livers (Fig S26E).

Altogether, these results indicate that increased DCR2 is characteristic of detrimental post-transplantation phenotypes such as NAS. Senescence ablation, through senolytic intervention prior-to-transplantation, improves preservation of the liver, maintaining morphology of the biliary tract and improving proliferation whilst decreasing DCR2 abundance.
Discussion

Regarded as the ‘Achilles heel of orthotopic liver transplantation’ (69), non-anastomotic strictures (NAS) are responsible for significant morbidity and mortality, frequently resulting in graft loss (45.8%), re-transplantation, or death (25%) (7). Surgical advances and incorporation of techniques such as endoscopic retrograde cholangio-pancreatography (ERCP) and percutaneous transhepatic cholangiogram (PTC) offer temporising treatments (69). However, these are not definitive treatments, optimal management has not been defined, and NAS continue to represent one of the most challenging complications after liver transplantation.

Although the pathological mechanisms remain unknown, previous studies have suggested two potential groups of risk factors: preservation injury-related factors and variables related to immunological processes (70). Prolonged ischemia prior to transplantation has been hypothesized to be a leading cause for NAS development; in fact, liver grafts recovered from Donations following Circulatory Death (DCD), which have an additional warm-ischemic phase in comparison with livers obtained from Donations following Brain Death (DBD), present up to 44% increase of NAS (compared to only 3% in grafts retrieved from DBD) (7, 71, 72). Similar to warm ischemia, extended cold ischemia prior to transplantation has been associated with increased NAS rates (72). At the end of the preservation phase, a high percentage of livers already present biliary damage (8-10), indicating that this period might contribute to the deterioration of the biliary tract and highlighting the absence/insufficient biliary regeneration after transplantation.

Cellular senescence provides an irreversible barrier to cell cycle progression to prevent undesired proliferation. However, under pathological circumstances, senescence can adversely affect organ function and viability by impacting the regenerative response of the cell and its environment (13). Cellular senescence has been previously associated with kidney allograft dysfunction (73) and cardiac failure after transplantation (23), whereas the ablation of the senescent response (by genetic (24) or pharmacological approaches (23)) has proved to be beneficial for the survival of the graft and transplant outcome.

Regarding the induction of senescence, in the context of liver preservation, ischemia-reperfusion is an important source of oxidative stress (74), a well-known inductor of senescence in a variety of tissues (13, 75). Moreover, the discrepancy between the number of donor livers and recipients has forced an expansion of the donor pool by accepting donor grafts from elderly donors, which present substantially increased NAS rates with donor age over 70 years (76). Senescent cells accumulate in ageing organisms (77) being a critical determinant for liver function in elderly patients (78). However, the exact mechanisms of senescence onset during cold storage remain to be elucidated.

Although the presence of senescent cholangiocytes has been associated with increased rejection after liver transplant (22), little is known about the underlying molecular mechanisms that lead to biliary damage during cold storage, hindering the development of potential therapies for transplanted patients. Here, we propose cellular senescence as a detrimental mechanism that would onset during the preservation phase and negatively impact the performance and regenerative abilities of the biliary tract post-transplantation. Presence of senescence, primarily assessed by DCR2, might provide a rational explanation for the lack of regenerative mechanisms in bile ducts post-transplantation.

Decoy Receptor 2 (DCR2) is a well-known marker of senescence (13), and a crucial element of the extrinsic apoptotic response (16). The cell surface decoy receptor proteins (DCR1 also known as TRAIL-R3, and DCR2 or TRAIL-R4) belong to the tumour necrosis factor receptor (TNFR) superfamily, characterized by the ability to bind tumour necrosis factors (TNFs) via an extracellular cysteine-rich domain, in order to induce caspase-dependent cell death. As opposed to the other members of this family, DCR1 and DCR2 inhibit Fas-ligand, TRAIL and TNFα death signals. However, DCR2 confers a complete long-term resistance to death (whereas DCR1 only offers 30% inhibition at short term),
through the combination of a truncated death domain incapable of signalling and the recruitment of adaptor proteins that form an heterocomplex which might result in an incomplete death-inducing signalling complex (79, 80).

DCR2 has long been considered a biomarker of senescence (13, 14), being a direct target of p53 (18) and associated with the activation of NFκB (80) (a major transcription factor that determines the induction of the senescence-associated secretory phenotype (SASP) (81)). DCR2 is therefore a pivotal factor between senescence and apoptotic crossroads, protecting senescent cells from death (19) while maintaining the senescent phenotype (13).

Our results indicate that DCR2 exerts differential roles in cholangiocytes and hepatocytes during cold storage, as indicated by the functional interactions of DCR2 with other senescent factors in cholangiocytes, and with apoptotic-related factors in hepatocytes. Altogether, this suggests that cholangiocytes are particularly prone to become senescent during the stress conditions of cold preservation while hepatocytes are directed towards apoptosis. However, the regenerative capacity of the liver parenchyma, in which the remaining hepatocytes after a 70% partial hepatectomy are able to restore the whole organ (82), might indicate that the presence of a minimal group of viable hepatocytes is sufficient to retain liver function, while proliferating to repair damaged structures and resume normal activity.

Our results also suggest that, in homeostasis, DCR2 is already present in a high percentage of cholangiocytes, suggesting that DCR2 provides a protective mechanism towards endogenous/exogenous toxic substances such as bile. Other epithelial surfaces (including those of stomach, bladder, and gut among others) also presented elevated constitutive abundance of DCR2. Whether this represents a senescent response, a degree of protection from potentially damaging substances, or a standby position of the cells to implement senescence when confronted to stressful signals requires further study. Our results also indicate that DCR2 abundance is reduced during biliary proliferation (such as in ductular reaction response during liver damage), and that DCR2-knockdown results in increased proliferation. Altogether, these results suggest that DCR2 loss is required for cholangiocytes to start a regenerative response. In fact, lack of expression of DCR1 and DCR2 is characteristic of adult and paediatric neuroblastomas (83), characterised by rapid uncontrolled proliferation of nerve cells.

The main limitation of our study is that DCR2 is poorly conserved at the protein level between mouse and human (54). The lack of structural similarities has, to date, hindered comparative studies and the use of murine models for the study of this marker in the context of senescence. However, our results indicate that, at a functional level, DCR2 interacts with the same factors in mouse and human, suggesting its use as a universal senescence marker and potential treatable target in experimental models. However, because abundance of MIF and TRIM28 did not change during cold storage, our data suggest that DCR2 might be a pivotal factor in the development of the phenotype. Furthermore, despite the low homology between mouse and human DCR2, the similarity of interactors found in both species indicate that, at functional level, the role of DCR2 might be conserved between these two species. However, the functional and mechanistic effects of DCR2 interactions are not yet fully understood and limited in this study by potential secondary interactors, antibody specificity and sensitivity of the techniques used. Further experimental work will be required to fully elucidate these interactions.

DCR2 also possess a particular interest for the detection of senescent cells: as a cell surface marker it would allow identification, quantification and isolation without disrupting the cell. Furthermore, in diabetic nephropathy (characterised by the presence of senescent renal tubular epithelial cells), DCR2 ectodomain has been shown to shear, being detected in urine (84), highlighting the properties of DCR2 as a non-invasive biomarker of senescence.
Several studies have suggested that depletion of senescent cells increases overall survival and function of the allograft (23, 24). Our results suggest that the p21KO model (unable to implement a full senescent response) have a better preserved biliary architecture during cold storage. The adaptation of this knockout strategy to the clinic is not feasible, however, the rapid development of senotherapies might contribute to the elimination of senescent cells and their detrimental effects in liver transplantation. Our experimental settings suggest that the use of the agent ABT737 or the combination of Dasatinib plus Quercetin (D+Q) improves biliary preservation during cold storage.

Last, the current global trend to implement machine perfusion for liver transplantation represents an exciting leap forward for the reconditioning of donor livers (for example, administration of cell therapy for biliary disease (11, 12, 68). To prove the feasibility of this approach for the administration of drugs to manipulate the senescent phenotype, we applied a proof-of-concept continuous perfusion of senolytics (D+Q). Although this is a preliminary approach that will require further experimentation in the clinical setting, our results indicate that D+Q perfusion may alleviate cold storage-dependent biliary damage, improving the overall preservation of the liver.

In summary, we have identified cellular senescence as a detrimental mechanism during liver preservation, with opposing DCR2 roles in cholangiocytes and hepatocytes. Furthermore, our data show that senolytic treatment may be efficacious as an intervention prior to liver transplantation.

Materials and Methods

Study Design. This study addressed the hypothesis that senescent cells of cold storage-preserved livers negatively impact the ability of the cholangiocytes to regenerate post-transplantation, and that their therapeutic depletion prior to transplantation would therefore be protective. All studies were conducted in accordance with ARRIVE guidelines. Power calculations are not routinely performed; however, animal numbers were chosen to reflect the expected magnitude of response considering the variability observed in previous experiments. Mice were randomly allocated to each experimental group and males/females were equally distributed. All animal experiments were carried out under procedural guidelines, severity protocols and within the UK with ethical permission from the Animal Welfare and Ethical Review Body (AWERB) and the Home Office (UK). No available data was excluded from the analysis. Primary and secondary endpoints were pre-specified. Each mouse represented one experimental unit. In all experiments, there was randomised allocation to groups. Tissue was collected coding each animal for subsequent blinded analysis, with unblinding only being performed after completion of data acquisition. qPCR experiments were performed in technical triplicates of multiple biological replicates. For representative images 3-4 liver lobes were examined histologically in at least 3 biological replicates; numbers of biological replicates for each experiment are in figure legends. Further methods details are available in the supplementary materials.

Clinical material. Human liver intrahepatic bench biopsies from a clinical series of transplanted livers were obtained from the Quality in Organ Donation (QUOD) national biobank (RAP 039). 7 of 19
recipients developed postoperative biliary complications (including ischemic cholangiopathy and postoperative anastomotic strictures) assessed by radiological pattern and clinical presentation at the Scottish Liver Transplant Unit, Royal Infirmary, Edinburgh, UK. Paraffin embedded samples were anonymized, assessed histologically and later correlated with the clinical outcome.

Animal models
The animals used in this study were C57BL/6 background, a mix of males and females aged 8–12 weeks at the start of the experiments. All animal genotyping was outsourced commercially to Transnetyx, Inc using the primers stated in table S3. All animal experiments were carried out under procedural guidelines, severity protocols and within the UK with ethical permission from the Animal Welfare and Ethical Review Body (AWERB) and the Home Office (UK).

**DBD-like liver procurement and cold static preservation**. Mice were culled by cervical dislocation and livers perfused through the portal vein with 10 ml of Belzer MPS UW solution whilst inferior and superior vena cava were transected. Liver was extracted en bloc and gall bladder excised to allow bile drainage. Perfused livers were submerged in a bag of UW solution and placed in a second bag with saline (Baxter). Livers were stored in an ice box for 1, 5 or 10 hours. 0-hour controls represent the livers perfused, extracted en bloc, and immediately processed for subsequent procedures.

**DCD-like liver procurement and cold storage preservation**. Mice were asphyxiated with CO2 to model the agonal phase observed in DCD donors, then culled by cervical dislocation. Livers were perfused with UW solution after 10 min of warm ischemia and then subjected to cold static preservation.

**Experimental hypothermic perfusion without oxygenation in mouse DCD livers (HMP-DCD)**. A peristaltic pump transported cooling water (4°C) into a counterflow wort chiller that kept the temperature of the perfusion chamber below 10°C. A second peristaltic pump drove perfusion media (Belzer, 4°C) into the DCD-like procured mouse liver at 5 ml/min. A bag of ice was placed on top of the chamber to cool the surface. Temperature of the surface of the chamber and the solutions was maintained below 10°C for the whole perfusion.

**Ischemia-reperfusion injury**. The ischemia was caused by clamping the hepatic artery, portal vein and common bile duct using a vascular clamp. Following 45 min of hepatic ischemia, the clamp was removed allowing reperfusion of the ischemic tissues. Tissue was analysed at different points of reperfusion: immediately following IRI (0 hours) and at 24 and 48 hours.

**Senolytic treatment**. Senolytics were administered to C57BL/6 mice prior liver procurement; 75 mg/kg of ABT737 (SelleckChem) administered by intraperitoneal injection for 7 consecutive days in 100 µl of the vehicle solution: 10% Ethanol (Sigma-Aldrich), 30% PEG-400 (Sigma-Aldrich), 60% Phosal-50 (Lipoid AG). 5 mg/kg Dasatinib (LC Laboratories) and 50 mg/kg Quercetin (Sigma-Aldrich) were administered in combination by oral gavage in 100 µl of the vehicle solution for 3 consecutive days.

**Statistical analysis**. A minimum of four mice were included per experimental group. Power calculations were not routinely performed; however, animal numbers were chosen to reflect the expected magnitude of response considering the variability observed in previous experiments. Mice were randomly allocated to each experimental group and males/females equally distributed. For cell culture experiments, a minimum of four independent biological replicates were performed. Prism software version 9 (GraphPad Software, Inc) was used for all statistical analyses. Normal distributions were determined using D’Agostino and Pearson Omnibus normality test with Welch’s correction if variances differed (f test). For parametric data, data significance was analysed using a two-tailed unpaired Student’s t-test. Non-parametric data was analysed using Mann-Whitney test. When more than two groups were being compared, a one-way ANOVA (with Bonferroni correction) was used. Statistical significance was assumed at $p<0.05$. Data is presented as mean ± standard error of the mean (SEM). n refers to biological replicates.
Supplementary Materials

Materials and Methods

Fig S1-S27
Tables S1-S6
MDAR Reproducibility Checklist
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References


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Author contributions.
SFG and SJF conceived and planned the project. SFG, TYM, HE, RA, AMK, DRT, NY, LC, VLG, BD, NA, LB, MTM, YW, KJM, DAF, TJK, WYL, JCA, DK, SO’N, PIK, performed experiments and SFG, TYM, AMK, DRT, LC, JCA, GCO analysed and interpreted the results. JMB provided the cell lines. SFG, AMK, DRT wrote the manuscript and SFG, AMK, DRT, SJF reviewed it. All authors critically read and approved the paper.

Competing interests. The authors declare that they have no competing interests. SF is founder and scientific advisor of Resolution Therapeutics Ltd (not related to this study).

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Figure Captions

Fig 1. Cold storage conditions induce morphological changes in the anatomy of murine bile ducts.

(A) Experimental setting for cold storage. Briefly, the mouse was culled by cervical dislocation (CD) and the liver was immediately perfused with UW solution and placed in a bag with UW. This bag was placed in a second bag with cold saline and surrounded by ice. Livers were maintained under these conditions for 1, 5 or 10 hours. n= 4-5 mice per experimental group.

(B) Whole mouse liver after 1, 5 or 10 hours in cold storage. Scale bar =1 cm.

(C) H&E staining of mouse livers at 0, 1, 5 or 10 hours in cold storage. Scale bar = 120 µm. Far right, digital magnification of the morphological changes in the biliary architecture.

(D) Immunofluorescence of Keratin-19 (K19) positive cholangiocytes (green). Scale bar = 250 µm. Isotype-negative control is shown in the upper left quadrant. Far right, digital magnification.

(E) H&E staining at 10 hours shows cholangiocyte detachment in the biliary lumen. Right, digital magnification. Far right, immunofluorescence confirms the detached cells express the cholangiocyte marker K19 (green). Scale bar = 60 µm.

(F) Number of bile ducts (BD) with detached K19-positive cholangiocytes during cold storage. ** denotes p<0.01 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(G) Bile ducts isolated from livers maintained 0 or 10 hours in cold storage (CS), grown as organoids in culture and photomicrographed over 7 days. Scale bar = 120 µm.

(H) Quantification of the average organoid size (pxl²) grown from bile ducts isolated from livers maintained for 0 or 10 hours in cold storage. ** denotes p<0.01 (mean ± SEM), Student’s t test for Day 7 (n=4 mice each with 3 technical replicas in culture).

(I) Percentage of total Ki67, PCNA and LGR5 per DAPI-positive cells in organoids grown from bile ducts isolated from livers cold stored for 0 or 10 hours. ** denotes p<0.01, *** p<0.001 (mean ± SEM), Student’s t test.
Fig 2. Differential senescence, apoptotic and proliferative response of mouse cholangiocytes and hepatocytes during static cold storage.

(A) Ki67-positive proliferating (green) K19-positive cholangiocytes (red) at 0, 1, 5 or 10 hours of cold storage. Isotype-negative control is shown in the upper left quadrant. Far right, digital magnification. Scale bar= 60 µm.

(B) H2A.X-positive (red) K19-positive (green) cholangiocytes. Far right, digital magnification: upper quadrant K19 and γH2A.X, lower quadrant H2A.X and DAPI. Scale bar= 60 µm.

(C-F) Comparison of mRNA abundance in bile ducts (BD, purple) and hepatocytes (H, grey) isolated from mouse livers after 0 or 10 hours of cold storage. Data normalised to Ppia1. * denotes p<0.05, ** p<0.01, *** p < 0.001 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test. n=7 biological replicates for each individual group. n = 7 biological replicates. Genes relate to: (C) the cell cycle, (D) apoptosis, (E) Senescence induction, or (F) SASP.
Fig 3. Murine hepatocytes implement apoptosis during cold storage whereas cholangiocytes upregulate pro-senescent, anti-apoptotic factor DCR2.

(A) Caspase-3 (red) is not present in K19-positive cholangiocytes (upper panel) despite increasing in hepatocytes during cold storage (lower panel). Below, digital magnification of hepatocytes showing Caspase-3 expression. Scale bar = 60 µm. n=4-5 biological replicates per group.

(B) Immunoblot of Caspase-3 (Casp3) and the activated form of Caspase-3 (Cleav-Casp3) in hepatocytes (H) and bile ducts (BD). Far right, immunoblot densitometry quantification (normalized to bActin control). * denotes $p < 0.05$ (mean ± SEM), Student’s $t$-test.

(C) Upper panel: DCR2 immunostaining (green) in K19-positive cholangiocytes at different time points of cold storage. Lower panel: DCR2 in hepatocytes. Scale bar = 60 µm. n=4-5 biological replicates per group.

(D) qRT-PCR analysis of *Tnfsrf10D* (DCR2) in hepatocytes (H) and bile ducts (BD) isolated at 0 and 10 hours of cold storage, normalized to housekeeping gene *Ppia1*. n=3 per group. *** denotes $p < 0.001$ (mean ± SEM) Student’s $t$-test.

(E) Immunoblot of DCR2 in hepatocytes (H) and bile ducts (BD) at 0 and 10 hours of cold storage. Far right, immunoblot densitometry quantification (normalized to bActin control). * denotes $p < 0.05$, ** $p<0.01$ (mean ± SEM), Student’s $t$-test.
Fig 4. DCR2 binds to senescence-associated factors in cholangiocytes while interacting with apoptotic factors in hepatocytes during cold storage.

(A) Venn diagram illustrating DCR2 interactors specific to hepatocytes (blue) and cholangiocytes (yellow) at 0 hours of cold storage. These proteins were identified through immunoprecipitation of DCR2 and mass spectrometry analysis. Below, list of selected interactors (blue for hepatocytes and yellow for cholangiocytes) based on published-peer reviewed literature that describe associations with cell-cycle, senescence and apoptosis. For the full list of DCR2 interactors at 0 hours see Data File S1.

(B) Venn diagram illustrating DCR2 interactors specific to hepatocytes (blue) and cholangiocytes (yellow) after 10 hours of cold storage. Below, list of selected interactors based on published-peer reviewed articles. For the full list of DCR2 interactors at 10 hours see Data File S2.

(C) Image depicting the potential interaction of DCR2 with TRIM28 in cholangiocytes (top) and hepatocytes (bottom) at 0 and 10 hours of cold storage.

(D) Immunoblot of TRIM28 in bile ducts at 0 and 10 hours of cold storage. Below, densitometry quantification (normalized to bActin control). Student’s t-test.

(E) Quantification of TRIM28 immunostaining in cholangiocytes (top) and hepatocytes (bottom) at different time points of cold storage. * denotes p<0.05 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(F) Image depicting the potential DCR2 interaction with MIF in cholangiocytes (top) and hepatocytes (bottom) at 0 and 10 hours of cold storage.

(G) Immunoblot of MIF in bile ducts at 0 and 10 hours of cold storage. Below, densitometry quantification (normalized to bActin control). Student’s t-test.

(H) Quantification of MIF immunostaining in cholangiocytes (top) and hepatocytes (bottom) at different time points of cold storage. * denotes p<0.05 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(I) Rosetta-predicted structure of DCR2 protein.

(J) DelPhi electrostatic interaction potential map at ±1 kT/e with positive potentials coloured blue and negative potentials indicated by red shading.
Fig 5. DCR2 knockdown increases proliferation in cholangiocytes.

(A) qRT-PCR analysis of TNFRSF10D (DCR2) in normal human cholangiocytes (NHC) DCR2-Wild Type (DCR2-WT) and DCR2-Knockdown (DCR2-KO), normalized to housekeeping gene PPIA1. n=9 per group. *** denotes p < 0.001 (mean ± SEM). Student’s t test.

(B) qRT-PCR analysis of MKI67 (Ki67, marker of proliferation), TP53 (p53), BCL2 (antiapoptotic Bcl2) and CDKN1A (p21), normalized to housekeeping gene PPIA1, in DCR2-WT and DCR2-KO NHC. n=5-8 per group. * denotes p < 0.05, ** p < 0.01 (mean ± SEM). Student’s t test.

(C) BrdU immunofluorescence for DCR2-WT and DCR2-KO NHC human cholangiocytes. Scale bar = 120 µm. Right, percentage of proliferating (BrdU-positive) DCR2-WT and DCR2-KO NHC in vitro. ** p<0.01 (mean ± SEM), Student’s t-test.

(D) qRT-PCR analysis of TNFRSF10D (DCR2) in human hepatocytes (HepG2) DCR2-Wild Type (DCR2-WT) and DCR2-Knockdown (DCR2-KO), normalized to housekeeping gene PPIA1. n=6 per group. *** denotes p < 0.001 (mean ± SEM). Student’s t test.

(E) Morphological assessment of DCR2-WT and DCR2-KO cells using immunofluorescence (K19 for NHC cholangiocytes and CYP2D6 for HepG2 hepatocytes). Scale bar = 60 µm.

(F) PanCK immunohistochemistry depicting ductular reaction. From left healthy murine liver, sham-operated control showing no regeneration and therefore no ductular reaction, 48 hours post-partial hepatectomy, DDC diet, and CDE diet.. Scale bar = 250 µm.

(G) EpCAM (magenta) and DCR2 (yellow) immunofluorescence in the different models displaying different degrees of ductular reaction. Scale bar = 250 µm.

(H) Percentage of EpCAM-positive cells in different models of liver damage. * denotes p<0.05, ** p<0.01, (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(I) Percentage of DCR2-positive cholangiocytes (left) and hepatocytes (right) in different models of liver damage. *** denotes p<0.001 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(J) Correlation between DCR2 in cholangiocytes and total EpCAM in different models of liver damage including healthy, sham control, partial hepatectomy at 48 hours (PH 48h), and DDC and CDE diets. Pearson correlation for a 95% confidence interval displaying linear regression slope. Right, correlation between DCR2 in hepatocytes and total EpCAM.
Fig 6. Inhibition of cellular senescence decreases DCR2 and improves preservation of the biliary anatomy during cold storage.

(A) Immunofluorescence for the cholangiocyte marker K19 in human healthy livers and livers with non-anastomotic strictures (NAS) development post-liver transplantation. Scale bar = 250 µm.

(B) Left, percentage of Ki67-positive cholangiocytes and hepatocytes in healthy (H) and NAS human livers. Right, percentage of DCR2-positive cholangiocytes and hepatocytes. *** denotes $p<0.001$, * $p<0.05$ (mean ± SEM). Student’s $t$ test.

(C) Haematoxylin and eosin staining of $p21^{KO}$ livers subjected to 0 or 10 hours of cold storage. Scale bar = 60 µm.

(D) Left, percentage of Ki67-positive cholangiocytes and hepatocytes at 0 and 10 hours of cold storage in $p21^{KO}$ livers. Right, percentage of DCR2-positive cholangiocytes and hepatocytes. * $p<0.05$ (mean ± SEM). Student’s $t$ test.

(E) Haematoxylin and eosin staining of livers extracted from animals treated with vehicle or ABT737 senolytic regime and cold stored for 10 hours. Scale bar = 120 µm.

(F) Percentage of total DCR2, Ki67, PCNA and LGR5 per DAPI-positive cells in organoids grown from bile ducts isolated from vehicle or ABT737-mice livers cold stored for 10 hours. *** denotes $p<0.001$, ** $p<0.01$, * $p<0.05$ (mean ± SEM). Student’s $t$ test.

(G) Immunofluorescence for K19 (green) and DCR2 (red) in human segments perfused with vehicle or D+Q, SB= 120 µm. Right, magnification Scale bar = 60 µm.

(H) Percentage of DCR2-, TUNEL-, and Ki67-positive cholangiocytes and hepatocytes, in vehicle (VEH) or D+Q perfused segments from 4 livers (L1, L2, L3, L4). Student’s $t$ test.
Materials and Methods

**Human Liver anatomical segments II and III normothermic perfusion with senolytics.** Seven human livers were initially accepted for transplantation and procured with the intent to transplant by a team of the UK National Retrieval Service. Following procurement, the liver grafts were deemed unsuitable by the consultant surgeon and declined by all UK liver transplant centers (Table S1, S2). All grafts were initially preserved in Belzer MPS UW preservation solution (Bridge-to-life Ltd) at 4°C. Livers were offered via NHS Organ Donation and Transplantation national research offer process. Ethical approval for the use of human livers was received from Lothian Research and Ethics Committee (LREC) reference number 15/SS/0218, Lothian Research and Development (Project No. 2015/0408), National Health Service Blood and Transplant (NHSBT) ethics committee and Research Innovation and Novel Technologies Advisory Group (RINTAG). Organ procurement and research utilisation of the livers was undertaken in accordance with the United Kingdom’s Human Tissue Act (Scotland, 2006) and registered with NHS Lothian Tissue Governance.

For livers L1-L4, upon arrival, the left lateral segments (Couinaud Segments II and III) were split and prepared for perfusion. One segment was perfused at 37°C for 2 hours with 1L of sterile Phosphate Buffered Saline (PBS) supplemented with 5 mg/kg Dasatinib (LC Laboratories) and 50 mg/kg Quercetin (Sigma-Aldrich) dissolved in 1 ml of DMSO. The other segment was perfused with PBS and DMSO.

For livers L5-L7, segments II and III were kept together and cannulated through all visible portal vein branches. Biopsies were obtained prior and after perfusion.

**Animal models**

*K19CreER^Mdm2^{fl/fl}*, recombination of LoxP sites was induced with three doses of 4 mg of tamoxifen (Sigma-Aldrich) in sunflower seed oil (Sigma-Aldrich) by intraperitoneal injection on alternate days. A single dose of tamoxifen was administered every 10 days after the initial induction for the duration of the experiment (2, 3 months). Controls received only sunflower seed oil.

*P21^{ko} model* was a kind gift by Dr Tom Bird, Institute of Cancer Sciences, University of Glasgow, UK.

Tissue from the *p16INK4A^{ko} model* was sourced from our collaborators Katie Mylonas, David Ferenbach (University of Edinburgh) and Paul Krimpenfort (Netherlands Cancer Institute).

**For DCR2 histological study,** 3 C57BL/6 mice (2 males and one female 8 weeks old) were culled by cervical dislocation and all organs collected.

**Serum analysis.** Blood was collected via cardiac puncture after confirming mouse death and centrifuged at 6000xg 10 min at 4°C. Serum analysis used commercial kits according to the manufacturer’s instructions: serum albumin (Alb), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) (Randox laboratories), Alanine transaminase (ALT), total bilirubin and Amylase (Alpha Laboratories). All kits were adapted for use on a Cobas FARA centrifugal analyser (Roche Diagnostics).

**Isolation of murine bile ducts.** Livers were perfused with saline, dissected down to approximately 2-4 mm³ portions and covered with a mix of 0.125 mg/ml Collagenase (Sigma-Aldrich) and Dispase (Life Technologies) in 2% FCS Advanced DMEM/F-12 (Thermo Fisher). This mix was incubated 1 hour at 37°C and individual ductular structures were manually picked up, cleaned twice with 5%FCS DMEM/F-12 (Thermo Fisher) and transferred to TRIzol (Invitrogen) for mRNA analysis, or to Matrigel (Corning) for subsequent culture.

**Isolation of murine hepatocytes.** Livers were perfused with Liver Perfusion Medium (Gibco) and Liver Digest Medium (Gibco). The liver was then disrupted to yield a cell suspension, filtered through a 70 μm filter (BD Biosciences) and hepatocytes were pelleted by centrifugation at 135xG 1 min. Hepatocytes were purified using a Percoll gradient (Sigma-Aldrich). Briefly, cells were underlayered with 1.06, 1.08 and 1.12 g/ml Percoll in PBS. The 1.08 mg/ml was spiked with phenol red to aid
visualization. Cells were spun at 750xG 20 min. Fraction between the 1.08 and 1.12 mg/ml Percoll layers was harvested and transferred to TRIzol.

**RNA isolation, RT-qPCR and gene expression analysis.** RNA was isolated from bile ducts and hepatocytes using TRIzol and purified using RNeasy RNA extraction kit (Qiagen) according to the manufacturer’s instructions. RNA concentration and quality was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher). Reverse transcription was performed using QuantiTect Reverse transcription kit (Qiagen). Real time-qPCR was performed on a LightCycler 480 II (Roche) with commercial primers from Qiagen’s QuantiTect (stated in Table S4). Each gene expression was assessed with its own standard curve and normalized using Peptidylprolyl Isomerase A (PPIA) as housekeeping gene. Samples were run in triplicate.

**Cell culture.** Isolated bile ducts were mixed with Matrigel (Corning) and cultured for 7 days. Medium composition was DMEM/F-12 (Thermo Fisher) supplemented with B27 and N2 (Invitrogen), N-acetylcysteine (1.25 μM, Sigma-Aldrich), gastrin (10 nM, Sigma-Aldrich), EGF (50 ng/ml, Peprotech), 10% RSPO1 (Peprotech), FGF10 (100 ng/ml, Peprotech), nicotinamide (10 mM, Sigma-Aldrich) and HGF (50 ng/ml, Peprotech). Normal Human Cholangiocytes (NHC) were kindly provided by Dr JM Banales, Biodonostia Health Research Institute, Donostia, Spain and grown in collagen-coated plates with DMEM/F-12 supplemented with fetal bovine serum (Life Technologies), MEM non-essential Amino acid solution (10 mM, Lonza), lipid mixture 1 (100x, Sigma-Aldrich), MEM vitamin solution (100x, Gibco), Penicillin/Streptomycin (Life Technologies), Soybean Trypsin inhibitor (5 mg/ml, Life Technologies), Insulin Transferrin Selenium (100x, Gibco), bovine pituitary extract (13 mg/ml, Life technologies), dexamethasone (4 mg/ml, Sigma-Aldrich), T3-3, 3’ 5-triiodo-L-thyronine (34 mg/ml, Sigma-Aldrich), EGF (0.25 mg/ml, Sigma-Aldrich), and Forskolin (40 mg/ml, Ascent scientific). Human hepatocytes HepG2 (ATCC HB-8065) were grown as per manufacturer instructions. EpCAM-positive murine cholangiocytes were isolated after mechanical disruption of the liver, enzymatic digestion (0.125 mg/ml Collagenase (Sigma-Aldrich) and 0.125 mg/ml Dispase (Life Technologies)) and FACS sorting using BD FACSARia Fusion (BD Biosciences), by positive selection for EpCAM (eBiosciences) and negative selection for CD45, CD31 and Ter119 (all from Biolegend). Sorted cells were collected in Advanced DMEM/F12 (Thermo Fisher) supplemented with 10% fetal bovine serum and cultured for 24 hours in either 3D (Matrigel, Corning) or 2D (Collagen coated plates, Sigma) conditions. HPCs of murine and human origin were isolated as previously described (39) FACS sorting for CD45-/CD31-/EpCAM+/CD24+/CD133+ profile. Primary murine hepatocytes were isolated as previously described (see isolation of murine hepatocytes section) and cultured in collagen-coated plates (Sigma-Aldrich) for 24 hours prior staining.

For proliferation studies, 3 mg/ml of BrdU were added to the media and incubated for three hours at 37°C.

**FAS-ligand treatment, DRAQ7 labelling and flow cytometry analysis.** NHC or HepG2 cells were treated for 6, 12 or 24 hours with 0, 10, 50 or 100 μM of human recombinant Fas-ligand (R&D Systems), harvested, counted and resuspended in PBS (w/o Ca²⁺ and Mg²⁺, Gibco) at a concentration of 500,000 cells/ml. 50,000 cells were set aside as unstained controls. DRAQ7 (Abcam) was added to the cell suspension at 1:500 and cells plated in a 96-well U bottom, low adherence plate (Costar) for flow cytometry analysis using the ACEA Novocyte 3000 (Agilent). Unstained sample was set as negative threshold for each cell line. Each condition was tested in triplicate. Data was analysed using the NovoExpress software (ACEA, Agilent).

**Rhodamine 123 transport assay.** Organoids grown from isolated bile ducts were incubated with 100 μM of Rhodamine 123 (Sigma-Aldrich) for 5 minutes at 37°C, washed 3 times with media and then kept in fresh media for 1 hour. After 1 hour multiple fluorescence measurements were performed between the organoid lumen and the surrounding background. Rhodamine 123 fluorescence in the
organoid lumen was normalised over the background measured in the surrounding areas and expressed as Fluorescence Units (FU).

**SAβGal,** was performed using the commercial kit #9860 from Cell Signalling, according to the manufacturer's instructions.

**Histology, immunohistochemistry and immunofluorescence.** Tissues were fixed O/N with formalin, washed and transferred to tissue cassettes and paraffin blocks using standard methods. **Organoids** were covered in the well with room-temperature 2% Agarose (Sigma-Aldrich) dissolved in PBS, flip over after solid, and covered again with agarose. Agarose coins were fixed O/N with formalin, washed and transferred to tissue cassettes and paraffin blocks. **2D-Cells** were fixed with 4% Formaldehyde and 1% Glutaraldehyde (Sigma-Aldrich) for 10 min, washed with PBS and permeabilised with 0.1% Tween-20 (Sigma-Aldrich) for 10 min. For tissue immunohistochemistry 4 µm paraffin sections were treated with sodium citrate pH 6.0 or Tris-EDTA pH 9.0 for antigen retrieval, blocked for endogenous peroxidase and alkaline phosphatase activity (BLOXALL, Vector Laboratories), endogenous Avidin/Biotin (Life Technologies) and nonspecific protein binding (Protein Block, Spring Bioscience). Primary antibodies, followed by species-specific secondary biotinylated antibodies (Vector Laboratories), VECTASTAIN ABC reagent, R.T.U. (Vector Laboratories) and DAB chromogen (Dako) were sequentially applied. Cells were stained in the same well (incubated with Protein Block (Spring Bioscience), primary antibody, Alexa Fluor fluorescent-secondary antibody (Life Tech) and DAPI (Sigma-Aldrich)). For immunofluorescence, primary antibodies were detected using fluorescent-conjugated secondary antibodies (Alexa Fluor, LifeTech).

Sectioning with DAPI-containing media (Southern Biotech) or counterstained with DAPI. Isotype controls were used at the same concentration as the corresponding primary antibody. Antibody details and staining conditions are outlined in Table S5. **Haematoxylin and Eosin staining.** Sections were stained routinely and mounted in fluorescence-free DPX mountant media (Sigma-Aldrich). **PicroSirius Red.** Sections were stained using picric acid (P6744-1GA), fast green (F7258-25G) and direct red (365548-25G, all from Sigma-Aldrich).

**TUNEL** Apoptosis was assessed using In Situ Cell Death Detection Kit (Roche) according to the manufacturers’ instructions. Positive control was performed using 10 units/ml of DNase-I (Qiagen).

**Microscopy and cell counting.** Images were acquired using a Nikon Eclipse e600 and Retiga 2000R camera (Q-Imaging, ImagePro premier software). Histological sections were assigned a randomized blinded code prior to quantification, and the randomization decoded at the time of the final data analysis. For single-cell quantification in tissue, images were acquired at x20 magnification, and analysed using Fiji Image J. Cells were identified based on DAPI stained-nuclei, morphology and specific staining for each population. For each experiment identical thresholds were used in all images for assigning nuclei to a specific population. For pixel analysis, ImagePro premier software was used to select regions of positivity and automatically analysed using a macro-instruction. For biliary organoid growth images were acquired with EVOS Cell imaging microscope (Thermo Fisher) over the course of 7 days. Average size of each organoid was assessed using Fiji Image J (for n=4 biological replicates and N=3 technical replicates) and normalised to Day 1 post-isolation. Results are expressed as average size (in pxl²). For SAβGAL assay, cells were scanned with the Opera Phoenix Plus High-Content Screening System (PerkinElmer, HH14001000) and subsequently analyzed with Signals Image Artist software (PerkinElmer, 1.0) to quantify SAβGAL. For SAβGAL quantification SAβGAL, K19/CYP2D6 and DAPI stained slides were scanned. First, a brightfield image was taken at x20, and then an immunofluorescent image was taken over the same area. Using the Signals Image Artist software cells were identified using nuclear (DAPI) and cytoplasmonic stain (K19 or CYP2D6). Using the software’s linear classifier system an algorithm to identify SAβGAL-positive cells was established and trained. Subsequently cells were classified into SAβGAL-positive and SAβGAL-negative cells.
**DCR2 CRISPR-editing.** TNFRSF10D Oligos were selected using the GeCKO v2.0 human library sgRNA sequence catalogue (85,86) and synthesised as single-stranded oligos (see Table S6). The LentiGuide-Puro (Addgene #52963) backbone was digested with Esp3I (New England Biolabs) to produce staggered ends, and dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific). Plasmid digestion products were size separated by gel electrophoresis in 2% agarose gel for 45-60 minutes (120V, constant), and extracted (Zymoclean Gel Recovery Kit, Zymo Research). sgRNA oligos were annealed and phosphorylated with T4 Polynucleotide Kinase (New England Biolabs) for 30 minutes at 37°C. Ligation of plasmid backbone and insert was carried out at 16°C overnight with T4 ligase (New England Biolab). Ligation reactions were heat-inactivated and transformed into Stbl3 cells as per the manufacturer’s instructions (One Shot Stbl3 Chemically Competent E.coli; ThermoFisher Scientific) and grown on Luria Broth agar at 37°C overnight. Sanger sequencing of colonies (MRC Human Genetics Unit Technical Services, University of Edinburgh, UK) identified clones with correctly ligated plasmids which were subsequently picked, cultured and extracted (QIAfilter Plasmid Maxi Kit, QIAGEN). Plasmid yield was assessed on the NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific). **LentiGuide-Puro-sgDCR2 Lentivirus.** 7.5μg of psPAX2 helper vector (Addgene #12260), 2.5g of pCMV-VSV-G envelope plasmid (Addgene), 10μg of lentiviral vector in 425μl Advanced DMEM (ThermoFisher Scientific), and 75μl 1mg/ml polyethylenimine (PEI linear, 25K molecular weight, Polysciences Inc) were combined and added to HEK293 cells which were cultured overnight. The following day media were collected and lentivirus was concentrated using the Lenti-X lentiviral concentrator (Takara Bio) as per manufacturer’s instructions. **Lentiviral transduction** was performed in 70% confluent NHC with 0.25 µl of the virus per ml of NHC media and 5 µg/ml Polybrene (Sigma) O/N. Cells were then treated for one week with selection media (NHC media supplemented with 5 µg/ml puromycin (Life Technologies) prior mRNA analysis).

**Immunoblotting.** Ductular structures were manually picked up, cleaned with 5% FCS DMEM and protein extracted with RIPA (Sigma-Aldrich) according to the manufacturer’s instructions. Similarly, hepatocytes were isolated, pelleted and treated with RIPA to extract protein. Protein content was quantified using a BSA standard and Pierce reagent before being resolved using SDS/PAGE and blotted onto nitrocellulose membrane. Membranes were blocked with 5% BSA on Tween-PBS during 1 h at room temperature. Membranes were incubated at 4°C overnight with primary antibody (mouse anti-β actin (Sigma-Aldrich), Caspase 3 (Cell Signalling), DCR2 (Abcam), TRIM28/KAP1 (Abcam) or MIF (Abcam)). Appropriate HRP-labelled secondary antibodies were used (Dako or CST) and signal detected with ECL reagent (Amersham). In order to use the same membrane with different antibodies, stripping was performed with mild stripping buffer during 10 min at room temperature. Membrane was blocked again with 5% BSA on T-PBS during 1 h and re-incubated with the desired antibody overnight. Protein band quantification was performed using ImageJ. All uncropped western blots can be found in Fig S27.

**Immunoprecipitation.** Bile ducts or hepatocytes were lysed in 250 µl T-PER protein extraction reagent (Thermo Fisher), containing protease inhibitor cocktail, for 1 hour at 4°C on a rotating mixer. Lysates were spun at 12,000 rpm for 15 min at 4°C and supernatant collected. Protein concentration was measured using the DC Protein Assay (Bio-Rad). For each sample 50µL of Protein G Dynabeads (Thermo Fisher) were labelled with 5µg of DCR2 antibody (Abcam) and rotated at room temperature for 45 min. Whole cell lysates (1mg protein for hepatocytes and 300µg protein for cholangiocytes) were incubated with the antibody coupled with Dynabeads at 4°C overnight, with rotation. Samples were placed on a magnet and supernatant removed. Beads were washed twice with lysis buffer and then stored at -80°C.

**Proteomics analysis.** ‘On-bead’ tryptic digestion and LC MS identification of proteins. Washed beads were resuspended in 60µL of urea-based digestion buffer 1 (2M urea, 50mM Tris-HCl pH 7.5)
containing 6μg/mL of trypsin (Promega sequencing grade) at room temperature for 30 min with gentle vortexing. The beads were then separated from the supernatant and washed twice with 25μL of buffer 2 (2M urea, 50mM Tris-HCl pH 7.5, 1mM Dithiothreitol). The supernatants were combined and allowed to digest overnight at room temperature. Iodoacetamide was added to a final concentration of 40mM to the sample and incubated for 30 min in the dark. The digested peptides were cleaned up using StageTips by following standard protocols. NanoflowLC-MS/MS was performed on a microOTOF-II mass spectrometer (Bruker) coupled to a RSLCnano LC system (Thermo Fisher). Tryptic digest was delivered to a trap column (Acclaim PepMap100, 5 μm, 100 Å, 100 μm i.d. × 2cm) at a flow rate of 20 μL/min in 100% solvent A (0.1% formic acid in LCMS grade water). After 4 min of loading and washing, peptides were transferred to an analytical column (Acclaim PepMap100, 3 μm, 100 Å, 75 μm i.d. × 25 cm) and separated at a flow rate of 300 nL/min using a 60-min gradient from 7% to 35% solvent B (solvent B, 0.1% formic acid in acetonitrile). The eluted peptides from LC were electrosprayed directly on to the mass spectrometer for MS and MS/MS analysis in a data-dependent mode of acquisition. The m/z values of tryptic peptides were measured using a MS (300-2000 m/z), followed by MS/MS scans of the six most intense ions. Rolling collision energy for fragmentation was selected based on the precursor ion mass and a dynamic exclusion was applied for 30 sec. Raw spectral data were processed with DataAnalysis (Bruker) software and the resulting peak lists were searched using Mascot 2.4 server (Matrix Science, London, UK) against Uniprot Mouse sequence database containing 53216 entries. Mass tolerance on peptide precursor ions was fixed at 25 ppm and on fragment ions at 0.08 Da. The peptide charge was set to 2+ and 3+. Carbamidomethylation of cysteine was selected as a fixed modification and oxidation of methionine and de-amidation were chosen as variable modifications. False discovery rate was limited to < 1% for peptide IDs after searching decoy databases. All proteomic analysis can be found in Data Files 1-3.

**Bioinformatic analysis. Putative gene homologs** were identified using NCBI HomoloGene (Release 68). Additional identification of homologs was carried out using the HGNC Genenames database (87). Sequence analysis of homologous genes was performed with NCBI blastp (v2.10.0+). Protein conserved domain architectures were extracted from the NLM Conserved Domain Database (88). Enrichment analysis with the PANTHER classification system (89) was used to identify Gene Ontology (GO) overrepresented terms. Fold enrichment of GO terms was computed by Fisher’s Exact Test; terms with FDR<0.05 were considered to be significantly enriched. **Computational proteomic analysis of DCR2 interactors** was performed using curated and peer-reviewed pathway databases: STRING v11.0 (90) for protein-protein interaction networks and functional enrichment analysis, paired with Reactome v75 (91) to determine protein relationship organized into biological pathways and processes. Known human DCR2-interactors were analysed using the BioGrid 4.2 (92) biomedical interaction repository database, and manually compared with mouse DCR2 proteomic profile (see Data File S4). **Predicted structure and equipotential map** for mouse DCR2 was computed using computational protein inference provided by trRosetta (93). Representation of DCR2 interactions with MIF and TRIM28 were imaged using BioRender.
Supplementary Figures

Fig S1. Injection via the portal vein as a method for liver perfusion in mouse.

(A) Left (Control), mouse liver before perfusion with Indian ink. Right, the same liver after perfusion via the portal vein. Far right, digital magnification of a lobe showing the degree of perfusion. Scale bar= 1 cm.
Fig S2. Increasing cold storage time increases the number of dead hepatocytes and inflammatory cells in the hepatic parenchyma.
Fig S2. Increasing cold storage time increases the number of dead hepatocytes and inflammatory cells in the hepatic parenchyma.

(A) H&E of mouse liver parenchyma at different times of cold storage. Scale bar= 250 µm.

(B) H&E staining of parenchyma with presence of granulomas. Far right, digital magnification. Scale bar= 120 µm.

(C) Representative section of a human liver affected by non-anastomotic strictures (NAS), showing detached K19-positive cholangiocytes (grey). Far right, digital magnification of selected area, Scale bar= 60 µm. n=5 human livers.

(D) Representative growth of a bile duct isolated from a control mouse liver (0 hours cold storage), grown as an organoid in culture for the course of 7 days (days are indicated in the top left). Scale bar= 120 µm.
Fig S3. Increasing cold storage time activates an α-SMA-positive fibroblast response and alters the liver macrophage landscape.
Fig S3. Increasing cold storage time activates an α-SMA-positive fibroblast response and alters the liver macrophage landscape.

(A) Immunofluorescence of α-SMA-positive fibroblasts (red) in the proximities of K19-positive cholangiocytes (green) at 0, 1, 5 or 10 hours in cold storage. Isotype-negative control is shown in the upper left quadrant. Scale bar= 120 µm. Far right, quantification. * denotes $p<0.05$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(B) Collagen I deposition. Scale bar= 60 µm.

(C) Quantification of PiSR shows no significant changes during cold storage. $p=0.2863$(mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(D) F4/80-positive macrophages (red) in the proximities of K19-positive cholangiocytes (green). Scale bar= 250 µm.

(E) Quantification of F4/80-positive macrophages per field. * denotes $p<0.05$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(F) Clusters of Ki67-positive proliferating cells (green) in close proximity to K19-positive cholangiocytes (red). Right square, Ki67 without DAPI. Scale bar= 250 µm.

n= 4-5 mice per experimental group.
Fig S4. DBD and DCD murine models, comparative study.

A

**HUMAN**

- **DBD**
  - Death Confirmed
  - Procurement
  - Transport
  - Recipient surgery

- **DCD**
  - Treatment withdrawal
  - Asystole

**MOUSE**

- **DBD**
  - Death Confirmed
  - Procurement
  - Transport

- **DCD**
  - Asphyxia
  - Procurement
  - Transport

B

- **H&E Bile Duct**
  - DBD
  - DCD

C

- **H&E Parenchyma**
  - DBD
  - DCD

D

- **DCD**
  - % PCNA / Cholang
  - % PCNA / Hepatocyt

E

- **DCD**
  - % H2A.X / Cholang
  - % H2A.X / Hepatocyt

F

- **DCD**
  - % DCR2 / Cholang
  - % DCR2 / Hepatocyt
Fig S4. DBD and DCD murine models, comparative study.

(A) Schematic representation of liver procurement in a DBD (Donation after Brain Death) and DCD (Donation after Circulatory Death) scenario, with an additional period of warm ischemia in DCD-livers. Cold storage times were extracted from the Annual report on liver transplantation NHS England for 2019-2020 (1st April 2019 to 31st March 2020).

Below, schematic representation of our DBD and DCD procurement in murine models.

(B) Haematoxylin and eosin staining of bile duct areas in DBD and DCD models. Scale bar= 120 µm.

(C) Haematoxylin and eosin staining of hepatic parenchyma in DBD and DCD models. Scale bar= 120 µm.

(D) Percentage of PCNA-positive cholangiocytes and hepatocytes in DCD procurement during the course of the experiment: 10 min of Warm Ischemic Time (WIT), WIT+5 hours of cold storage and WIT+10 hours of cold storage. Student’s t test.

(E) Percentage of H2A.X-positive cholangiocytes and hepatocytes in DCD procurement during the course of the experiment. Student’s t test.

(F) Percentage of DCR2-positive cholangiocytes and hepatocytes in DCD procurement during the course of the experiment. Student’s t test.
Fig S5. DBD and DCD murine models, proliferation, senescence, and apoptosis.
Fig S5. DBD and DCD murine models, proliferation, senescence and apoptosis.

(A) Left, percentage of proliferating cholangiocytes (PCNA-positive K19-positive) in DBD (blue) and DCD (orange) procurement at baseline (DBD: 0 hours of cold storage; DCD: 10 min warm ischemic time + 0 hours cold storage), 5 and 10 hours after cold storage. Student’s t test.
Right, immunofluorescence for K19 (green) and PCNA (red) at baseline for DBD and DCD procurement. Scale bar= 60 µm.

(B) Left, percentage of H2A.X-positive K19-positive cholangiocytes. Student’s t test.
Right, immunofluorescence for K19 (green) and H2A.X (red). Scale bar= 60 µm.

(C) Left, percentage of DCR2-positive K19-positive cholangiocytes. Student’s t test.
Right, immunofluorescence for K19 (green) and DCR2 (red). Scale bar= 60 µm.

(D) Left, percentage of TUNEL-positive cholangiocytes. Student’s t test.
Right, immunofluorescence for TUNEL (red) in cholangiocytes. Scale bar= 60 µm.
Fig S6. Senescence profile characterization in bile ducts during cold storage.

A. % p21 / Chilling

B. % p16 / Chilling

C. Isotype 10h CS p21KO 14 m.o. p16KO 10h CS WT

D. Isotype 10h CS p21KO 14 m.o. p16KO 10h CS WT

E. Isotype 10h CS p21KO 14 m.o. p16KO 10h CS WT

F. Isotype 10h CS p21KO 14 m.o. p16KO 10h CS WT
Fig S6. Senescence profile characterization in bile ducts during cold storage.

(A) Left, percentage of Ki67-positive cholangiocytes per total number of cholangiocytes. Right, percentage of Ki67-positive hepatocytes per total number of hepatocytes. * denotes $p<0.05$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(B) Left, percentage of H2A.X-positive cholangiocytes per total number of cholangiocytes. Right, percentage of H2A.X-positive hepatocytes per total number of hepatocytes. * denotes $p<0.05$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(C) p21 immunohistochemistry, Scale bar= 60 µm. From left to right, representative images of isotype negative control; p21KO mouse livers after 10 hours of cold storage (CS); 14 months old p16KO mice, and wild type (WT) mice liver after 10 hours of cold storage.

(D) p16 immunohistochemistry, Scale bar= 60 µm.

(E) H2A.X immunohistochemistry, Scale bar= 60 µm.

(F) DCR2 immunohistochemistry, Scale bar= 60 µm.

For (C-F): $n = 4-5$ mice per experimental group.
Fig S7. Isolation of bile ducts and hepatocytes from mouse cold-stored livers.

A

B
Bright Field
Bile Ducts

C
EpCAM αSMA DAPI
K19 F4/80 DAPI

D
Bright Field
Hepatocytes

E
HNF4α αSMA DAPI CYP2D6 F4/80 DAPI

F

Krt19/Ppi1

Hnf4a/Ppi1

Igami/Ppi1
Fig S7. Isolation of bile ducts and hepatocytes from mouse cold-stored livers.

(A) Experimental setting for the isolation of bile ducts and hepatocytes. Briefly, bile ducts were isolated from control livers (t=0 h) or livers subjected to 10 hours of cold storage (t=10 h) by mechanical/enzymatic disruption of the tissue, followed by manual selection. Hepatocytes were isolated as previously described, by enzymatic disruption (39). RNA was isolated from both bile ducts and hepatocytes and used for subsequent gene expression analysis.

(B) Bile ducts isolated from 0 hours cold stored livers. Scale bar = 250 µm.

(C) Left, immunofluorescence for EpCAM (green) and αSMA (red) in bile ducts isolated from 0 hours cold stored livers. Right, immunofluorescence for K19 (green) and F4/80 (red). SB= 60 µm.

(D) Hepatocytes isolated from 0 hours cold stored livers. Scale bar = 60 µm.

(E) Left, immunofluorescence for HNF4α (green) and αSMA (red) in hepatocytes isolated from 0 hours cold stored livers. Right, immunofluorescence for CYP2D6 (green) and F4/80 (red). Scale bar= 60 µm.

(F) qRT-PCR of Krt19 (cholangiocyte marker), Hnf4α (hepatocytes) and Itgαm (macrophages) in bile ducts (BD, purple) and hepatocytes (H, grey) following cold storage (t=10h). Data normalised to Ppia1. * denotes p<0.05 (mean ± SEM). Student’s t test for each individual gene. n = 3-7 biological replicates.
Fig S8. Functional analysis of bile ducts isolated from DBD/DCD murine models, grown as organoids in culture.
Fig S8. Functional analysis of bile ducts isolated from DBD/DCD murine models, grown as organoids in culture.

(A) Representative images demonstrating the efflux of MDR1-fluorescent substrate Rhodamine 123, detected in the lumen of Control organoids (grown from bile ducts isolated from 0 hours cold stored livers) and DBD organoids (from bile ducts isolated from DBD procurement followed by 10 hours of cold storage). Scale bar= 250 µm. Fluorescence intensity is measured along the area indicated by the red line.

(B) Left, mean intraluminal fluorescence intensity, normalized over the background for organoids grown from bile ducts isolated from: Control, DBD and DCD livers.*** denotes p<0.001 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test. Three biological replicas (three livers) were used for this quantification and the results from each individual liver are presented separately in the far right graph.

(C) Senescence Associated β galactosidase activity (SAbGal) in organoids grown from bile ducts isolated from 0 hours cold stored livers (0h Control), 10 hours after DBD procurement and 10 hours after DCD procurement. Scale bar= 200 µm

(D) Left, immunofluorescence for DCR2+K19, p16, and p21+K19 at 0 hours control, 10 hours DBD and 10 hours DCD procurement. Scale bar= 120 µm. Right percentage of cells expressing DCR2, p16 or p21. * denotes p<0.05 (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.
Fig S9. Hepatocytes engage pro-apoptotic factors in livers subjected to 10 hours of cold storage preservation.
Fig 59. Hepatocytes engage pro-apoptotic factors in livers subjected to 10 hours of cold storage preservation.

(A) Canonical Death-Receptor (DR4/5)-mediated apoptosis signalling pathway. Upon binding of DR4/5 with the ligand (FasL, TRAIL or TNFα) the pathway activates caspases, leading to cell death. Unlike DR4/5, DCR2 has a truncated domain that prevents engagement with the death-ligands, determining the cell sensitivity to the killing signals. It also blocks the caspase activation through direct interaction with FADD.

(B) Caspase-3 (red) is not present in K19-positive cholangiocytes. Left, Merge. Right, same image without K19. Scale bar= 60 µm. n= 4-5 biological replicas per group.

(C) Left, percentage of Caspase-3 (Casp3)-positive cholangiocytes per total number of cholangiocytes. Right, percentage of Casp3-positive hepatocytes per total number of hepatocytes. * denotes $p<0.05$, ** $p<0.01$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(D) Left, immunofluorescence for cholangiocyte marker K19 (green) and TUNEL (red) after 10 hours of cold storage. Digital magnification on the merge indicates TUNEL+ cholangiocytes (white arrows). Scale bar= 60 µm. Right, quantification.

(E) Left, immunofluorescence for hepatocytes (K19-negative) and TUNEL (red) after 10 hours of cold storage. Scale bar= 60 µm. Right, quantification. * denotes $p<0.05$ (mean ± SEM) Student’s $t$ test. $n≥4$ biological replicates for each group.
Fig S10. FAS-Associating Death Domain expression during cold storage.

(A) FADD immunohistochemistry at 0 and 10 hours of cold storage. Scale bar= 60 µm. Red arrows indicate presence of nuclear FADD and black arrows indicate absence.

(B) Percentage of nuclear FADD-positive cholangiocytes and hepatocytes at different time points of cold storage. ** denotes \( p < 0.01 \), *** denotes \( p < 0.001 \) (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(C) Representative image of loss of hepatocyte’s nuclear FADD and cytoplasmic expression. Red arrows indicate nuclear expression. Black arrow indicate cytoplasmic accumulation and nuclear loss. Scale bar= 60 µm.
Fig S11. Cholangiocytes constitutively express high DCR2 compared to hepatocytes.
Fig S11. Cholangiocytes constitutively express high DCR2 compared to hepatocytes.

(A) DCR2 (green) in K19-positive cholangiocytes (grey). Upper image, Merge. Below same image without K19. Scale bar = 60 µm. n= 4-5 biological replicas per group.

(B) Percentage of DCR2-positive cholangiocytes and hepatocytes at different time points of cold storage. * denotes $p<0.05$, ** $p<0.01$ (mean ± SEM). ANOVA, Sidak’s multiple comparisons test.

(C) Percentage of DCR2-positive cells in organoids grown from bile ducts isolated from 0 or 10 hours cold stored livers. *** denotes $p<0.001$ (mean ± SEM), Student’s t test.

(D) Immunofluorescence of Keratin-19 (K19) and DCR2 (red) in organoids grown from bile ducts isolated from 0 or 10 hours cold stored livers. Scale bar= 60 µm.

(E) Immunofluorescence in normal human cholangiocytes (NHC) and HepG2 human hepatocytes. From top: DCR2, cholangiocyte marker Keratin-19 (K19) and hepatocyte markers HNF4α, CYP2D6 and Albumin (ALB). Scale bar = 120 µm.
Fig S12. DCR2 expression confers cholangiocytes resistance to FasL-related apoptosis.
Fig S12. DCR2 expression confers cholangiocytes resistance to FasL-related apoptosis.

(A) Immunofluorescence of Keratin-19 (K19, grey) and DCR2 (red) in isolated murine EpCAM-positive cells and murine hepatic progenitor cells of biliary origin (HPC). Scale bar= 120 µm.

(B) DCR2 immunofluorescence in human cholangiocytes grown as three-dimensional organoids. Top: cholangiocyte marker Keratin-19 (K19, red) and DCR2 (green). Bottom: hepatocyte marker (HNF4α, red) and DCR2 (green). Scale bar= 120 µm.

(C) DCR2 immunofluorescence in primary murine hepatocytes. Murine hepatocytes express CYP2D6 and HNF4α but not Keratin-19 (K19, cholangiocyte marker). Scale bar= 120 µm.

(D) Percentage of DCR2 in murine HPC grown in 2D, EpCAM-positive cholangiocytes (Col) grown in 2D and 3D and murine hepatocytes. *** denotes p<0.001 (mean ± SEM), ANOVA, Sidak’s multiple comparisons test.

(E) Number of DRAQ7-positive (dead) HepG2 human hepatocytes after treatment with 0, 10, 50 or 100 µM of human recombinant FasL for 6, 12 or 24 hours. * denotes p<0.05, ** p<0.01, *** p<0.001 (mean ± SEM), ANOVA, Sidak’s multiple comparisons test.

(F) Percentage of DRAQ7-positive (dead) NHC cholangiocytes after treatment with 0, 10, 50 or 100 µM of human recombinant FasL for 6, 12 or 24 hours.
Fig S13. DCR2 is widely expressed across tissue types in mouse (I).
Fig S13. DCR2 is widely expressed across tissue types in mouse (I).

(A) Left, Haematoxylin and Eosin of different murine tissues. Right, DCR2 (green) expression in PanCK-positive epithelial cells (red). Scale bar= 120 µm.
Fig S14. DCR2 is widely expressed across tissue types in mouse (II).

B

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Fig S14. DCR2 is widely expressed across tissue types in mouse (II).

(A) Left, Haematoxylin and Eosin of different murine tissues. Right, DCR2 (green) expression in PanCK-positive epithelial cells (red). Scale bar= 120 µm.

(B) Summary table indicating presence of DCR2 in different tissues, including epithelial (PanCK-positive) and non-epithelial (PanCK-negative) areas. n=3 mice.
Fig S15. DCR2 presents cell type-specific protein-protein interactions at 0 hours of cold storage.

### Hepatocytes 0h

#### Biological Process (Gene Ontology)

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#### Reactome pathways

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### Cholangiocytes 0h

#### Biological Process (Gene Ontology)

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#### Reactome pathways

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### C

#### Hepatocytes t=0h

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<th>Pathway name</th>
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#### Cholangiocytes t=0h

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Fig S15. DCR2 presents cell type-specific protein-protein interactions at 0 hours of cold storage.

(A) Representative STRING-Functional protein association network of some DCR2 interactors found only in hepatocytes at 0 hours of cold storage. Below, Gene Ontology (GO) indicating biological processes significantly associated with these interactors, including processes relating to senescence, cell cycle and apoptosis, and STRING-Reactome pathways for the full network.

(B) Representative STRING-Functional protein association network of some DCR2 interactors found only in cholangiocytes at 0 hours of cold storage. Below, GO biological processes significantly associated with these interactors and STRING-Reactome pathways.

(C) Extended Reactome analysis identified pathways associated with DCR2-interactors in hepatocytes and cholangiocytes at 0 hours of cold storage. The analysis included the matched identifier in the pathway (Curated Found) and the main interactors in the pathway for the selected molecular type (Interactor Found) within the dataset.

In hepatocytes, biological pathways associated with DCR2 pulldown are associated with death receptor signalling, cell cycle and programmed cell death, among others. In cholangiocytes, pathways are associated with suppression of apoptosis and regulation of necrosis, among others.
Fig S16. DCR2 presents cell type-specific protein-protein interactions at 10 hours of cold storage.

### Hepatocytes 10h

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#### Reactome pathways

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### Cholangiocytes 10h

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### Hepatocytes t=10h

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### Cholangiocytes t=10h

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<td>Cellular senescence</td>
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Fig S16. DCR2 presents cell type-specific protein-protein interactions at 10 hours of cold storage.

(A) Representative STRING-Functional protein association network of some DCR2 interactors found only in hepatocytes after 10 hours of cold storage. Below, GO biological processes significantly associated with these interactors including senescence, cell cycle and apoptosis and STRING-Reactome pathways for the full network.

(B) Representative STRING-Functional protein association network of some DCR2 interactors found only in cholangiocytes at 10 hours of cold storage. Below, GO biological processes significantly associated with these interactors and STRING-Reactome pathways.

(C) Extended Reactome analysis identified pathways associated with DCR2-interactors in hepatocytes and cholangiocytes after 10 hours of cold storage. The analysis included the matched identifier in the pathway (Curated Found) and the main interactors in the pathway for the selected molecular type (Interactor Found) within the dataset.

In hepatocytes, biological pathways associated with DCR2 pulldown are associated with apoptosis (2 hits, 3 interactors) and senescence (1 interactor). In cholangiocytes, pathways are associated with Ras activation/signalling (2 interactors) and cellular senescence (4 hits and 7 interactors).
Fig S17. Computational protein structure prediction identifies potential areas for DCR2 interaction with TRIM28 and MIF.

(A) Diagram illustrating protein domain architectures conserved between mouse DCR2 and different members of the human Death Receptor family including Death Receptor 4 (DR4) and 5 (DR5), Decoy Receptor 1 (DCR1) and 2 (DCR2). * The death domain in human DCR2 is annotated as ‘non-specific’. The table presents identity (%) and positive (%) results from protein BLAST analysis of mouse DCR2 against the human proteins, representing the proportion of identical and similar amino acids, respectively. Although protein domains are broadly conserved between mouse and human, sequence conservation is low.

(B) List of DCR2 interactions that are conserved between mouse and human (including MIF and TRIM28). For the full list of published human DCR2 known interactors (UCSD-Signaling gateway database) see Data File S4.

(C) Diagram illustrating protein domain architectures conserved between human and mouse TRIM28 sequences.

(D) Diagram illustrating protein domain architectures conserved between human and mouse MIF sequences.
Fig S18. DCR2 protein interactions in hepatocytes and cholangiocytes.

A

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Fig S18. DCR2 protein interactions in hepatocytes and cholangiocytes.

(A) Immunohistochemistry of TRIM28/KAP1 at 0 and 10 hours of cold storage. Cholangiocytes maintain high levels of TRIM28 (red arrows) after 10 hours of cold storage, whilst hepatocytes significantly increase TRIM28. TRIM28-negative cells are indicated with white arrows. Scale bar= 60 µm.

(B) Immunohistochemistry of MIF at 0 and 10 hours of cold storage. Cholangiocytes have low levels of MIF that do not increase over the course of cold storage (see white arrows). Hepatocytes increase MIF after 10 hours of cold storage (MIF-positive cells are indicated with red arrows). Scale bar= 60 µm.

(C) 2D predicted inter-residue information generated by trRosetta in predicting the model of DCR2 protein structures (Fig 4I). Main parameters include contact (darker colours represent stronger predicted inter-residue contact) and distance (darker colours represent smaller predicted inter-residue distance).
Fig S19. DCR2 knockdown in human cholangiocytes and hepatocytes in vitro.

(A) To induce cellular senescence, cells were exposed for 24 hours to 1 µM etoposide, washed, and then stained 5 days later for SAbGal activity.

(B) Response of human cholangiocytes (NHC, left) and human hepatocytes (HepG2, right) to etoposide exposure in presence or absence of DCR2. * denotes $p < 0.05$ (mean ± SEM). One way ANOVA, Sidak’s multiple comparisons test.

(C) Percentage of DRAQ7-positive (dead) human cholangiocytes (NHC, orange) and human hepatocytes (HepG2, green) after treatment with 100 µM of human recombinant FasL for 24 hours. *** denotes $p < 0.001$ (mean ± SEM), Student’s t test.

(D) Relative mRNA abundance for CASP3, TP53, CDKN1A, CDKN2A and BLC2 in DCR2WT and DCR2KO cholangiocytes (NHC, up) and hepatocytes (HepG2, down) exposed to 100 µM FasL for 24 hours. Data normalised to PPIA1. * denotes $p < 0.05$, *** $p < 0.001$ (mean ± SEM), Student’s t test.

(E) Left, Percentage of DRAQ7-positive (dead) DCR2-WT and DCR2-KO NHC human cholangiocytes, after exposure to 100 µM of human recombinant FasL for 24 hours. *** $p < 0.001$ (mean ± SEM), Student’s t-test. Right, Percentage of DRAQ7-positive (dead) DCR2-WT and DCR2-KO HepG2 human hepatocytes, after exposure to 100 µM of human recombinant FasL for 24 hours.
Fig S20. Mdm2-driven senescence expression in cholangiocytes induces alterations in biliary architecture.
Fig S20. Mdm2-driven senescence expression in cholangiocytes induces alterations in biliary architecture.

(A) Experimental setting for the induction of the K19-Mdm2\textsuperscript{flox/flox} model. Three doses of tamoxifen were administered on alternate days. Mice were culled at day 2 post final administration. Scale bar= 60 µm.

(B) Immunofluorescence for K19-positive cholangiocytes (green) and senescence marker p21 (red) in the K19-Mdm2\textsuperscript{flox/flox} model. Left, isotype negative control. Middle, vehicle (oil) at day 2 after final administration. Right, tamoxifen administration (TM) at day 2.

(C) Representative images of the K19-Mdm2\textsuperscript{flox/flox} model 12 weeks after oil administration (negative control, Oil 12 weeks), 8 weeks after tamoxifen induction (TM 8 weeks) and 12 weeks after tamoxifen (TM 12 weeks).
Top, Haematoxylin and eosin staining. Scale bar= 250 µm;
Middle, immunofluorescence for K19 (green) and p21 (red), Scale bar= 120 µm;
Bottom, immunofluorescence for K19 (green) and DCR2 (red), Scale bar= 120 µm.
Fig S21. Cold storage in p21KO mouse livers.

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Fig S21. Cold storage in p21KO mouse livers.

(A) K19 and DCR2 immunofluorescence in the p21KO model at 0 and 10 hours of cold storage. Scale bar= 60 µm.

(B) Percentage of cholangiocytes (left) and hepatocytes (right) expressing H2A.X in the p21KO model at 0 and 10 hours of cold storage. ** denotes p<0.01 (mean ± SEM) Student’s t test.

(C) Percentage of cholangiocytes (left) and hepatocytes (right) TUNEL+ in the p21KO model at 0 and 10 hours of cold storage. Student’s t test.

(D) Immunoblot of DCR2 and Caspase-3 (CASP3) in bile ducts isolated at 0 and 10 hours of cold storage. Each lane represents the bile ducts from 2 mouse (1+2 and 3+4, with 4 biological replicas in total for each time point). Right, Immunoblot densitometry quantification (normalized to bActin control).
Fig S22. Senolytic administration decreases DCR2 and increases proliferation *in vitro* and *in vivo*, in a model of cold-storage preservation.
Fig S22. Senolytic administration decreases DCR2 and increases proliferation *in vitro* and *in vivo*, in a model of cold-storage preservation.

(A) Experimental setting for the administration of senolytic ABT737, by intraperitoneal injection for 7 consecutive days, followed by culling and 10 hours of liver cold storage. Below, administration of Dasatinib-Quercetin (D+Q) in 3 oral gavage doses in consecutive days.

(B) Quantification of the average organoid size (pxl$^2$) grown from bile ducts isolated from livers of vehicle or ABT737-treated mice and maintained for 10 hours in cold storage. * denotes $p<0.05$ (mean ± SEM), Student’s t test for Day 7.

(C) Quantification of the average organoid size (pxl$^2$) grown from bile ducts isolated from livers of vehicle or D+Q-treated mice and maintained for 10 hours in cold storage. * denotes $p<0.05$ (Mean ± SEM), Student’s t test for Day 7.

(D) Immunofluorescence for p21 (green) and p16 (red) in organoids grown from bile ducts isolated from the livers of vehicle or ABT737-treated mice, cold stored for 10 hours. For p16, digital magnification of the p16-positive foci. Scale bar = 120 µm.

(E) Immunofluorescence for K19 (green) and DCR2 (red) in organoids grown from bile ducts isolated from the livers of vehicle or ABT737-treated mice, cold stored for 10 hours. Right, digital magnification of selected areas. Scale bar = 120 µm.

(F) Percentage of total DCR2, Ki67, PCNA and LGR5 per DAPI-positive cells, in organoids grown from bile ducts isolated from vehicle or D+Q-mice livers cold stored for 10 hours. * $p<0.05$, ** $p<0.01$ (mean ± SEM). Student’s t test.

(G) p21 immunohistochemistry of livers extracted from animals treated with vehicle or ABT737 senolytic regime and cold stored for 10 hours. Scale bar = 60 µm.

(H) H2A.X immunohistochemistry of livers extracted from animals treated with vehicle or ABT737 senolytic regime and cold stored for 10 hours. Scale bar= 60 µm.
Fig S23. Senolytic treatment for ischemia reperfusion injury (IRI) in vivo.

A

ABT737
VEH

Reperfusion
0h 24h 48h

Ischemia
(45 min)

B

IRI + Vehicle
IRI + ABT737

H&E

C

ALT (u/l)

0h 24h 48h

p=0.4747

p=0.8243

**

AST (u/l)

0h 24h 48h

p=0.1249

p=0.0927

***

Cholesterol (mmol/l)

0h 24h 48h

p=0.8413

*  

Alb (g/l)

0h 24h 48h

p=0.7670

p=0.4684

p=0.8023

D

IRI + Vehicle
IRI + ABT737

PCNA K19

E

% Casp3 / Cholang

Veh ABT

***

% Casp3 / Hepatob

Veh ABT

***

F

% DCR2 / Cholang

Veh ABT

**

% DCR2 / Hepatob

Veh ABT

p=0.2111
Fig S23. Senolytic treatment for ischemia reperfusion injury (IRI) in vivo.

(A) Schematic representation of the experiment: we administered senolytic ABT737 or vehicle for a week before the IRI model (45 minutes of ischemia followed by 0, 24 or 48 hours of reperfusion).

(B) Haematoxylin and eosin staining of IRI mice treated with vehicle or senolytic ABT737 at 48 hours of reperfusion. Scale bar= 60 µm.

(C) Transaminases in IRI pre-treated with vehicle (blue), or with ABT737 (orange). * denotes p<0.05, *** p<0.001 (mean ± SEM) Student’s t test for each time group. n≥5 biological replicates for each group.

(D) Left, immunofluorescence for K19 (green) and PCNA (red) in IRI pre-treated with vehicle or senolytic at 48 hours of reperfusion. Scale bar= 60 µm. Right, percentage of cholangiocytes or hepatocytes that proliferate (PCNA-positive) in vehicle (Veh) or ABT737 (ABT)-treated mice at 48 hours of reperfusion. *** p<0.001 (mean ± SEM) Student’s t test.

(E) Percentage of Caspase 3-positive cholangiocytes or hepatocytes at 48 hours of reperfusion. ** p<0.005, *** p<0.001 (mean ± SEM) Student’s t test.

(F) Percentage of DCR2-positive cholangiocytes or hepatocytes at 48 hours of reperfusion. * p<0.05 (mean ± SEM) Student’s t test.
(A) Schematic representation of the senolytic ABT737 administration during procurement: the liver is perfused in situ with ABT737 and placed in a bag submerged in Universal Wisconsin (UW) preservation solution supplemented with ABT737.

(B) Quantification of the number of PCNA-positive proliferating cholangiocytes (left) and hepatocytes (right) in livers perfused with ABT737 and kept in a bag filled with UW+ABT737 for 10 hours of cold storage. *p<0.05, **p<0.01 (mean ± SEM), Student’s t test.

(C) Quantification of the number of DCR2-positive cholangiocytes (left) and hepatocytes (right) in livers perfused with ABT737 and kept in a bag filled with UW+ABT737 for 10 hours of cold storage. *p<0.05 (mean ± SEM), Student’s t test.
Fig S25. Hypothermic machine perfusion (HMP) on cold stored livers from mouse DCD procurement.
Fig S25. Hypothermic machine perfusion (HMP) on col- stored livers from mouse DCD procurement.

(A) Schematic representation of the experiment. We applied a pre-clinical, proof of concept, hypothermic machine perfusion (HMP) to mouse DCD procured livers. This group is then compared it with a DCD procured liver without perfusion.

(B) The experimental setting includes a peristaltic pump that transport cooling water (4°C) into a counterflow wort chiller that keeps the temperature of the perfusion chamber below 10°C. A second peristaltic pump drives prechilled perfusion media (Belzer, 4°C) into the DCD liver at 5 ml/min. A bag of ice is placed on top of the chamber to cool the surface.

(C) Image depicting the experimental setting, indicating the cooling water network (blue) and the perfusion system (orange). Arrows indicate flow direction.

(D) Temperature on the surface of the chamber and the solutions is maintained below 10°C for the whole perfusion.

(E) Haematoxylin and eosin staining of bile duct areas in DCD and HMP-DCD livers. Scale bar= 120 µm.

(F) Left, immunofluorescence for K19 (green) and DCR2 (red) HMP-DCD livers. Scale bar= 120 µm. Right, percentage of cholangiocytes that proliferate (Ki67-positive) or express DCR2 in DCD or HMP-DCD livers. Right, same quantification for hepatocytes. * denotes $p<0.05$, ** $p<0.005$ (mean ± SEM) Student’s $t$ test. n=4 biological replicates for each group.
Fig S26. Senolytic (D+Q) administration in human Couinaud segments II and III.

(A) Portal vein branches indicate Couinaud segments II and III in human liver number 1 (L1).

(B) Segments II and III divided and cannulated in human liver L1.

(C) Left, human liver number 5 (L5) anatomical segments II and III ready for perfusion with D+Q normothermic solution. Control biopsies were obtained at this point, before perfusion. Right, L5 after D+Q perfusion.

(D) Haematoxylin and eosin staining of human liver (L5) pre and post D+Q perfusion. Scale bar= 120 µm.

(E) Percentage of TUNEL, Ki67 and DCR2-positive cholangiocytes and hepatocytes, pre (Control) and after D+Q perfusion from 3 human livers (L5, L6, L7). Student’s t test.
Fig S27. Original immunoblots.

1. **Fig 3B.** Caspase-3 and bActin in murine hepatocytes and cholangiocytes at 10 hours of cold storage.

2. **Fig 3E.** DCR2 and bActin in murine hepatocytes and cholangiocytes at 0 and 10 hours of cold storage.

3. **Fig 4D.** TRIM28 in cholangiocytes at 0 and 10 hours of cold storage.

4. **Fig 4G.** MIF in cholangiocytes at 0 and 10 hours of cold storage.

5. **Fig S21D.** DCR2 and Caspase 3 in p21KO bile ducts after 0 and 10 hours of cold storage.
### Table S1. Donor liver characteristics and reasons for declining transplantation.

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**DBD**: Donation after Brain Death; **DCD**: Donation after Circulatory Death. **WIT**: Warm Ischemic Time if DCD. **CIT**: Cold Ischemic Time. **Sex**: M (Male); F (Female). **BMI**: Body Mass Index.

### Table S2. Donor liver characteristics: liver function biochemistry prior to donation

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**AST**: Aspartate transaminase. **ALT**: Alanine Aminotransferase. **ALP**: Alkaline Phosphatase. **GGT**: Gamma-Glutamyl Transferase.

### Table S3. Primers for genotyping.

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<td>HNF4a mouse</td>
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<td>Itgam mouse</td>
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Table S5. Antibodies

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<td>αSSMA</td>
<td>A2547 Sigma-Aldrich RRID: AB_476701</td>
<td>M mAb</td>
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<td>BrdU</td>
<td>ab6326 Abcam RRID: AB_305426</td>
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<td>ab34710 Abcam RRID: AB_731684</td>
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<td>CYP2D6</td>
<td>Roland Wolf *</td>
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**Isotype Controls**

| DAPI | D9542 Sigma-Aldrich | 1:1000 |

* M, Mouse; R, rat; Rb, Rabbit; G, Goat, Dk, Donkey; H, Human. mAb, monoclonal antibody; pAb, polyclonal antibody.
* Antigen retrieval: C, Citrate 1x; T, Tris-EDTA 1x; HP, High Power; PK, 20 µg/ml proteinase K.*
* CYP2D6 was a kind gift from Prof Roland Wolf, Division of Cancer Research, School of Medicine, University of Dundee.
Table S6. *TNFRSF10D* Oligos.

<table>
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<th>Name</th>
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