The transcription factor ETS1 promotes apoptosis resistance of senescent cholangiocytes by epigenetically up-regulating the apoptosis suppressor BCL2L1

Received for publication, July 11, 2019, and in revised form, October 15, 2019. Published, Papers in Press, October 28, 2019, DOI 10.1074/jbc.RA119.010176

Steven P. O’Hara1,2, Patrick L. Splinter1, Christy E. Trussoni, Maria Eugenia Guicciardi, Noah P. Splinter, Mohammed S. Al Suraih, Navine Nasser-Ghodsi, Deborah Stollenwerk, Gregory J. Gores, and Nicholas F. LaRusso

From the Division of Gastroenterology and Hepatology and the Mayo Clinic Center for Cell Signaling in Gastroenterology, Mayo Clinic, Rochester, Minnesota 55905

Edited by Joel M. Gottesfeld

Primary sclerosing cholangitis (PSC) is an idiopathic, progressive cholangiopathy. Cholangiocyte senescence is important in PSC pathogenesis, and we have previously reported that senescence is regulated by the transcription factor ETS proto-oncogene 1 (ETS1) and associated with overexpression of BCL2 like 1 (BCL2L1 or BCL-xl), an anti-apoptotic BCL2-family member. Here, we further explored the mechanisms regulating BCL-xl-mediated, apoptosis resistance in senescent cholangiocytes and uncovered that ETS1 and the histone acetyltransferase E1A-binding protein P300 (EP300 or p300) both promote BCL-xl transcription. Using immunofluorescence, we found that BCL-xl protein expression is increased both in cholangiocytes of livers from individuals with PSC and a mouse model of PSC. Using an in vitro model of lipopolysaccharide-induced senescence in normal human cholangiocytes (NHCs), we found increased BCL-xl mRNA and protein levels, and ChIP-PCRs indicated increased occupancy of ETS1, p300, and histone 3 Lys-27 acetylation (H3K27Ac) at the BCL-xl promoter. Using co-immunoprecipitation and proximity ligation assays, we further demonstrate that ETS1 and p300 physically interact in senescent but not control NHCs. Additionally, mutagenesis of predicted ETS1-binding sites within the BCL-xl promoter blocked luciferase reporter activity, and CRISPR/Cas9-mediated genetic deletion of ETS1 reduced senescence-associated BCL-xl expression. In senescent NHCs, TRAIL-mediated apoptosis was reduced ~70%, and ETS1 deletion or RNAi-mediated BCL-xl suppression increased apoptosis. Overall, our results suggest that ETS1 and p300 promote senescent cholangiocyte resistance to apoptosis by modifying chromatin and inducing BCL-xl expression. These findings reveal ETS1 as a central regulator of both cholangiocyte senescence and the associated apoptosis-resistant phenotype.

Bile ducts are lined by epithelial cells, termed cholangiocytes, that transport and modify primary bile as it transits from the liver to the small intestine. Primary sclerosing cholangitis (PSC)3 is an idiopathic, progressive fibro-inflammatory disease whereby inflammation and fibrotic stricturing ultimately destroy these intra- and/or extrahepatic bile ducts (1, 2). Approximately 25,000 adolescents and adults in the United States suffer from PSC and the median liver transplant-free survival remains at ~12 years (2, 3). Currently, no effective pharmacotherapy exists to slow progression of the disease. Therefore, a better understanding of PSC pathogenesis and effective, targeted pharmacologic treatment are critically needed. Our recent studies have implicated cholangiocyte senescence as an etiologic feature of PSC and a potential therapeutic target.

The senescent cell fate is characterized by permanent withdrawal from the cell cycle in phase G1 or G2 (4, 5). This cellular phenotype is increasingly recognized as an important pathological feature in a variety of conditions including aging, type 2 diabetes, atherosclerosis, osteoarthritis, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (6–10). The inciting events triggering withdrawal from the cell cycle include strong mitogenic or oncogenic signals, and telomeric or nontelomeric DNA damage (9, 11–13). We previously demonstrated that PSC liver tissue exhibits increased numbers of senescent cholangiocytes, characterized by increased histone marks of DNA damage (γH2AX) and elevated expression of the inhibitor of cyclin-dependent kinases, CDKN2A (p16INK4a) (14). Moreover, these cells expressed markers of the senescence-associated secretory phenotype (SASP) (14), a potentially pathologic state of proinflammatory cytokine, chemo-

3 The abbreviations used are: PSC, primary sclerosing cholangitis; Ets, V-Ets avian erythroblastosis virus E26 oncogene homolog 1; EP300, E1A-binding protein p300; BCL2L1, BCL2-like 1; SASP, senescence-associated secretory phenotype; LPS, lipopolysaccharide; H3K27Ac, histone 3 lysine 27 acetylation; SA-β-gal, senescence-associated β-galactosidase; PLA, proximity ligation assay; qPCR, quantitative PCR; PCNA, proliferating cell nuclear antigen; β-gal, β-galactosidase; fmk, fluoromethyl ketone; POLR2, RNA polymerase 2; DAPI, 4′,6-diamidino-2-phenylindole; EV, empty vector; FL, full-length; OE, overexpression; shRNA, short hairpin RNA; MOMP, mitochondrial outer-membrane permeabilization; BCL2, B cell lymphoma 2; CREB, cAMP-response element-binding protein; NHC, normal human cholangiocyte; SDM, site-directed mutagenesis; HAT, hypoxanthine/aminopterin/thymidine medium; IP, immunoprecipitation; LPS-Is, LPS-induced senescence; Ctrl, control.
kine, and growth factor hypersecretion (15). In previous work, we demonstrated that lipopolysaccharide (LPS) promotes Toll-like receptor 4-dependent activation of the small GTPase, NRAS (16, 17), and persistent LPS treatment of cultured cholangiocytes induces NRAS-dependent expression of p16INK4a and senescence in cultured cholangiocytes (14). Our data also support that the interaction of a Ras responsive transcription factor, ETS1, with the promoter elements of p16INK4a is required for p16INK4a expression and the senescent cholangiocyte cell fate in both LPS-induced and NRAS overexpression culture models (18). The relevance of these in vitro observations was supported by in vivo data showing that phospho-ETS1 protein expression was increased in cholangiocytes of both human PSC liver samples and in the ABC subfamily B member 4 genetic knockout (Abcb4−/−); also known as multidrug-resistant 2 (Mdr2−/−) mouse, an animal model of PSC (18). Although these data enhanced our understanding of the molecular mechanisms of cholangiocyte senescence, they did not address other phenotypic features of senescent cholangiocytes.

Senescence is frequently associated with resistance to apoptosis, which may account for the persistence of senescent cells in tissues and associated deleterious consequences (19–21). The BCL2 protein family plays a central role in mitochondrial-dependent apoptosis (22). This family includes the mitochondrial pore forming effector proteins, BAK and BAX, as well as pro-apoptotic activators and anti-apoptotic mediators, the balance of which determines cell survival or death (22, 23). We recently demonstrated in vitro that the anti-apoptotic protein, BCL2L1 (BCL-xL), is up-regulated in senescent cholangiocytes, and pharmacological inhibition of BCL-xL with the small molecule inhibitor, A1331852, selectively kills cultured senescent cholangiocytes. Moreover, pharmacological inhibition of BCL-xL in the Mdr2−/− mouse diminished the number of senescent cholangiocytes and decreased liver fibrosis (24). Although ETS1 has been implicated in promoting the expression of prosurvival proteins and resistance to apoptosis (25, 26), whether ETS1 promotes apoptosis resistance of senescent cells in general, and of senescent cholangiocytes in particular is unclear and is the focus of our work here. Our collective data suggest that ETS1 not only promotes cholangiocyte senescence via the up-regulation of p16INK4a, but also drives the expression of BCL-xL via the recruitment of the chromatin remodeling histone acetyltransferase, p300. These novel results provide mechanistic insight into senescent cholangiocyte apoptosis resistance, and suggest a potential pathophysiological role in the development and progression of PSC and perhaps other diseases. Moreover, pharmacologic targeting of this pathway may provide a new therapeutic strategy for PSC and other conditions where apoptosis-resistant senescent cells likely contribute to disease progression.

Results

Cholangiocytes from PSC patient and Mdr2−/− mouse liver tissue exhibit increased BCL-xL expression

We previously published that BCL-xL inhibition in the Mdr2−/− mouse model of PSC-depleted senescent cholangiocyte number and improved fibrosis. To extend this observation, we assessed BCL-xL protein expression by immunofluorescent confocal microscopy and confirmed that cholangiocytes from nondiseased human liver (normal control) express very little BCL-xL protein, whereas cholangiocytes from PSC patient liver tissue expressed increased BCL-xL (Fig. 1A). Quantitation of fluorescent intensity was performed and confirmed increased BCL-xL expression (~5-fold) in PSC cholangiocytes compared with normal control cholangiocytes (Fig. 1B). Given that Mdr2−/− mice exhibit increased senescent cholangiocytes (14) and that BCL-xL inhibition improves fibrosis (24), we next assessed cholangiocyte BCL-xL expression in wildtype (WT) C57bl6 and Mdr2−/− mouse liver tissue. As with human specimens, BCL-xL was minimally expressed in cholangiocytes from WT mice, whereas expression was increased ~3-fold in Mdr2−/− mice (Fig. 1, C and D). Thus, these in vivo data confirm and extend our previous work by demonstrating up-regulated expression of the prosurvival protein, BCL-xL, in cholangiocytes in samples of liver from PSC patients and the Mdr2−/− mouse.

LPS treatment increases BCL-xL expression in cultured human cholangiocytes

The anti-apoptotic Bcl-2 proteins may be up-regulated in senescent cells and prevent senescent cell death (20). In an effort to assess the expression of potential pro- and anti-apoptotic mediators in senescent cholangiocytes, we utilized our culture model of LPS-induced cholangiocyte senescence (14). Quantitative PCR (qPCR) demonstrated a selective increase in BCL-xL mRNA (~8-fold), in senescent versus nonsenescent controls (Fig. 2A); other anti-apoptotic BCL2 family members were not affected. We further found that the pro-apoptotic effector, BAK1, increased ~2-fold in senescent cholangiocytes, whereas the proapoptotic activator, Noxa, decreased (Fig. 2A). Western blotting analyses confirmed the selective up-regulation of BCL-xL and BAK1, as well as the decreased expression of BAX and NOXA in senescent cholangiocytes (Fig. 2B). To confirm the induction of cholangiocyte senescence in our culture model, we performed qPCR and Western blotting on senescence-associated gene products. qPCR confirmed that both CDKN2A (p16) and CDKN1A (p21) were up-regulated in our culture system, whereas Western blotting confirmed the up-regulation of CDKN2A, CDKN1A as well as the senescence-associated DNA damage histone mark, γH2A.X (Fig. 2C). The proliferation-associated gene product, proliferating cell nuclear antigen (PCNA) also decreased (Fig. 2C). Furthermore, and as demonstrated by us previously (18), ETS1 is up-regulated in senescent cholangiocytes (Fig. 2C), whereas pharmacologic inhibition of Ras/MAPK prevents ETS1 up-regulation and also diminishes BCL-xL mRNA and protein up-regulation in our senescent cholangiocyte culture model (Fig. 5I). Collectively, these data suggest that the mitochondrial apoptotic pathway in senescent cholangiocytes is primed for mitochondrial cell death via up-regulation of the pro-apoptotic effector protein, BAK1, and support that the increased expression of the anti-apoptotic mediator, BCL-xL, may be essential to sequester BAK1 and confer the apoptosis-resistant phenotype of senescent cholangiocytes.
BCL-xL up-regulation correlates with the induction of cholangiocyte senescence and ETS1 expression

We previously demonstrated that the Ras-responsive transcription factor, ETS1, promoted CDKN21 (p16) expression and cholangiocyte senescence following LPS treatment (18). We therefore asked whether the expression of BCL-xL dynamically correlated with the induction of cholangiocyte senescence and ETS1 expression in our experimentally-induced senescent cholangiocytes. Western blotting demonstrated that BCL-xL up-regulation occurred following 6 days of repeated LPS treatment, which correlates with ETS1 up-regulation and the induction of senescence as demonstrated with the induction of CDKN2A and CDKN1A (Fig. 3A). To directly assess whether the up-regulated expression of BCL-xL correlated with apoptosis resistance and senescence, we utilized our cell culture model of induced senescence and assessed apoptosis by measuring activation of caspase-3/7 and senescence by β-galactosidase positivity. Although untreated normal human cholangiocytes (NHCs) exhibited minimal apoptosis, we found that exposure to LPS induced cultured NHC apoptosis at days 1–4, but decreased on days 6–10 (Fig. 3B). Moreover, and as demonstrated previously (14), β-gal activity increased at 6 days post-repeated LPS treatment, and persisted through 10 days post-repeated LPS treatment (Fig. 3C). To confirm the apoptosis-resistant phenotype we treated nonsenescent or induced senescent NHC and that the induction of apoptosis in nonsenescent NHC was prevented by the caspase inhibitor Z-VAD-fmk (Fig. 3, D and E). Senescence detection remained elevated in LPS-LS cholangiocytes in both the presence and absence of TRAIL and the presence and absence of Z-VAD-FMK (Fig. 3F). Together, these data continue to support an essential role of ETS1 in cholangiocyte senescence and demonstrate further that BCL-xL up-regulation correlates with the expression of ETS1, senescence induction, and the apoptosis-resistance phenotype.

BCL-xL interacts with the cell death effectors BAK and BAX in senescent cholangiocytes

Anti-apoptotic Bcl-2 proteins (e.g. BCL-xL) either inhibit the pro-apoptotic effectors (BAK and BAX) via direct binding of
their activated form or by sequestering “activator” BH3-only proteins (e.g. BIM, NOXA) and preventing their activation of BAK and BAX (22). We therefore assessed whether the anti-apoptotic protein BCL-xL directly interacted with apoptotic effectors or activators in senescent cholangiocytes. We immunoprecipitated BCL-xL from control and senescent cholangiocytes and performed immunoblotting for apoptosis activators and effectors. We found that BCL-xL formed an immunoprecipitable complex with BAK and BAX, but not BIM or NOXA (Fig. 4A). It is proposed that BCL-xL interacts with activated BAK and/or BAX, blocks oligomerization of these proapoptotic molecules, and thus prevents apoptosis (23, 24). Therefore, we next immunoprecipitated activated BAK and BAX and immunoblotted for BCL-xL. We detected BCL-xL only in senescent cholangiocytes and not in control cholangiocytes (Fig. 4B). Finally, to determine whether the antiapoptotic protein BCL-xL interacts with BAX and BAK in a human PSC liver tissue, we performed proximity ligation assays on formalin-fixed paraffin-embedded tissue. This fluorescence-based approach supports that active BAK and BAX in senescent cholangiocytes and that this interaction is a likely mechanism for the apoptosis-resistant phenotype of senescent cholangiocytes. Furthermore, the data supports that this process occurs in cholangiocytes from PSC liver tissue.

**ETS1 and the histone acetyltransferase, EP300 (p300), occupy the BCL-xL proximal promoter during induced cholangiocyte senescence**

The DNA elements upstream of the BCL-xL transcriptional start site were analyzed for potential ETS1-binding sites and also analyzed for potential epigenetic regulation (i.e. histone acetylation marks). To perform these in silico analyses, we used the Encyclopedia of DNA Elements (ENCODE) annotation data (30) through the UCSC Genome Browser (http://genome.ucsc.edu/) and the Blast-like alignment tool (BLAT) (31). This search tool identified a number of putative Histone-3 lysine 27 acetylation (H3K27ac) (permissive chromatin) marks (not shown) and three potential ETS1-binding sites throughout the proximal BCL-xL promoter region (Fig. 5A). Next, we...
performed ChIP-PCR (Fig. 5A) and quantitative (q)ChIP-PCR (Fig. 5B) on nonsenescent control NHCCs and cells from our induced senescence model. The BCL-xL promoter PCR ampli-
con is increased in senescent cholangiocytes after 10 days of LPS treatment (Fig. 5, A and B), suggesting that this transcription factor occupies the BCL-xL proximal promoter upon the induction of senescence. We next performed ChIP-PCR for the histone acetyltransferase, p300 and H3K27ac marks. We found that the chromatin modifying enzyme, p300, and the associated histone modification mark, H3K27ac, were both increased at the BCL-xL promoter locus upon senescence induction (Fig. 5, A and B). We also performed qChIP-PCR for activated RNA Polymerase 2 (POLR2), using an antibody specific for phosphorylated serine 2 of the POLR2 C-terminal domain (p-Ser-2 POLR2). Again, we observed increased PCR amplification of the BCL-xL promoter following the induction of senescence supporting active transcription from this locus (Fig. 5, A and B). To confirm co-occupancy of ETS1 and p300 at the BCL-xL locus during cholangiocyte senescence, we performed sequential ChIP-PCR. Initially, an antibody against p-ETS1 was used for ChIP and PCR amplification was performed for the BCL-xL promoter locus; as previous, amplification was seen only in senescent cholangiocytes. Subsequent immunoprecipitation of the pETS1 chromatin immunoprecipitate was performed with an antibody against p300; again, amplification of the BCL-xL promoter locus occurred only in senescent cholangiocytes (Fig. 5C). The converse, i.e. initial ChIP with p300 followed by subsequent ChIP of the immunoprecipitate with pETS1, again revealed amplification of the BCL-xL locus only in senescent cholangiocytes. Together, these data support that ETS1 and p300 occupy the BCL-xL promoter locus, the permissive chromatin mark H3K27ac accumulates at this locus, and active transcription occurs at this region (p-Ser-2 POLR2) during cholangiocyte senescence.
increased amplification of the distal (H9004 of a luciferase reporter (FL, 1009 bp). Site-directed mutagenesis was performed and was performed to detect simultaneous interaction of ETS1 and p300 with the transcription factor-binding sites (H11002 1/2, 167) or combinations of these mutated predicted ETS1-BCL-xL (H11002 1/2, 167 bp) predicted ETS1-binding sites. Combinations of these mutants (Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3) were also generated. The FL construct in nonsenescent (Ctrl) cholangiocytes did not increase luciferase activity above the EV control. Persistent treatment with LPS (LPS-UIS) induced luciferase activity (−3-fold) in both full-length and the Δ3 construct (n = 8; *, p < 0.05 versus Ctrl FL). Luciferase activity was not induced in LPS-UIS cholangiocytes transfected with the Δ1, Δ2, Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3 constructs.

**Predicted ETS1-binding sites promote reporter gene expression in senescent cholangiocytes**

To further address the role of ETS1 in our induced cholangiocyte senescence model, we generated a BCL-xL promoter-driven luciferase reporter construct. Approximately 1000 bp (full-length, FL) of the BCL-xL proximal promoter were cloned into the luciferase reporter plasmid, pGL4.22 (BCL-xL-pGL4.22). Constructs with the individual ETS1 sites mutated by site-directed mutagenesis (Δ1 (−861), Δ2 (−341), and Δ3 (−167)) or combinations of these mutated predicted ETS1-binding sites (Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3) were also generated. We found that the FL BCL-xL promoter induced luciferase expression upon the induction of cholangiocyte senescence compared with nonsenescent control cholangiocytes (Fig. 5D). Moreover, the individual mutations Δ1 and Δ2, or combinations with these mutations present (Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3) significantly decreased induction of the BCL-xL promoter-induced luciferase in senescent cholangiocytes (Fig. 5D). Although these data do not specifically identify ETS1 as the driver of transcription, as all members of the ETS family interact with similar sequence motifs (33), the data identify ETS1-binding sites within the BCL-xL promoter show that they are functional, and that they are required for efficient transcription of the luciferase reporter.

**ETS1 and p300 physically interact in senescent cells and in PSC cholangiocytes**

Having demonstrated that ETS1 and p300 co-occupy the BCL-xL promoter in senescent NHC, we next assessed whether ETS1 and p300 formed a complex and whether this complex was required for BCL-xL transcription. First, we immunoprecipitated p300 from nonsenescent control and senescent cholangiocytes and performed immunoblots for ETS1 and p300 (Fig. 6A). We found that ETS1 formed an immunoprecipitable complex with p300 only in senescent cholangiocytes, whereas the p300 immunoblot confirms efficient immunoprecipitation (Fig. 6A). ETS1 was also immunoprecipitated from nonsenescent control and senescent NHC cellular lysates and immunoblotting for p300 and p-ETS1 was performed on the immunoprecipitate. We again found

**Figure 5. Phospho-ETS1 and p300 drive BCL-xL promoter-mediated transcription.** A, the BCL-xL putative promoter region contains 3 potential ETS1 transcription factor-binding sites (−861, −314, and −167). Cholangiocytes were treated for 10 days in the presence or absence of LPS (LPS-IS and Ctrl, respectively). We performed IP with IgG control antibody (IgG ctrl) or with ChIP quality antibodies against p-ETS1, p300, H3K27ac, and p-POLR2 and PCR amplification of BCL-xL promoter loci. ChIP-PCR reveals minimal amplification of the BCL-xL promoter locus from all four immunoprecipitations in Ctrl cholangiocytes and increased amplification from LPS-IS cholangiocytes. B, qChIP-PCR using p-ETS1 confirms the increased amplification of the BCL-xL promoter (−8-fold), p300 (−4.5-fold), H3K27ac (−4-fold), and p-POLR2 (−4-fold) in LPS-IS versus Ctrl cholangiocytes (n = 3; *, p < 0.05). C, sequential ChIP-PCR was performed to detect simultaneous interaction of ETS1 and p300 with the BCL-xL locus. As previously shown, ChIP-PCR using a p-ETS1 antibody shows increased amplification of the BCL-xL promoter in LPS-IS versus Ctrl cholangiocytes. Subsequent p300 ChIP-PCR of the p-ETS1 IP product reveals amplification of the BCL-xL promoter locus in LPS-IS but not Ctrl cholangiocytes. The converse, p300 ChIP-PCR, with sequential pETS1 ChIP-PCR for BCL-xL further supports the simultaneous occupancy of these proteins at the BCL-xL promoter locus in LPS-IS cholangiocytes. D, the BCL-xL proximal promoter locus was cloned upstream of a luciferase reporter (FL, 1009 bp). Site-directed mutagenesis was performed and BCL-xL promoter-driven luciferase constructs were generated to eliminate the distal (Δ1; −861 bp), mid (Δ2; −314 bp), and proximal (Δ3; −167 bp) predicted ETS1-binding sites. Combinations of these mutants (Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3) were also generated. The FL construct in nonsenescent (Ctrl) cholangiocytes did not increase luciferase activity above the EV control. Persistent treatment with LPS (LPS-UIS) induced luciferase activity (−3-fold) in both full-length and the Δ3 construct (n = 8; *, p < 0.05 versus Ctrl FL). Luciferase activity was not induced in LPS-UIS cholangiocytes transfected with the Δ1, Δ2, Δ1/2, Δ1/3, Δ2/3, and Δ1/2/3 constructs.
that p300 and ETS1 formed an immunoprecipitatable complex, which was increased in senescent compared with nonsenescent control NHC; the pETS1 blot confirms efficient immunoprecipitation (Fig. 6B). Having previously demonstrated that senescent cholangiocytes are enriched in PSC patient bile ducts, we next assessed whether we could detect the physical interaction between ETS1 and p300 in bile ducts of PSC patients. Proximity ligation assays (PLA) were performed in tissue derived from either nondiseased human bile ducts (normal) or in PSC; DAPI was used to identify nuclei. PLA fluorescence was not detected in normal bile ducts, whereas PSC patient bile ducts exhibited increased PLA-associated fluorescence (Fig. 6C), suggesting that the proteins physically interact in diseased bile ducts. Together, these data suggest that ETS1 and p300 physically interact in senescent cholangiocytes and in PSC patient bile ducts, which are enriched in senescent cholangiocytes.

**ETS1 depletion prevents p300 occupancy at the BCL-xL promoter in senescent cholangiocytes**

Given that ETS1 and p300 co-occupy the BCL-xL promoter and physically interact in senescent cells, we assessed whether ETS1 was required for p300 localization at the BCL-xL promoter locus. To test this, ETS1-deficient cultured cholangiocytes were generated using CRISPR Cas9 technology. The BCL-xL locus was successfully amplified following ChIP with antibodies against ETS1, p300, and H3K27ac in senescent NHCs, but PCR amplification was diminished in the absence of ETS1 (ΔETS1) (Fig. 7, A and B). ChIP-PCR was also performed on nonsenescent and NHCs cultured in our in vitro senescence model, which were depleted of p300 (p300 shRNA). In these p300-depleted cells, ChIP-PCR revealed decreased amplification of the BCL-xL promoter following ChIP with p300 and H3K27ac antibodies, however, ChIP-PCR with the ETS1 antibody resulted in BCL-xL promoter amplification similar to senescent NHC, suggesting that ETS1 can bind to the BCL-xL promoter locus in the absence of p300 (Fig. 7, C and D). Similarly, treatment of cultured cholangiocytes with an inhibitor of p300 histone acetyltransferase activity (C646) diminished BCL-xL and acetylated H3K27 expression, and suppressed LPS-induced senescence (Fig. S2, A–C). Together, these data support that ETS1 is required for p300 occupancy and H3K27ac accumulation at the BCL-xL promoter locus.

**Depletion of NHC ETS1 prevents senescence-associated BCL-xL expression**

To further demonstrate the functional role of ETS1 in cholangiocyte senescence-associated BCL-xL expression, we used ETS1-deficient cultured cholangiocytes. Western blotting demonstrated that ETS1-deficient cholangiocytes exhibited diminished BCL-xL expression and did not show up-regulated BCL-xL expression in our senescent cholangiocyte model, whereas Bcl2 and Mcl1 expression remained unchanged in both NHC- and ETS1-deficient cholangiocytes (Fig. 8, A and B). Quantitative PCR further demonstrated that BCL-xL, but not BCL2 or MCL1, is up-regulated in our senescent cholangiocyte model, but expression is diminished in cells lacking ETS1 (Fig. 8C). Next, we performed BCL-xL promoter luciferase assays in ΔETS1 cholangiocytes. The BCL-xL promoter did not induce luciferase expression in our LPS-induced senescence model in the ΔETS1 cholangiocytes. Moreover, reconstitution of ΔETS1 cholangiocytes with constructs encoding either ETS1 lacking the transactivation domain (ETS-ΔTrans) or a nonphosphorylatable mutant (ETS1-T38A) were resistant to induced senescence-associated BCL-xL promoter luciferase expression (Fig. 8D). In contrast, forced overexpression (OE) of ETS1 induced luciferase expression in NHC with or without LPS treatment, with more robust expression in the LPS-treated (i.e. senescent) NHC (Fig. 8D). These data continue to support that ETS1 is required for the induced expression of BCL-xL in our culture model of senescence.
Deletion of NHC ETS1 prevents senescence-associated NHC apoptosis resistance whereas overexpression of ETS1 or BCL-xL rescues this phenotype

Having demonstrated that ETS1 is required for increased BCL-xL expression in senescent cholangiocytes, we next assessed whether ETS1 and BCL-xL are required for senescent cholangiocyte apoptosis resistance. First, we demonstrated, using ΔETS1 cholangiocytes in our in vitro NHC senescence model, that forced overexpression of ETS1 restored BCL-xL and p-ETS1 expression (Fig. 9A). Using our in vitro senescence model and the caspase-3/7 and annexin V positivity assays, we demonstrated that ΔETS1 cholangiocytes are more susceptible to apoptosis (ΔETS1 transfected with the empty vector (EV)) and that overexpression of ETS1 (ETS1 OE) rescues apoptosis resistance (Fig. 9, B and C). As demonstrated previously, ETS1-deficient cholangiocytes are resistant to induced senescence (18), whereas forced expression of ETS1 in ΔETS1 cholangiocytes promoted senescence as demonstrated by fluorescent β-gal detection and quantification (Fig. 9D). Next, we overexpressed BCL-xL in ΔETS1 cholangiocytes (Fig. 9E). Using our in vitro senescence model and apoptosis assays, we demonstrated that ΔETS1 cholangiocytes transfected with the EV were more susceptible to apoptosis, whereas forced expression of BCL-xL (BCL-xL OE) restores apoptosis resistance, but did not promote senescence (Fig. 9, F–H). Together, these data further support that ETS1 promotes the expression of BCL-xL and is required for cholangiocyte senescence, and that BCL-xL expression is sufficient to promote the apoptotic-resistant phenotype of senescent NHCs.

RNAi-induced suppression of BCL-xL in senescent cholangiocytes promotes apoptosis

To further confirm the role of BCL-xL in senescent NHC apoptosis resistance, we transfected NHC with a doxycycline-inducible BCL-xL shRNA. Nonsenescent control or LPS-in
The BCL family member, BCL-xL, providing mechanistic insight into the transcriptional regulation of BCL-xL expression, and the resultant resistance to apoptosis of senescent cholangiocytes (Fig. 11). Our in vivo results demonstrate that BCL-xL is up-regulated in cholangiocytes of human PSC and Mdr2−/− mouse liver. Using an in vitro model of stress-induced cholangiocyte senescence, we further demonstrated that: (i) BCL-xL mRNA and protein is increased and pharmacologic inhibition of RAS/MAPK signaling prevented the senescence-associated expression of BCL-xL; (ii) up-regulated BCL-xL physically interacts with the apoptosis effector proteins BAK and BAX; (iii) ETS1 and the histone acetyltransferase p300 physically interact with each other at the BCL-xL promoter locus, promote H3K27 acetylation and active POLR2 (p-Ser-2) occupation at the BCL-xL locus; (iv) site-directed mutagenesis or deletion of the predicted distal ETS-binding sites within the BCL-xL promoter prevented induced luciferase reporter detection; (v) genetic deletion of ETS1 prevented BCL-xL expression and promoted apoptosis, whereas reconstitution of ETS1 restored BCL-xL expression, senescence, and apoptosis resistance; and (vi) induced suppression of BCL-xL (shRNA) resulted in increased apoptosis and reduced detection of senescence. Moreover, we demonstrated the relevance of our mechanistic in vitro data by demonstrating in vivo, with proximity ligation assays in human PSC tissue, that BCL-xL interaction

Discussion

Our results reveal that ETS1 promotes cholangiocyte resistance to apoptosis by selectively up-regulating transcription of the BCL family member, BCL-xL, providing mechanistic insight into the transcriptional regulation of BCL-xL expression, and the resultant resistance to apoptosis of senescent cholangiocytes (Fig. 11). Our in vivo results demonstrate that BCL-xL is up-regulated in cholangiocytes of human PSC and Mdr2−/− mouse liver. Using an in vitro model of stress-induced cholangiocyte senescence, we further demonstrated that: (i) BCL-xL mRNA and protein is increased and pharmacologic inhibition of RAS/MAPK signaling prevented the senescence-associated expression of BCL-xL; (ii) up-regulated BCL-xL physically interacts with the apoptosis effector proteins BAK and BAX; (iii) ETS1 and the histone acetyltransferase p300 physically interact with each other at the BCL-xL promoter locus, promote H3K27 acetylation and active POLR2 (p-Ser-2) occupation at the BCL-xL locus; (iv) site-directed mutagenesis or deletion of the predicted distal ETS-binding sites within the BCL-xL promoter prevented induced luciferase reporter detection; (v) genetic deletion of ETS1 prevented BCL-xL expression and promoted apoptosis, whereas reconstitution of ETS1 restored BCL-xL expression, senescence, and apoptosis resistance; and (vi) induced suppression of BCL-xL (shRNA) resulted in increased apoptosis and reduced detection of senescence. Moreover, we demonstrated the relevance of our mechanistic in vitro data by demonstrating in vivo, with proximity ligation assays in human PSC tissue, that BCL-xL interaction

Figure 8. Genomic deletion of ETS1 suppresses BCL-xL expression. A, control cholangiocytes (NHC) or NHC cells stably transfected with a CRISPR/Cas9 double nickase to ETS1 (ΔETS1) were treated in the absence or presence of LPS for 10 days (Ctrl and LPS-IS, respectively). Immunoblotting demonstrated an increase in both ETS1 and BCL-xL in LPS-IS NHC versus Ctrl NHC, but neither protein was detected in Ctrl and LPS-IS ΔETS1 cells. Conversely the prosurvival proteins, BCL2 and MCL1, were not changed. B, semiquantitative densitometry of BCL-xL immunoblots demonstrates an increase in BCL-xL detection in LPS-IS NHC versus Ctrl NHC and a decrease (−6.5-fold) of BCL-xL in LPS-IS ΔETS1 compared LPS-IS NHC (n = 3). C, quantitative PCR shows increased BCL-xL mRNA in LPS-IS NHC versus Ctrl NHC, and decreased BCL-xL mRNA (−5.7-fold) in LPS-IS ΔETS1 compared with LPS-IS NHC. BCL2 and MCL1 showed no change in ∆ETS1 cells in any of the conditions (n = 3; *, p < 0.05). D, the pGL4.22 (EV) control or the full-length BCL-xL promoter-luciferase construct were transfected into ∆ETS1 cells. No increase in luciferase detection over EV control was observed in Ctrl and LPS-IS ∆ETS1 cells. Additionally, transfection and forced expression of the phospho-mutant ETS1 (T38A) and the transactivation domain mutant ETS1 (ΔTrans) into ∆ETS1 cells showed no responsiveness in both Ctrl and LPS-IS cells (n = 6). Transfection and OE of WT ETS1 in ∆ETS1 cells resulted in increased BCL-xL promoter-dependent luciferase detection in both Ctrl ETS1 OE (−5.4-fold) and LPS-IS OE (−11-fold) compared with LPS-IS ∆ETS1 cells (n = 6; *, p < 0.05).
with BAK and BAX and phospho-ETS1 interaction with p300 are likely increased in cholangiocytes of human PSC tissue. These data show for the first time that cholangiocyte ETS1 and p300-dependent chromatin modification of the BCL-xL promoter locus induces BCL-xL expression, which is required for apoptosis resistance of senescent cholangiocytes in vitro, and

**Figure 9.** ETS1 and BCL-xL overexpression promote the prosurvival phenotype of LPS-induced senescent cholangiocytes. A, immunoblots demonstrate that transfection and OE of ETS1 rescues the expression of BCL-xL in ΔETS1 cells. Although BCL-xL was detected in Ctrl ETS1 OE cells, it is robustly up-regulated in LPS-IS ETS1 OE cells. Phospho-ETS1 is also expressed in LPS-IS ETS1 OE cells, but was not detected in nonsenescent (Ctrl) ETS1 OE cells. B and C, apoptosis, as measured by caspase-3/7 assay and annexin V staining, is increased (~4.9- and ~13.6-fold, respectively) in the EV-transfected LPS-IS ΔETS1 versus LPS-IS NHC (n = 6; *, p < 0.05). Apoptosis, assessed by both assays, was reduced in cholangiocytes overexpressing ETS1 (ETS1 OE) in ΔETS1 cells (~7.4- and ~5.8-fold decrease, respectively) versus EV-LPS-IS ΔETS1 (n = 6; *, p < 0.05). D, the senescence marker fluorescent β-gal, decreased (~6.9-fold) in EV-LPS-IS ΔETS1 cholangiocytes versus LPS-IS NHC (n = 6). However, senescence increased (~7-fold) in LPS-IS ΔETS1 cholangiocytes overexpressing ETS1 (ETS1 OE) versus EV-transfected LPS-IS ΔETS1 cholangiocytes (n = 6; *, p < 0.05). E, immunoblots show that transfection and forced overexpression of BCL-xL (BCL-xL OE) in ΔETS1 cells re-establishes BCL-xL expression, but not phospho-ETS1, CDKN2A (p16), or CDKN1A (p21) expression. F and G, apoptosis, as measured by caspase-3/7 assay and annexin V staining of cholangiocytes, is increased (~5.1- and ~4.9-fold, respectively) in EV-transfected LPS-IS ΔETS1 versus LPS-IS NHC (n = 6). However, apoptosis, as assessed by these methods, was reduced in LPS-IS ΔETS1 overexpressing BCL-xL (BCL-xL OE; ~4.6- and ~2.2-fold decrease, respectively) versus EV-transfected LPS-IS ΔETS1 (n = 6; *, p < 0.05). H, the senescence marker fluorescent β-gal is decreased (~6-fold decrease) in EV-transfected ΔETS1 cells compared with LPS-IS NHC. Senescence was slightly increased (~2.6-fold) in LPS-IS ΔETS1 rescued with BCL-xL OE compared with EV-transfected LPS-IS ΔETS1 (n = 6; *, p < 0.05).

**Figure 10.** Inducible BCL-xL-shRNA increased apoptosis and decreased apoptosis resistance. A, doxycycline (Dox)-induced expression of a stably transfected BCL-xL shRNA resulted in decreased BCL-xL protein expression in both LPS-IS and nontreated (Ctrl) cholangiocytes versus non-Dox-treated cells. ETS1 was not affected by the BCL-xL shRNA; β-actin was used as a loading control. B and C, apoptosis, as measured by caspase-3/7 assay and annexin V detection, is increased (~6.4- and 6.1-fold, respectively) in LPS-IS cholangiocytes following induced BCL-xL-shRNA induction versus LPS-IS cells without shRNA induction (n = 6; *, p < 0.05). D, the senescence marker, fluorescent β-gal, is decreased (~2.2-fold) in LPS-IS cholangiocytes upon the induction of BCL-xL shRNA expression versus LPS-IS cholangiocytes in the absence of doxycycline (n = 6; *, p < 0.05).

**Figure 11.** Conceptual framework of BCL-xL expression during persistent LPS senescence induction. Persistent cholangiocyte stress results in the activation of NRAS and subsequently the MAPK/ERK signaling pathway. ETS1 is up-regulated, phosphorylated, and translocates to the nucleus. In previous work we demonstrated that pharmacologic inhibition of Ras/MEK/ERK (manumycin A, PD98059, or UO126) and molecular inhibition of ETS1 is up-regulated, phosphorylated, and translocates to the nucleus. In previous work we demonstrated that pharmacologic inhibition of Ras/MEK/ERK (manumycin A, PD98059, or UO126) and molecular inhibition of ETS1 (Ets1-shRNA) or genetic deletion of ETS1 prevents p16 (CDKN2A) and p21 (CDKN1A) expression, and ultimately senescence-associated apoptosis resistance, likely via sequestration of BAK and BAX and prevention of MOMP. This pathway is likely operative in cholangiocytes in PSC liver. Thus, our results provide mechanistic insight into stress-induced (i.e. LPS treatment) senescent cholangiocyte apoptosis resistance, a cellular process that may promote the persistence of senescent cholangiocytes and the potential deleterious consequences of the cholangiocyte SASP in PSC. Hence, this cellular phenotype and the identified pathways may contribute to the pathogenesis of PSC, and are novel potential pharmacologic targets for PSC therapy.
ETS1/p300 and BCL2L1 expression

The Ets family of transcription factors, including ETS1 and ETS2 are effectors of the RAS/MAPK signaling pathway (34–36). Moreover, ETS1 was previously implicated in CDKN2A (p16INK4a) expression and senescence in human diploid fibroblasts (37). Additionally, our previous in vitro and in vivo studies identified ETS1, but not ETS2, as a central mediator of persistent LPS-induced cholangiocyte CDKN2A (p16INK4a) expression and hence, senescence (18). More recently, we demonstrated, in vitro, that the anti-apoptotic protein BCL-xL is up-regulated in irradiation-induced senescent cholangiocytes, and pharmacological inhibition of BCL-xL with the small molecule inhibitor, A1331852, selectively kills cultured senescent cholangiocytes. Moreover, pharmacological inhibition of BCL-xL in the Mdr2−/− mouse with A1331852 diminished the number of senescent cholangiocytes and decreased liver fibrosis (24). However, the molecular mechanisms of BCL-xL up-regulation in senescent cholangiocytes remained unclear and were the focus of our efforts in this manuscript. Although there is no consensus regarding the role of ETS1 in apoptosis regulation, reports have implicated this transcription factor in the induced expression of anti-apoptotic Bcl2 family members (25, 26). Together with our previous data, here we demonstrate that ETS1 not only induces cholangiocyte withdrawal from the cell cycle via p16INK4a expression, but promotes the phenotypic transition to apoptosis resistance via induced expression of BCL-xL.

The B cell lymphoma 2 (BCL2) gene family regulates mitochondrial-dependent apoptosis. This family comprises both pro- and anti-apoptotic members that interact with each other and the mitochondrial membrane to either prevent or promote mitochondrial outer-membrane permeabilization (MOMP). Pro-apoptotic members of this family promote MOMP, which releases several activators of apoptosis into the cytosol. Anti-apoptotic BCL2 proteins (e.g. BCL2 and BCL-xL) antagonize proapoptotic BH3-only proteins (e.g. BID, BIM, and NOXA) or directly sequester the effector proteins, BAK and BAX. Hence, the balance of pro- and anti-apoptotic BCL2 family members determines cell survival or death (22). Although the short-term presence of senescence can be beneficial in certain circumstances, such as embryonic development (38, 39) and wound healing (40), increasing evidence supports that long-term retention of senescent cells can have detrimental effects on tissue health (9, 41–43). Apoptosis resistance of senescent cells likely contributes to their long-term retention in tissues in aging animals as well as in senescence-associated disease states. Importantly, we show that BCL-xL physically interacts with the effector proteins, BAK and BAX, in senescent-cultured cholangiocytes and in human PSC. This interaction of BCL-xL with these apoptosis effector proteins prevents BAK and BAX-dependent MOMP, and hence the induction of apoptosis. Collectively, the data support that induced BCL-xL expression promotes apoptosis resistance of senescent cholangiocytes, and apoptotic-resistant senescent cholangiocytes are a prominent feature of PSC. These data also implicate BCL-xL up-regulation and the resultant apoptotic-resistant phenotype of senescent cholangiocytes as potential therapeutic targets for PSC.

Mechanisms promoting senescent cell apoptotic resistance are currently being explored, but these remain unclear and likely differ among cell types (42). Together with our previous finding that targeted killing of senescent cholangiocytes via targeting BCL-xL improved the Mdr2−/− mouse phenotype, our work presented here implicating ETS1 in the up-regulation of BCL-xL, is the only information regarding apoptosis resistance in senescent cholangiocytes. We demonstrated the transition from repressive to permissive chromatin at the BCL-xL promoter over time in our culture model of senescence. The histone acetyltransferase, p300, and its parologue, CREB-binding protein (CREBBP, CBP), modulate locus-specific transcription via direct lysine acetyltransferase catalytic activity, and acetylation of histones, promoting accessibility for the transcription apparatus. Our current data demonstrates not only the accumulation of ETS1 and the transcriptional co-activator, p300, to the BCL-xL promoter locus, but the likely physical interaction of these transcriptional regulators. Although the physical interaction between phospho-ETS1 and p300 has been described previously (44, 45), as has the likely requirement of ETS1 for the occupancy of CBP at enhancer regions in activated T-cells (39), our data presented herein suggests that ETS1 directs p300 to the BCL-xL locus during senescence induction. Indeed, genetic depletion of ETS1 prevented the accumulation of p300 at the BCL-xL locus; conversely, depletion of p300 did not prevent ETS1 accumulation. Therefore, the activation of a Ras-responsive transcription factor, ETS1, and the interaction of this transcription factor at specific sites in the genome, may promote loci-specific chromatin modification and gene expression via recruitment of p300. It should be noted that not every cholangiocyte in the PSC patient liver tissue revealed positivity for the PLA products demonstrating BCL-xL:BAK/BAX or ETS1:p300 proximity. This outcome likely reflects the heterogeneity of the cholangiocytes, and the presence of both senescent and non-senescent cells within bile ducts (14). Although not addressed in this manuscript, the concept of ETS1 promoting global transcriptional modification during cholangiocyte senescence via recruitment of chromatin-modifying enzymes to specific loci merits further investigation as does the question of why some cholangiocytes transition to a senescent phenotype, whereas others do not.

Senescent cells frequently transition to the SASP, a potentially pathologic state of proinflammatory cytokine, chemokine, and growth factor hypersecretion (15). Surprisingly, we previously demonstrated that cells lacking ETS1 express increased expression of the SASP-associated components, interleukin 6 and interleukin 8, both in the presence and absence of LPS (18). We propose that whereas ETS1 is essential for senescence induction and the apoptotic-resistant phenotype via up-regulated gene expression, this transcription factor may function as a negative regulator of cholangiocyte proinflammatory cytokine production. Hence, cholangiocyte senescence, apoptosis resistance, and SASP are regulated by ETS1-dependent gene expression regulation via distinct mechanisms. We are actively interrogating this interesting observation in additional model systems and via unbiased RNAseq and ChIPseq studies in senescent versus non-senescent cholangiocytes.

In summary, we have defined a mechanism regulating the apoptotic-resistant phenotype of LPS-induced cholangiocyte senescence in vitro and provided compelling evidence for the
function of this pathway in human and animal models of PSC. Our findings support the central role of ETS1 not only in senescence via the regulated expression of CDKN2A (18), but also in the apoptosis-resistant phenotype of senescent cholangiocytes via up-regulated BCL-xL expression. These insights provide a better mechanistic understanding of the relationship between cholangiocyte senescence and the cholangiopathies, and identify targets for molecular interventions including pathways (RAS/MAPK), transcriptional regulators (ETS1/p300), and phenotypic features (apoptosis resistance via up-regulated BCL-xL) to selectively prevent or eliminate senescent cholangiocytes (senolytics) as a therapeutic approach for PSC. Moreover, these observations may be generalizable to other progressive, fibroinflammatory diseases especially those that implicate cellular senescence in pathogenesis, for example, idiopathic pulmonary fibrosis (10).

**Experimental procedures**

This study was approved by the Mayo Clinic Institutional Review Board and abides by the Declaration of Helsinki principles. The Mayo Clinic Institutional Animal Care and Use Committee also approved this study.

**Liver tissues**

Upon Institutional Animal Care and Use Committee approval, C57b16 background Mdr2−/− mice and C57b16 WT mice were housed at the Mayo Clinic animal care facility with a standard 12:12 h light/dark cycle and ad libitum access to water and standard rodent diet.

Following IRB approval, human PSC patient samples, which fulfilled clinical, serological, histological, and/or cholangiographic criteria for stage IV PSC, were obtained at the time of transplant. Normal liver samples from surgical resection or explant were also utilized. Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned (4 μM) for immunofluorescence.

**Immunofluorescence**

Briefly, tissue sections were deparaffinized in xylene and rehydrated through a series of increasing ethanol dilutions. An antigen retrieval step was performed by immersing slides in boiling sodium citrate buffer (pH 6.0) for 10 min. Slides were then washed in buffer containing Tris base, NaCl, and Triton X-100, blocked in 4% BSA solution, and incubated with primary antibodies to BCL-xL (54H6, Cell Signaling) and CK19 (Santa Cruz Biotechnology) overnight at 4 °C. After primary incubation, slides were washed in PBS and incubated with Alexa Fluor secondary antibodies (Life Technologies) for 30 min at room temperature. Slides were washed again in PBS and mounted using Prolong-Gold Antifade with DAPI (Life Technologies). Slides were analyzed using a Zeiss 780 Laser Scanning Confocal Microscope.

**Cell culture and in vitro model of senescence**

The well-characterized normal human cholangiocyte (NHC) cell line derived from normal liver was provided by Dr. Medina (University of Navarra, Pamplona, Spain) (46). The NHC cells were routinely monitored for mycoplasma by using the Lonza MycoAlert™ and culturing the cells in the presence of Primocin (Invivogen). Our in vitro model of senescence was performed as previously described (14, 18).

**Total RNA extraction and quantitative RT-PCR**

TRIzol (Invitrogen) was used to extract total RNA from the NHC cells according to the manufacturer’s protocol. Following RNA extraction, 2.0 μg of total RNA was used as template for reverse transcription using the SuperScript III First Strand Synthesis system (Invitrogen) according to the manufacturer’s directions. The cDNA was then used as template for PCR amplification (primers listed in Table 1), mixed with the Rotor-Gene SYBR Green PCR Master Mix (Qiagen), and analyzed with the Rotor-gene quantitative PCR instrument (Qiagen). All qPCR samples were normalized to 18s RNA.

**Protein isolation and Western blotting**

Protein lysate was harvested from NHC cells using the Mammalian Protein Extraction Reagent (M-PER) (Roche) containing cComplete ULTRA Tablets, Mini, Protease Inhibitor Mixture Tablet (Roche), and PhosSTOP Phosphatase Inhibitor Mixture Tablet (Roche) in 10 ml of M-PER reagent. Protein lysate was subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking in 5% milk or 5% BSA, the membranes were incubated with primary antibodies at 1:1000 dilution and incubated overnight at 4 °C. The following primary antibodies used: Bcl2L1, Bcl2, Mcl1, Bcl2A1, and ActB (Sc-1615; Santa Cruz Biotechnology); Bax (clone D2E11; 5023), Bik (4592), Bim (clone C34C5; 2933), Bid (human specific; 2002), Bad (clone D24A9; 9239), Noxa (clone D8L7u; 147667), Puma (clone D30C10; 12450), and Bak (clone D4E4; 12105; Cell Signaling); phosphorylated-p16INK4a (ab135552), phosphorylated-ETS1 (ab59179), p21 (ab7960–1; Abcam), and p16 (clone G175–405; 550834; BD Biosciences). The membranes were washed and incubated with secondary antibodies conjugated to HRP (1:2000 dilution, Cell Signaling). Western blotting bands were detected using Enhanced Chemiluminescent plus detection system (ECL Plus; Promega).

**Cloning of the BCL-xL promoter luciferase construct**

Genomic DNA was extracted from NHC cells using the DNA Blood and Cell Culture DNA Midi Kit (Qiagen) according the manufacturer’s protocol. PCR was performed using the primer pairs found in Table 1 that contain a Nhel restriction enzyme site in the forward primer (underlined) and a HindIII site in the reverse primer (bold type). The reverse primer was used in the generation of all the constructs. Following PCR amplification (95 °C for 10 min; 30 cycles of 95 °C for 30 s and 55 °C for 60 s; and 72 °C for 30 s) the PCR amplicons were digested with Nhel and HindIII, electrophoresed, and gel-purified by QIAquick gel extraction kit (Qiagen). These digested inserts were then ligated into the XhoI- and HindIII-linearized pGL4.22 luciferase plasmid (Promega). To obtain the ETS1 site-directed mutagenesis (SDM) clones in the BCLXL promoter, we used a Q5 SDM kit (New England BioLabs). Briefly, PCR primers were designed using the NEBaseChanger (New England BioLabs) algorithm. The ΔSDM constructs deleted 2–3 bp within the three putative Ets1-binding sites containing the GGAA motif.
ETS1/p300 and BCL2L1 expression

Table 1

| Primer sequences |
|------------------|
| Gene-specific primers | Sequences (5’ to 3’)
| qPCR primer | BCL-xL fwd | NGAGAGCTTCCTCCTTTGTAAG |
| | BCL-xL rev | CCGGTTGCTCTGAGACTT |
| | BCL2 fwd | GGAGAGCTTTGACGCTTCT |
| | BCL2 rev | GGGCCAAATGACGAGATG |
| | MCL1 fwd | TCCCTAGTGAAGTCTGCTG |
| | MCL1 rev | AAATTCCTCCGACATGTAAT |
| | BCLXL1A1 fwd | ACAAGGGAAGCCCTGTCG |
| | BCLXL1A1 rev | GATGTCAGCTGCTGAGGAT |
| | Bid fwd | TTTCCTAGGCAACCTGCACT |
| | Bid rev | GGTGGAGAGGCTGAGGCTT |
| | Bad fwd | TTTCCTAGGCAACCTGCACT |
| | Bad rev | GGTGGAGAGGCTGAGGCTT |
| | Bak fwd | TCCCTGGCCGACAAATTC |
| | Bak rev | ATGGGAGATCCCTGCTCCT |
| | NOXA fwd | TTTGGCCAAGAGACGTTA |
| | NOXA rev | TTTGGCCAAGAGACGTTA |
| | PUMA fwd | AACTCAACAAACACGAGG |
| | PUMA rev | CTCTGGGGGGGCAAAATTC |
| | CDKN2A fwd | CAGACTGAGGCTTGGTAC |
| | CDKN2A rev | AGTCAGCCGAAGGCTCCAT |
| | CDKN1A fwd | AGTCAGCCGAAGGCTCCAT |
| | CDKN1A rev | AGTCAGCCGAAGGCTCCAT |
| | BCL-xL promoter luciferase primers | GCTGCTTCCTCCTTGACA |
| Full-length f/wd | GTGACTGCTGACGCTTGGTAAG |
| Full-length rev | CGGAAAGCTTCCTCCTTTG |
| Mutagenesis 1 (Δ1) f/wd | GGGCCGGTCTTCTCCGAAATG |
| Mutagenesis 1 (Δ1) rev | CCGGTTGCTCTGAGACTT |
| Mutagenesis 2 (Δ2) f/wd | AGGGGGCTGGCGCGCTGAG |
| Mutagenesis 2 (Δ2) rev | AGGGGGCTGGCGCGCTGAG |
| Mutagenesis 3 (Δ3) f/wd | CTCTGGGGGGGCAAAATTC |
| Mutagenesis 3 (Δ3) rev | ATGGGAGATCCCTGCTCCT |
| ChIP primers | BCL-xL fwd | CCGGGCTGCTGCTTAAATA |
| | BCL-xL rev | GAATTGCGAAGCTCAGGAAC |
| | Core promoter f/wd | CACGAGGACCTGCTGAGGAT |
| | Core promoter rev | CCGGGCTGCTGCTTAAATA |

BCL-xL promoter luciferase primers

Full-length fwd

Full-length rev

Mutagenesis 1 (Δ1) fwd

Mutagenesis 1 (Δ1) rev

Mutagenesis 2 (Δ2) fwd

Mutagenesis 2 (Δ2) rev

Mutagenesis 3 (Δ3) fwd

Mutagenesis 3 (Δ3) rev

ChIP primers

BCL-xL fwd

BCL-xL rev

Core promoter fwd

Core promoter rev

(--861 bp (Δ1), --314 bp Δ2, 167 bp (Δ3)). Primers used for SDM are found in Table 1 with mutated nucleotides underlined and in bold. PCR for the site-directed mutants was carried out using the following parameters: 98 °C for 10 s, 25 cycles of 98 °C for 10 s, 72 °C (Δ1) or 68 °C (Δ2) or 68 °C (Δ3) for 60 s, 72 °C for 3 min, and 72 °C for 30 s. The Δ2 SDM construct was then used as template for Δ1/2 SDM and Δ2/3 SDM by targeting the Δ1 ETS1 or Δ3 ETS1 sites as above, respectively. The Δ1 SDM construct was then used as template for Δ1/3 SDM by targeting the Δ3 ETS1; finally, the Δ1/2 SDM plasmid was used as template and Δ3 ETS1 was targeted by SDM for the Δ1/2/3 ETS1 plasmid construct. DNA sequencing was performed on all plasmid constructs at the Mayo Clinic DNA Sequencing Core Facility.

Transfection and generation of stable cell lines

NHCs were transfected with EV-pGL4.22, full-length (FL) BCLXLprom-pGL4.22, BCLXLpom (Δ1 ETS1)-pGL4.22, BCLXLpom (Δ2 ETS1)-pGL4.22, BCLXLpom (Δ3 ETS1)-pGL4.22, BCLXLpom (Δ1/2 ETS1)-pGL4.22, BCLXLpom (Δ1/3 ETS1)-pGL4.22, and BCLXLpom (Δ1/2/3 ETS1)-pGL4.22. To achieve an inducible BCLXL shRNA cell line under the regulation of doxycycline, we transfected NHC cells with a BCLXL SMARTvector Inducible shRNA (Dharmacon). The NHC cells were grown to 60% confluent the day of transfection in a 6-well plate. FuGENE HD (Promega) was used for the transfections based on the manufacturer’s directions. Briefly, 2 μg of plasmid DNA was added to 100 μl of Opti-MEM (Promega) low-serum transfection media. Subsequently 8 μl of FuGENE HD was added to the mix to achieve a 4:1 (FuGENE HD:DNA) ratio. The transfection mix was allowed to incubate for 15 min at room temperature and was added to the NHC cells in H69 medium. Twenty-four hours following transfection, the media was replaced with H69 media containing 1.5 μg/ml of puromycin (Invitrogen). After 1 week of selection, only the selected cells remained viable, and the puromycin was removed from the cells and replaced with H69 media. To induce expression of the BCLXL shRNA, doxycycline (1 μg/ml) was added to the media.

Promoter luciferase assay

CDKN2A promoter luciferase constructs and TK-Renilla (Promega), an internal control, were cotransfected into NHC cells using FuGENE HD (Promega) when the cultured cells were 25% confluent. As a baseline control, EV pGL4.22 was cotransfected with TK-Renilla. After transfections, we utilized our in vitro model of senescence while changing the media every 48 h. At the end of the incubation, we used the Dual-Luciferase Reporter Assay System (Promega) to measure the BCLXL promoter-driven firefly luciferase and Renilla lucifer-
ase. The values are expressed as fold-change between the relative CDKN2A promoter-driven firefly luciferase compared with pGL4.22 empty vector firefly luciferase to Renilla luciferase.

**ChIP-PCR**

ChIP was performed according to the Abcam cross-linking ChIP protocol. Briefly, NHCs were treated with or without LPS using our in vitro model of senescence protocol. The cells were incubated at room temperature for 10 min with formaldehyde to cross-link the proteins to genomic DNA, and glycine was added at a final concentration of 125 mM to terminate the cross-linking reaction. The cells were then scraped, lysed, and subsequently sheared with a sonicator to generate genomic DNA fragments ranging in length from 500 to 1000 bp. An aliquot of this lysate was stored as “input” DNA and the remainder of the lysate was immunoprecipitated. Immunoprecipitations were performed with the following antibodies: anti-ETS1 (Thr(P)-38, AB59179; Abcam), EP300 (AB14984; Abcam), anti-RNA POLR2 (Ser(P)-2, MABE953; Millipore), and H3K27ac (39685; Active Motif). Upon completion of the immunoprecipitation, the lysate cross-links we reversed and elutions were purified using Chromatin IP DNA Purification Kit (Active Motif). The DNA from the ChIP was used as template for the respective PCR amplifications and the appropriate primers are shown in Table 1. For block PCR, a GeneAmp PCR System 9700 (PE Applied Biosystems) was utilized and the resulting amplicons were resolved on a 1.5% agarose gel stained with ethidium bromide. Quantitative PCR was performed in a Rotor-Gene Q (Qiagen) system using Rotor-Gene SYBR Green master mix (Qiagen) according to manufacturer’s directions.

**Histone acetyltransferase activity assay**

To measure the histone acetyltransferase activity, we used a HAT activity kit (ScienCell). This assay is based on the acetylation of a peptide substrate by cellular HATs and the subsequent release of CoA. CoA serves as a coenzyme of a NADH-generating enzyme to produce NADH. The NADH stoichiometry is spectrophotometrically quantified following the reaction with tetrazolium-1 (WST-1). The protocol was followed according to manufacturer’s directions and fluorescence was read with a fluorescence plate reader at OD360 nm (excitation)/OD465 nm (emission).

**qRT-PCR**

mRNA analysis was performed using the Light Cycler Fast Start DNA Master Plus SYBR Green I kit (Roche) as previously described. Primer sequences used are provided in Table 1. Samples were normalized to 18s rRNA. qRT-PCR was performed using the Light Cycler Fast Start DNA Master plus SYBR Green I kit (Roche) as previously described. Samples were normalized to 18s rRNA.

**Caspase 3/7 apoptosis assays**

Apoptosis was assessed biochemically by measuring caspase-3/7 activity in cell cultures using the Apo-ONE homogeneous caspase-3/7 kit (Promega) following the supplier’s instructions. Briefly, the NHC cells plus/minus senescence were grown in black 96-well plates. TRAIL (20 ng/ml, R&D Systems) and/or Z-VAD-FMK (10 μM, R & D Systems) were added to the cultured cells for 24 h. Next, 100 μl of Apo-ONE caspase-3/7 reagent was added including the blank and the reaction was incubated for 2 h at 37 °C. Finally following the incubation, fluorescence was measured using a spectrofluorometer at an excitation wavelength 485 nm and an emission wavelength range of 530 nm.

**Immunoprecipitations**

Immunoprecipitation was performed as previously reported (17). Briefly, total protein was extracted from non-senescent and LPS-induced senescent cholangiocytes. Lysates were pre-cleared with Protein A/G-Sepharose beads (Santa Cruz) at 4 °C and incubated with BAK (AB1, clone TC-100 Millipore Sigma), BAX (clone 6A7, Invitrogen), BCLXL (54H6, Cell Signaling), P300 (SC-48343 AC, Santa Cruz), or ETS1 (SC-55581 AC, Santa Cruz) antibodies overnight at 4 °C. Both the BAK and BAX antibodies recognize only the activated forms of the molecule under nondenaturing, native conditions (49, 50). Protein A/G-Sepharose beads were added to the samples, centrifuged, washed either in ice-cold CHAPS IP buffer (BAX/BCLXL and BAX/BCLXL IPs; 0.3% CHAPS, 40 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate and protease/phosphatase inhibitors (Pierce)), or RIPA IP buffer (p300/ETS1 IPs; 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris) and resuspended in Laemmli sample buffer.

**PLA**

PLAs were performed as an approach to determine protein-protein interactions in vivo. The Sigma Doulink PLA kit was
used to detect interactions between ETS1 and p300 and BCLXL with BAK or BAX. The PLA's were performed according to manufacturer’s directions using from normal or PSC human and WT or Mrdr2/−/− mouse paraffin sections. The following primary antibodies were used for the PLA assays; mouse monoclonal p300 antibody (AB54984; Abcam) and rabbit phosphor-T38 ETS1 (AB59179, Abcam) and rabbit BCLXL (54H6, Cell Signaling) and mouse monoclonal BAX (clone 6A7, Invitrogen) or BAK (AB1, clone TC-100, CalBiochem). After washing the slides, mouse-minus and rabbit-plus were incubated to the slides for 60 min at 37 °C. Next the ligase was diluted 1:40 and incubated for 30 min at 37 °C. Again washes were performed; polymerase was diluted 1:80 and incubated for 100 min at 37 °C. The slides were washed 2 x 10 min in 1 x wash buffer B and washed for 1 min with 0.01 x wash buffer B prior to mounting the samples with Duolink in situ mounting medium with DAPI. For dual PLA and Lamin A immunofluorescence, prior to mounting, the slides were washed 1 x with PBS, blocked with 3% BSA, and incubated with a primary antibody to Lamin A (133A2, Cell Signaling). After primary incubation, slides were washed in PBS and incubated with Alexa Fluor secondary antibody (Life Technologies) for 30 min at room temperature. Slides were washed again in PBS and mounted. The slides were analyzed using a Zeiss 780 laser scanning confocal microscope.

Statistical analysis

All data are reported as the mean (or fold-change in mean) ± S.D. from a minimum of three independent experiments. Statistical analyses were performed with Student’s t test or analysis of variance when appropriate. p < 0.05 was considered statistically significant.

Author contributions—S. P. O., P. L. S., M. E. G., G. J. G., and N. F. L. conceptualization; S. P. O. data curation; S. P. O., M. A. S. A., and N. N.-G. formal analysis; S. P. O. and N. F. L. supervision; S. P. O., P. L. S., C. E. T., N. P. S., M. S. A. S., N. N.-G., and D. S. investigation; S. P. O. writing-original draft; S. P. O. project administration; P. L. S., C. E. T., N. P. S., M. S. A. S., and D. S. methodology; P. L. S., C. E. T., M. E. G., G. J. G., and N. F. L. writing-review and editing; N. F. L. funding acquisition.

Acknowledgments—We thank Dr. Steven A. Johnsen for his encouragement and critical reading of the manuscript and we acknowledge D. Hintz for assistance in preparation of the manuscript.

References

1. Lazaridis, K. N., and LaRusso, N. F. (2016) Primary sclerosing cholangitis. N. Engl. J. Med. 375, 1161–1170 CrossRef Medline
2. Tabibian, J. H., and Lindor, K. D. (2013) Primary sclerosing cholangitis: a review and update on therapeutic developments. Expert Rev. Gastroenterol. Hepatol. 7, 103–114 CrossRef Medline
3. Farrant, J. M., Hayllar, K. M., Wilkinson, M. L., Karani, J., Portmann, B. C., Westaby, D., and Williams, R. (1991) Natural history and prognostic variables in primary sclerosing cholangitis. Gastroenterology 100, 1710–1717 CrossRef Medline
4. Campisi, J., and d’Adda di Fagagna, F. (2007) Cellular senescence: when bad things happen to good cells. Nat. Rev. Mol. Cell Biol. 8, 729–740 CrossRef Medline
5. Mao, Z., Ke, Z., Gorbunova, V., and Seluanov, A. (2012) Replicatively senescent cells are arrested in G1 and G2 phases. Aging (Albany, NY) 4, 431–435 CrossRef Medline
6. Armanios, M. Y., Chen, J. J., Cogun, J. D., Alder, J. K., Ingersoll, R. G., Markin, C., Lawson, W. E., Xie, M., Vulto, J., Phillips, J. A., 3rd, Lansdorp, P. M., Greider, C. W., and Loyd, J. E. (2007) Telomerase mutations in families with idiopathic pulmonary fibrosis. N. Engl. J. Med. 356, 1317–1326 CrossRef Medline
7. Burton, D. G. (2009) Cellular senescence, ageing and disease. Age (Dordr) 31, 1–9 Medline
8. Hecker, L., Logsdon, N. J., Kurundkar, D., Kurundkar, A., Bernard, K., Hock, T., Meldrum, E., Sanders, Y. Y., and Thannickal, V. J. (2014) Reversal of persistent fibrosis in aging by targeting N04x-Nrf2 redox imbalance. Sci. Transl. Med. 6, 231ra247 24718857
9. Tchkonia, T., Morbeck, D. E., Von Zglinicki, T., Van Deursen, J., Lustgarten, J., Scoble, H., Khosla, S., Jensen, M. D., and Kirkland, J. L. (2010) Fat tissue, aging, and cellular senescence. Aging Cell 9, 667–684 CrossRef Medline
10. Schafer, M. J., White, T. A., Iijima, K., Haak, A. J., Ligresti, G., Atkinson, E. J., Oberg, A. L., Birch, J., Salmonowicz, H., Zhu, Y., Mazula, D. L., Brooks, R. W., Fuhrmann-Stroissnigg, H., Pirtskhalava, T., Prakash, Y. S., et al. (2017) Cellular senescence mediates fibrotic pulmonary disease. Nat. Commun. 8, 14532 CrossRef Medline
11. Anderson, R., Lagnado, A., Maggiorani, D., Walaszczuk, A., Dookun, E., Chapman, J., Birch, J., Salmonowicz, H., Ogrodnik, M., Jurb, D., Proctor, C., Correia-Melo, C., Victorelli, S., Fielder, E., Berlinger-Palmiini, R., et al. (2019) Length-independent telomere damage drives post-mitotic cardiomyocyte senescence. EMBO J. 38, e100492 Medline
12. Jeyapalan, J. C., and Sedivy, J. M. (2008) Cellular senescence and organismal aging. Mech. Ageing Dev. 129, 467–474 CrossRef Medline
13. Victorelli, S., and Passos, J. F. (2017) Telomeres and cell senescence: size matters not. EBioMedicine 21, 14–20 CrossRef Medline
14. Tabibian, J. H., O’Hara, S. P., Splinter, P. L., Trussoni, C. E., and LaRusso, N. F. (2014) Cholangiocyte senescence by way of N-ras activation is a characteristic of primary sclerosing cholangitis. Hepatology 59, 2263–2275 CrossRef Medline
15. Coppé, J. P., Patil, C. K., Rodier, F., Sun, Y., Munoz, D. P., Goldstein, J. N., Nelson, P. S., Desprez, P. Y., and Campisi, J. (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. Plos Biol. 6, 2853–2868 Medline
16. O’Hara, S. P., Splinter, P. L., Trussoni, C. E., Gajdos, G. B., Lineswala, P. N., and LaRusso, N. F. (2011) Cholangiocyte N-Ras protein mediates lipopolysaccharide-induced interleukin 6 secretion and proliferation. J. Biol. Chem. 286, 30352–30360 CrossRef Medline
17. Trussoni, C. E., Tabibian, J. H., Splinter, P. L., and O’Hara, S. P. (2015) Lipopolysaccharide (LPS)-induced biliary epithelial cell NRas activation requires epidermal growth factor receptor (EGFR). PLoS ONE 10, e0125793 CrossRef Medline
18. O’Hara, S. P., Splinter, P. L., Trussoni, C. E., Pisarello, M. J., Loarca, L., Splinter, N. S., Schutte, B. F., and LaRusso, N. F. (2017) ETS proto-oncogene 1 transcriptionally up-regulates the cholangiocyte senescence-associated protein cyclin-dependent kinase inhibitor 2A. J. Biol. Chem. 292, 4833–4846 CrossRef Medline
19. Wang, E. (1995) Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res. 55, 2284–2292 Medline
20. Yosef, R., Pilpel, N., Tokarsky-Amiel, R., Biran, A., Ovadya, Y., Cohen, S., Vadai, E., Dassa, L., Shahar, E., Condotti, R., Ben-Porath, I., and Krizhnovsky, V. (2016) Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. Cancer Res. 76, 11190 CrossRef Medline
21. Zhu, Y., Doornebal, E. J., Oberg, A. L., Birch, J., Salmonowicz, H., Zhu, Y., Mazula, D. L., Brooks, R. W., Fuhrmann-Stroissnigg, H., Pirtskhalava, T., Prakash, Y. S., et al. (2017) Cellular senescence mediates fibrotic pulmonary disease. Nat. Commun. 8, 14532 CrossRef Medline
22. Armanios, M. Y., Chen, J. J., Cogun, J. D., Alder, J. K., Ingersoll, R. G., Markin, C., Lawson, W. E., Xie, M., Vulto, J., Phillips, J. A., 3rd, Lansdorp, P. M., Greider, C. W., and Loyd, J. E. (2007) Telomerase mutations in families with idiopathic pulmonary fibrosis. N. Engl. J. Med. 356, 1317–1326 CrossRef Medline
23. Adams, J. M., and Cory, S. (2007) Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr. Opin. Immunol. 19, 488–496 CrossRef Medline
24. Wang, C., and Youle, R. J. (2009) The role of mitochondria in apoptosis. Annu. Rev. Genet. 43, 95–118 CrossRef Medline
24. Moncsek, A., Al-Surait, M. S., Trussoni, C. E., O’Hara, S. P., Splinter, P. L., Zuber, C., Patensen, E., Valli, P. V., Fingas, C. D., Weber, A., Zhu, Y., Tchkonia, T., Kirkland, J. L., Gores, G. J., Mühllbaur, B., L’Russo, N. F., and Mertens, J. C. (2018) Targeting senescent cholangiocytes and activated fibroblasts with B-cell lymphoma-extra large inhibitors ameliorates fibrosis in multidrug resistance 2 gene knockout (Mdr2(−/−)) mice. *Hepatology* **67**, 247–259 CrossRef Medline

25. Li, R., Pei, H., Watson, D. K., and Papas, T. S. (2000) EAP1/Daxx interacts with ETS1 and represses transcriptional activation of ETS1 target genes. *Oncogene* **19**, 745–753 CrossRef Medline

26. Yu, Z., and Shah, D. M. (2007) Curcumin down-regulates Ets-1 and Bcl-2 expression in human endometrial carcinoma HEC-1-A cells. *Gynecol. Oncol.* **106**, 541–548 CrossRef Medline

27. Guacciardi, M. E., Malhi, H., Mott, J. L., and Gores, G. J. (2013) Apoptosis and necrosis in the liver. *Compr. Physiol.* **3**, 977–1010 Medline

28. Kurita, S., Mott, J. L., Cazanave, S. C., Fingas, C. D., Guacciardi, M. E., Bronk, S. F., Roberts, L. R., Fernandez-Zapico, M. E., and Gores, G. J. (2011) Hedgehog inhibition promotes a switch from type II to type I cell death receptor signaling in cancer cells. *PLoS ONE* **6**, e18330 CrossRef Medline

29. Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Kent, W. J. (2002) BLAT: the BLAST-like alignment tool. *Genome Res.* **12**, 656–664 CrossRef Medline

30. Kent, W. J. (2002) BLAT: the BLAST-like alignment tool. *Genome Res.* **12**, 656–664 CrossRef Medline

31. Wei, G. H., Badis, G., Berger, M. F., Kivioja, T., Palin, K., Enge, M., Monke, M., Jolma, A., Varjosalo, M., Gehrk, A. R., Yan, J., Talukder, S., Turunen, M., Taitale, M., Stummenberg, H. G., et al. (2010) Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J.* **29**, 2147–2160 CrossRef Medline

32. Cho, M. C., Choi, H. S., Lee, S., Kim, B. Y., Jung, M., Park, S. N., and Yoon, D. Y. (2008) Epiregulin expression by Ets-1 and ERK signaling pathway in Ki-ras-transformed cells. *Biochem. Biophys. Res. Commun.* **377**, 832–837 CrossRef Medline

33. Hollenhorst, P. C. (2012) RAS/ERK pathway transcriptional regulation in vivo. *Cell Death Differ.* **19**, 1067–1070 CrossRef Medline

34. Ohtani, N., Zebedee, Z., Huot, T. J., Stinson, J. A., Sugimoto, M., Ohashi, Y., Sharrocks, A. D., Peters, G., and Hara, E. (2001) Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* **409**, 1067–1070 CrossRef Medline

35. Hollenhorst, P. C., Chandler, K. J., Poulsen, R. L., Johnson, W. E., Speck, N. A., and Graves, B. J. (2009) DNA specificity determinants associate with distinct transcription factor functions. *PLoS Genet.* **5**, e1000778 CrossRef Medline

36. Joplin, R., Strain, A. J., and Neuberger, J. M. (1989) Immuno-isolation and culture of biliary epithelial cells from normal human liver. *In Vitro Cell Dev. Biol.* **25**, 1189–1192 CrossRef Medline

37. Jiménez, O., and Campisi, J. (2005) Cell senescence in cancer and aging-related disease: from mechanisms to therapy. *Nat. Med.* **11**, 1424–1435 CrossRef Medline

38. Jiménez, O., and Campisi, J. (2005) Cell senescence: a translational perspective. *EBioMedicine* **21**, 21–28 CrossRef Medline

39. Xu, M., Palmer, A. K., Ding, H., Wei, M., Pirtskhalava, T., White, T. A., Sepe, A., Johnson, K. O., Stout, M. B., Giorgadze, N., Jensen, M. D., LeBrasseur, N. K., Tchkonia, T., and Kirkland, J. L. (2015) Targeting senescent cells enhances adipogenesis and metabolic function in old age. *Elife* **4**, e12997 CrossRef Medline

40. Foulds, C. E., Nelson, M. L., Blaszczak, A. G., and Graves, B. J. (2004) Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. *Mol. Cell. Biol.* **24**, 10954–10964 CrossRef Medline

41. Tchkonia, T., Kirkland, J. L., and Campisi, J. (2017) Cellular senescence: a translational perspective. *EBioMedicine* **21**, 21–28 CrossRef Medline

42. Debacq-Chainiaux, F., Evers, M., Distler, A., and Soares, D. (2010) Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J.* **29**, 2147–2160 CrossRef Medline

43. Debacq-Chainiaux, F., Evers, M., Distler, A., and Soares, D. (2010) Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J.* **29**, 2147–2160 CrossRef Medline

44. Griffiths, G. I., Durenb, L., Morgan, C. J., Jones, N. A., Whitehouse, J., Corfe, B. M., Dive, C., and Hickman, J. A. (1999) Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *J. Cell Biol.* **144**, 903–914 CrossRef Medline

45. Hsu, Y. T., and Youle, R. J. (1998) Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *J. Biol. Chem.* **273**, 10777–10783 CrossRef Medline