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SOX17 Regulates Cholangiocyte Differentiation and Acts as a Tumor Suppressor in Cholangiocarcinoma

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Abstract

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Background & aims—Cholangiocarcinoma (CCA) is a biliary malignancy linked to genetic and epigenetic abnormalities, such as hypermethylation of *SOX17* promoter. Here, the role of SOX17 in cholangiocyte differentiation and cholangiocarcinogenesis was studied.

Methods—SOX17 expression/function was evaluated along the differentiation of human induced pluripotent stem cells (iPSC) into cholangiocytes, in the dedifferentiation process of normal human cholangiocytes (NHC) in culture and in cholangiocarcinogenesis. Lentiviruses for *SOX17* overexpression or knock-down were used. Gene expression and DNA methylation profiling were performed.

Results—*SOX17* expression is induced in the last stage of cholangiocyte differentiation from iPSC and regulates the acquisition of biliary markers. *SOX17* becomes downregulated in NHC undergoing dedifferentiation; experimental *SOX17* knock-down in differentiated NHC downregulated biliary markers and promoted baseline and Wnt-dependent proliferation. *SOX17* expression is lower in human CCA than in healthy tissue, which correlates with worse survival after tumor resection. In CCA cells, *SOX17* overexpression decreased their tumorigenic capacity in murine xenograft models, which was related to increased oxidative stress and apoptosis. In contrast, *SOX17* overexpression in NHC did not affect their survival but inhibited their baseline proliferation. In CCA cells, SOX17 inhibited migration, anchorage-independent growth and Wnt/β-catenin-dependent proliferation, and restored the expression of biliary markers and primary cilium length. In human CCA, *SOX17* promoter was found hypermethylated and its expression inversely correlates with the methylation grade. In NHC, Wnt3a decreased *SOX17* expression in a DNMT-dependent manner, whereas in CCA, DNMT1 inhibition or silencing upregulated *SOX17*.

Conclusions—SOX17 regulates the differentiation and maintenance of the biliary phenotype and functions as a tumor suppressor for CCA, being a potential prognostic marker and a promising therapeutic target.

Introduction

Cholangiocarcinoma (CCA) is a heterogeneous group of biliary malignancies with poor prognosis [1]. The etiopathogenesis remains largely unknown [1]. Incidence is rising worldwide and CCA already represents the second most common primary liver tumor and ~3% of all gastrointestinal cancers [1]. CCA is generally asymptomatic in early stages and is commonly diagnosed at advanced phases, when symptoms associated with biliary obstruction arise; this circumstance compromises the potential therapeutic options [1]. Recurrence after surgery and elevated chemoresistance of CCAs contribute to the limited prognosis [1]. Therefore, it is essential to investigate in detail the molecular mechanisms involved in the pathogenesis of CCA to find new potential targets for therapy.

CCA is characterized by genetic and epigenetic abnormalities [1]. Hypermethylation of certain gene promoters was previously reported in CCA tumors, including the promoter of SRY-related HMG-box 17 (*SOX17*) transcription factor [2]. The family of *SOX* genes plays key roles in tissue homeostasis, organogenesis and cell fate decision from embryonic to

adult stages [3]. In particular, SOX17 appears essential in the development of the biliary epithelium during embryogenesis since *Sox17*-deficient (*Sox17*–/–) mice exhibit premature lethality due to alterations in the formation of the definitive gut endoderm [4] and *Sox17* haploinsufficiency (*Sox17*+/–) in mice leads to congenital biliary atresia, acute embryonic hepatitis and high perinatal death [5]. Moreover, SOX17 is necessary for the development of the biliary primordium and loss of SOX17 leads to gallbladder agenesia and ectopic development of pancreatic tissue in the common bile duct [5, 6]. On the other hand, *SOX* genes are also involved in carcinogenesis, acting as oncogenes or tumor suppressor genes [7]. Little information is available about the role of SOX17 in carcinogenesis. Some reports suggest that it may act as a tumor suppressor in some types of cancer and that is able to antagonize the pro-tumorigenic Wnt/β-catenin signaling pathway [8–11].

In the present study, we have investigated the role of SOX17 in the differentiation and maintenance of the biliary phenotype and in cholangiocarcinogenesis. Moreover, the prognostic clinical value of *SOX17* expression was evaluated.

Material and methods

Complete transparent accurate and timely account (CTAT) methods are included as Supplementary CTAT table.

Human samples

Whole transcriptome profiling was performed using *humanRef-8v2 BeadChips* (Illumina) on 104 CCA surgical specimens (68 intrahepatic and 36 perihilar CCAs) and 6 normal intrahepatic bile ducts, as previously described (GEO: GSE26566) ("Copenhagen cohort" of patients) [12]. Additionally, laser microdissection (LMD) was performed using *LMD6000 system* (Leica) to generate 23 pairs of matched tumor epithelia and tumor stroma [12]. DNA promoter methylation was analyzed in 48 CCAs using *Infinium HumanMethylation27 BeadChips* (Illumina Inc.). To evaluate the relationship between *SOX17* expression and the outcome of patients, samples were grouped as high *vs* low *SOX17* expression in the resected tumor, which was obtained according to an arbitrary average expression level (cut-off).

Moreover, 13 CCA human biopsies and 14 normal human gallbladder tissues were obtained from the Biobank of Donostia University Hospital for the analysis of *SOX17* expression by qPCR ("San Sebastian cohort" of patients) (Supplementary Table 1). Research protocols were approved by the *Ethical Committees for Clinical Research* of supporting Institutions, and all patients signed written consents for the use of their samples for biomedical research.

CCA xenograft animal models

Immune-deficient CD-1 nude mice (Crl:CD1-*Foxn1*[nu]; strain 086, homozygous) were purchased from Charles River Company. As a first procedure, CCA (EGI1) cells (1×10^5 cells resuspended in 100 μL of PBS) infected either with Lent-control or Lent-SOX17 (MOI 3) were subcutaneously injected in the dorsal flanks of animals. In a second approach, 1×10^6 CCA (EGI1) cells were subcutaneously injected and once tumors were well-established (average size: ~175 mm^3) Lent-control or Lent-SOX17 (1×10^7 lentiviral particles) were intratumorally injected. Tumor size was measured every 2–4 days and tumor volume (TV)

was calculated as "TV = (D × d^2)/2", where "D" represents the largest diameter measured and "d" the shortest.

Isolation and reprogramming of human myofibroblasts into cholangiocytes

Human myofibroblasts were isolated from biopsy specimens and then cultured and reprogrammed as previously described (Supplementary data) [13].

Normal human cholangiocytes and CCA human cells

Normal human cholangiocytes (NHC) were isolated from normal liver tissue and characterized as we previously described [14–16]. The protocol of isolation and culture is summarized in Supplementary data. In addition, 3 CCA human cell lines (EGI1, TFK1 and Witt) were also used. Both EGI1 and Witt cell lines are KRAS mutated whereas TFK1 is wild-type. Cells were tested as mycoplasma negative along the experiments.

Quantitative polymerase chain reaction

RNA extraction, retrotranscription and quantitative polymerase chain reaction (qPCR) were performed as previously described [14–16]. Primer sequences are included in Supplementary Table 2.

Immunoblotting

Analysis of cell protein expression by immunoblotting was performed as described in Supplementary data. Antibodies are listed in Supplementary Table 3.

Immunofluorescence

Detection of protein expression by immunofluorescence (IMF) was performed as described in Supplementary data.

Cell transduction with lentiviral vectors

iPSC cells were differentiated to definitive endoderm (DE) and then cells were either left untreated (control) or infected with lentiviruses carrying small hairpin RNA (shRNA) against *SOX17* (Lent-shRNA-*SOX17*; Santa Cruz) or control shRNA (Lent-shRNA-control: Santa Cruz) at the first day of each differentiation phase (HS, HP, iDC) and with a multiplicity of infection (MOI) of 3. NHC were infected with Lent-shRNA-*SOX17* or Lent-shRNA-control at MOI 3. CCA cells (EGI1 or TFK1) and NHC were infected with lentiviruses carrying *SOX17* (Lent-SOX17) or empty viruses (Lent-control) at MOI 3. Lent-SOX17 production and lentiviral infections are described in Supplementary data.

Cell Death Analysis

Cell death was determined by using three different flow cytometry-based assays: Annexin-V (Invitrogen), Propidium Iodide (Invitrogen) and Caspase-3 activity (Phiphilux-G2D2, OncoImmunin® Inc), as described in Supplementary data.

Cell redox stress

Levels of oxidative stress were determined in CCA (EGI1) cells infected with Lent-SOX17 or Lent-control by using the *CellROX® "Deep Red" Flow Cytometry Assay Kit* (Invitrogen). Non-infected cells were also used as controls.

Cell proliferation

Proliferation of NHC and CCA (EGI1) cells was evaluated under Lent-shRNA-*SOX17* (or Lent-shRNA-control) and/or Lent-SOX17 (or Lent-control) infection. Non-infected cells were also used as controls. Protocol is described in Supplementary data.

Anchorage-independent growth in soft agar

CCA (EGI1) cell growth in soft agar was analyzed as described in Supplementary data.

Cell senescence

NHC were used to analyze cell senescence (β-galactosidase activity) over passages and under Lent-shRNA-*SOX17* or Lent-shRNA-control infections as described in Supplementary data.

Cell migration

CCA cell migration was evaluated in cells (EGI1) infected with Lent-SOX17 or Lent-control, as well as in non-infected cells, as described in Supplementary data.

Illumina mRNA expression array

Illumina Human HT12 v4 BeadChips (GPL10558) were chosen to characterize gene expression in NHC and CCA (EGI1) cells under Lent-shRNA-*SOX17*/Lent-shRNA-control or Lent-SOX17/Lent-control infection, respectively (Supplementary data). Non-infected cells were also used as controls.

Knock-down of DNMT1 mRNA expression with siRNAs and pharmacological inhibition of DNMT activity

DNA methyltransferase 1 (*DNMT1*) mRNA expression was knocked-down in CCA cells by using specific small interference RNA (siRNA) oligos (Supplementary data). SOX17 expression was measured in CCA cells under *DNMT1* knocked-down or under the presence or absence of 5-aza-2'-deoxycytidine (1 μM) (also known as decitabine) for 48 h. *SOX17* mRNA expression was measured in NHC under the presence or absence of Wnt3a (100 ng/mL, R&D systems) in DMEM/F12+Glutamax/1%P/S culture media for 48 h and with or without decitabine (pre-incubated for 18 h).

SOX17 promoter methylation

The methylation status of *SOX17* promoter was studied as described in Supplementary data. DNA methylation was quantified using beta (β)-value metric (range: 0–1; 0=0% methylation, 1=100% methylation).

To further investigate the extent of aberrant DNA methylation in *SOX17*, level 1 *Infinium HumanMethylation450 BeadChip* (Illumina Inc.) data were analyzed in specimens obtainable through the cancer genome atlas (TCGA) (Supplementary data).

Statistical analysis

Results were statistically analyzed using *GraphPad* program. For comparisons between two groups, parametric *t-Student* test or nonparametric *Mann-Whitney* test were used. For comparisons between more than two parametric groups, One-Way analysis of variance (ANOVA) with Tukey´s post-hoc was employed. In data correlation, nonparametric *Spearman* test was employed. Differences were considered significant when $p<0.05$.

Results

Role of SOX17 in cholangiocyte differentiation

iPSC were differentiated into mature cholangiocytes (i.e iDCs) by using a multistep process with exposure to biliary morphogens (Figure 1A). Similarly to the biliary markers cytokeratin 7 and 19 (CK7 and CK19, respectively), SOX17 protein expression is also highly induced in the last step of the differentiation process (from HP into iDC) (Figure 1B,C) and its partial experimental knock-down with Lent-shRNA-*SOX17* decreased CK7 and CK19 protein expression compared to cells infected with Lent-shRNA-control or non-infected cells (Figures 1B,C).

Involvement of SOX17 in the maintenance of the biliary phenotype

Normal primary epithelial cells progressively decrease the expression of differentiation markers along cell passages *in vitro* and gradually enter in senescence. Here, the expression of *SOX17*, similarly to *CK7* and *CK19*, decreases with NHC passages (Figure 2A). In contrast, the expression (p21, *p16ink4a*) and activity (β-galactosidase) of senescence markers increased, whereas the cell-cycle kinases [cyclin-dependent kinase 4 (*CDK4)]* decreased (Figures 2B–D). To further study the role of SOX17 in cholangiocyte phenotype and senescence, *SOX17* was experimentally downregulated in NHC at low passages with Lent-shRNA-*SOX17* (Figure 3A). Experimental *SOX17* knock-down prompted the downregulation of both *CK7* and *CK19* (Figure 3A), but did not affect NHC senescence (p21, *p16ink4a, CDK4* expression and β-galactosidase activity) compared to control conditions (Figures 3B–D). In addition, mRNA microarrays were carried out to test the effect of *SOX17* downregulation on the NHC mRNA profile. Heatmaps showed that most of the differentially-expressed genes were found overexpressed under *SOX17* downregulation; many of them were oncogenes, cell-cycle promoters, invasion/migration inducers and genes involved in drug resistance and cell survival (Supplementary Figure 1).

Then, we investigated the role of SOX17 and Wnt on NHC proliferation. Under baseline conditions, experimental SOX17 knock-down with Lent-shRNA-*SOX17* increased NHC proliferation compared to controls (Figure 3E). The presence of Wnt3a ligand did not modify the baseline proliferation of NHC infected with Lent-shRNA-control or non-infected. However, experimental SOX17 knock-down rendered these cells susceptible to Wnt3a-induced proliferation (Figure 3E); this event was associated with decreased secreted

frizzled-related protein 1 (*SFRP1*) mRNA levels (Figure 3E), which translate into a protein that is extracellularly secreted and may bind and inhibit Wnt ligands.

Expression of SOX17 in CCA human tissue and cells

The analysis of *SOX17* expression was performed in CCA human biopsies and controls by using 2 approaches (mRNA microarray and qPCR) and 2 different cohorts of patients (Copenhagen and San Sebastian), respectively. By using mRNA microarrays in samples from the Copenhagen cohort, *SOX17* expression was found downregulated in 104 CCA biopsies compared to 6 normal intrahepatic bile ducts (Figure 4A). *SOX17* expression was also found downregulated in CCA epithelia obtained by laser microdissection, compared with their matched tumor stroma [mainly composed by cancer associated fibroblasts (CAFs), immune cells such as tumor associated macrophages (TAMs) and endothelial cells] [1] (Figure 4A).

Next, the expression of *SOX17* was evaluated by qPCR in the San Sebastian cohort. Data revealed that *SOX17* levels are also lower in 13 CCA tissues compared to NHC and 14 normal gallbladder tissues (which is mainly composed by cholangiocytes but also by progenitor cells that express high SOX17 levels) [17] (Figure 4B). In addition, SOX17 protein expression was evaluated by IMF in normal human livers as well as in CCA human tissues. In normal human livers, CK19-positive cells (cholangiocytes) show high SOX17 expression (Figure 4C), which is mainly localized in the perinuclear and nuclear compartments. In contrast, SOX17 immunostaining was negligible in CK19-positive human iCCA tissue (Figure 4C). *SOX17* expression (mRNA and protein) was also very poor in three human CCA cell lines (EGI1, TFK1 and Witt) compared to NHC (Figures 4D,E, respectively).

Relationship of SOX17 expression in CCA tumors and the outcome of patients

SOX17 expression was retrospectively evaluated in 104 CCA patients (Copenhagen cohort) who had undergone tumor resection and were monitored for 5 years after surgery. Data showed that *SOX17* expression correlates with the outcome of patients. Thus, patients with lower *SOX17* mRNA levels in CCA tumors showed shorter overall survival time (25.5 months) than those with higher levels of *SOX17* (77.9 months) (Figure 4F).

Role of SOX17 in cholangiocarcinogenesis

In order to evaluate the role of SOX17 on biliary tumorigenesis, this transcription factor was overexpressed in CCA (EGI1) cells by using Lent-SOX17. First, we evaluated the MOI needed to overexpress SOX17 in CCA cells *in vitro*. Immunoblots revealed a MOI-dependent SOX17 overexpression in CCA cells after Lent-SOX17 infection (Figure 5A). IMF analysis confirmed higher SOX17 baseline levels in NHC than in CCA cells. SOX17 immunostaining was mainly located at the nucleus, although some staining was also found in the cytoplasm (Figure 5A). Experimental SOX17 overexpression in CCA cells resulted in its protein accumulation in the nucleus. The percentage of transduced cells was dependent on the MOI employed (1, 3 or 5) (Figure 5A). MOI 3 was used for further experiments as this was the minimal value able to transduce all cells with non-saturating SOX17 overexpression.

When implanted subcutaneously in immunodeficient nude mice, CCA (EGI1) cells pre-infected with Lent-control showed a continuous lineal growth overtime (Figure 5B), which was similar to that observed in non-infected CCA cells (data not shown). In contrast, overexpression of SOX17 in CCA human cells inhibited their tumorigenic capacity (implantation and growth) *in vivo* compared to cells infected with Lent-control (Figure 5B). Moreover, intratumoral injection of Lent-SOX17 in pre-existing CCA tumors also suppressed the tumor growth *in vivo* (Figure 5C). Elucidation of the mechanisms accounting for this anti-tumorigenic effect revealed that SOX17 overexpression promoted a MOI-dependent cell death in CCA cells compared to control conditions (Figure 5D). SOX17-induced cell death was associated with increased cellular levels of reactive oxygen species (ROS), increased pro-apoptotic caspase-3 activity, and phosphorylation of the pro-apoptotic p53 protein (Figure 5E). Notably, and in contrast to CCA cells, experimental overexpression of *SOX17* in NHC did not display deleterious effects but reduced their baseline proliferation promoting a quiescent phenotype (Figure 5F).

Since a fraction of CCA cells overexpressing SOX17 did not enter into apoptosis we used them to investigate several characteristics involved in carcinogenesis and malignancy, such as proliferation, invasion/migration and differentiation. The presence of Wnt3a promotes the proliferation of CCA (EGI1) cells in a dose-dependent manner (Supplementary Figure 2). Notably, overexpression of SOX17 in CCA cells (EGI1 and TFK1) inhibited the Wnt3a–dependent proliferation (Figure 6A and Supplementary Figure 3). This was associated with increased phosphorylation of β-catenin (key effector of the Wnt signaling pathway) in the ser33/37/thr41 residues that promotes its ubiquitin/proteasome-dependent protein degradation [18] (Figure 6A). Moreover, experimental overexpression of SOX17 impaired the anchorage-independent growth in soft agar and the invasion/migration properties of CCA cells (Figures 6B,C). SOX17-mediated effects were associated with increased mRNA levels of biliary/epithelial markers *CK7*, fibronectin (*FN*) and zonula occludens 1 (*ZO-1*), as well as with the normalization of the upregulated *CK19* levels and decreased expression of the mesenchymal and pro-tumor/metastatic marker S100 calcium-binding protein A4 (*S100A4*) compared to controls (Figures 6C,D and Supplementary Figure 4). Heatmaps obtained from mRNA microarrays analysis showed that most of the differentially-expressed genes in CCA cells under SOX17 overexpression were found downregulated. These were mainly oncogenes, cell-cycle promoters, invasion/migration inducers and genes involved in angiogenesis and cell survival (Figure 6E).

Role of SOX17 in CCA primary cilium length

CCA cells show shorter or even absent primary cilia due to histone deacetylase 6 (HDAC6) overexpression that de-acetylates the ciliary α-tubulin [19]. The decreased primary cilium length is in part responsible of the depolarization and hyperproliferation observed in CCA cells. Our data indicate that those CCA human cells overexpressing SOX17, but that did not enter apoptosis, show longer cilia than controls (Figure 6F). This event was associated with decreased *HDAC6* mRNA levels (Figure 6F).

Regulatory mechanisms of SOX17 expression in normal human cholangiocytes and CCA human cells

Epigenetic abnormalities have been described in CCA, including hypermethylation of some promoters of genes that inhibit Wnt/β-catenin signaling like SOX17 [2]. Here, we also confirmed in 48 CCAs from the Copenhagen cohort that the *SOX17* promoter is hypermethylated at probe cg02919422 compared to normal human intrahepatic bile ducts (Figure 7A). Moreover, we observed an inverse correlation between *SOX17* methylation and gene expression (Figure 7A). To further investigate the extent of such aberrant methylation, we also analyzed TCGA Infinium 450k data from 35 CCA patients and 6 surrounding normal samples. Remarkably, 2 large differentially methylated regions were uncovered – one localized at the *SOX17* promoter (chr8: 55,368,995–55,370,994 corresponding to 11 significant probes, including cg02919422) and other localized at the *SOX17* gene body (chr8: 55,370,495 – 55,373,448 corresponding to 10 significant probes).

In vitro studies aimed to investigate the role of promoter methylation in *SOX17* expression revealed that Wnt3a is able to inhibit *SOX17* expression in NHC in a DNA methyltransferase (DNMT)-dependent manner; treatment with the DNMT-inhibitor decitabine restored *SOX17* expression (Figure 7B). Since DNA methylation is carried out by several DNMT enzymes (DNMT1, 3a and 3b) [20] their expression in three CCA human cell lines (EGI1, TFK1 and Witt) was compared to that found in NHC. Both *DNMT1* and *DNMT3b* were found highly expressed in all three CCA cell lines compared to NHC, whereas no difference of *DNMT3a* was seen (Figure 7C and Supplementary Figure 5). The knock-down of *DNMT1*, which is considered the main responsible for constitutive DNA methylation [20], resulted in the upregulation of *SOX17* mRNA in all three CCA cell lines compared to controls (Figure 7D). Similarly, pharmacological inhibition of DNMT activity with decitabine increased SOX17 mRNA and protein expression in CCA cells (Figures 7E,F, respectively).

Discussion

The present study supports the concept that SOX17 plays an important role in the regulation of the phenotype of normal human cholangiocytes. Differentiation of iPSCs into cholangiocytes occurs *in vitro* through a well-established accurate protocol that results in the development of typical biliary features [13]. SOX17 expression, similarly to CK7 and CK19, is highly induced in the last step of this differentiation process and particularly regulates the expression of both biliary markers. These data suggest that this transcription factor is crucial during the last phase of the embryogenic biliary development. SOX17, similar to CK7 and CK19, becomes downregulated during the process of NHC dedifferentiation *in vitro* and this is associated with increased cellular senescence. Experimental downregulation of *SOX17* expression in low passage NHC resulted in decreased levels of biliary markers (CK7 and CK19) and in the downregulation of inhibitors of proliferation and migration, as well as in overexpression of different genes that promote tumorigenesis. However, SOX17 did not regulate cholangiocyte senescence and by contrast, its downregulation did increase the baseline and Wnt3a-dependent proliferation of NHC, events that are associated with decreased expression of the Wnt ligand inhibitor *SFRP1*. These data are consistent with

previous reports indicating that SOX17 may act as a negative regulator of the promitotic Wnt/β-catenin pathway and its downregulation may facilitate the activation of this pathway [9, 11]. Altogether, these results indicate that SOX17 is a key player regulating the differentiation process of cholangiocytes and the maintenance of the biliary phenotype, and its downregulation in cholangiocytes may promote their transformation into a pro-mitotic state.

Based on the aforementioned results and on a previous report showing that *SOX17* promoter is hypermethylated in CCA human tissues [2], we investigated in detail the expression of SOX17 in CCA and its potential epigenetic regulation. We found that SOX17 is downregulated in human CCA tissues and cell lines. In addition, we confirmed that the *SOX17* promoter is hypermethylated in CCA human tissues and found that its expression inversely correlates with the grade of methylation. These findings seem to constitute a universal event in cholangiocyte-derived CCAs, as CCA human samples independent of their anatomical location (intrahepatic, perihilar or distal) and CCA cell lines independent of being KRAS mutants or wild-types show SOX17 downregulation, pointing out the role of SOX17 in the etiopathogenesis of CCA. Indeed, overexpression of SOX17 reduced the tumorigenic capacity of CCA cells both *in vitro* and *in vivo*. These data are consistent with previous reports indicating the potential tumor suppressor capacity of SOX17 [8–11]. The Wnt/β-catenin pathway is very relevant in the promotion of cholangiocarcinogenesis [21, 22]. Wnt ligands are highly present in the tumor microenvironment and are released by both cancer-associated fibroblasts (CAFs) and activated macrophages [23]. Our data showed that Wnt3a ligand promotes proliferation of CCA cells in a dose-dependent manner. However, the Wnt3a-dependent proliferation was inhibited by SOX17 overexpression. SOX17 may inhibit the Wnt signaling by promoting the expression of the Wnt ligand inhibitor *SFRP1* and/or by inhibiting β-catenin expression and activity. Our data suggested that SOX17 increases the levels of β-catenin phosphorylated in residues ser33/37/thr41, indicating its inactivation/degradation in CCA cells. Furthermore, SOX17 also downregulated the expression of some genes involved in proliferation and inhibited the anchorage-independent growth and the invasion/migration capacity of CCA cells; this fact was associated with downregulation of different proinvasive/migration genes including *S100A4*, which is a key driver for CCA dissemination [24]. The analysis of the biliary phenotype in CCA cells revealed that SOX17 stimulates the expression of the biliary marker *CK7* and epithelial markers *FN* and *ZO-1*, whose deficiency has been related to cancer progression [25]. Moreover, SOX17 normalizes the upregulated CK19 expression in CCA cells.

Mature cholangiocytes have a single primary cilium extending from the apical membrane [26], which in addition to other physiological roles, functions as a structural cell cycle checkpoint. Thus, its loss or shortening induces proliferation in several cholangiopathies [26]. Human CCA cells show loss or decreased primary cilium length, which is dependent on the overexpression of HDAC6 that de-acetylates the cilium scaffold protein α-tubulin [19, 26]. SOX17 overexpression induced the downregulation of *HDAC6* together with the restoration of primary cilium structure and size, indicating improved cell polarity and differentiation. In this regard, the phenotype of *Sox17+/-*mice mimics human biliary atresia [5], which is a cholangiociliopathy that might predispose to the development of CCA [27]. The primary cilia of cholangiocytes in patients with biliary atresia are also shortened and

malformed, which could promote the development and progression of the disease [28]. Thus, future studies should evaluate in detail the mechanisms by which SOX17 regulates the primary cilium length in cholangiocytes.

The investigation of the molecular mechanisms triggering the downregulation of *SOX17* expression in CCA pointed to a role of Wnt3a. This ligand decreased *SOX17* expression in NHC, which was prevented by the presence of the demethylating agent decitabine. These data indicate that molecules present during chronic inflammatory processes and in the CCA tumor microenvironment are able to downregulate the expression of *SOX17* in cholangiocytes via a DNA methylation-dependent mechanism and thus promote cell transformation. Moreover, knocking-down of *DNMT1* expression or inhibition of DNMT activity with decitabine in CCA cells resulted in the upregulation of *SOX17* expression. These data indicate that restoration of *SOX17* expression in CCA with demethylating agents could have important therapeutic value. Decitabine was reported to inhibit CCA cell growth *in vitro* and in a mouse xenograft model [29] and is a drug approved by the food and drug administration (FDA) for the treatment of human diseases such as myelodysplastic syndromes [30]. So, decitabine could be a drug to be considered for CCA. On the other hand, gene therapy might be an alternative approach for overexpressing SOX17 in CCAs and deserves future consideration.

Regarding the potential prognostic value of this transcription factor in patients with CCA, *SOX17* expression in the tumor correlates with overall survival after surgical resection. This might be due to its aforementioned properties as a tumor suppressor and as key player in the regulation of cholangiocyte differentiation. Therefore, *SOX17* expression in CCA tumors can be considered as a potential prognostic marker but needs to be validated in other independent cohorts of patients. Owing to the urgent need of prognostic biomarkers for CCA, these findings are clinically relevant and should be confirmed in further prospective studies.

In summary, this study provides cellular, molecular and clinical evidence of the key role of SOX17 regulating the differentiation and maintenance of the biliary phenotype and its function as a tumor suppressor in CCA. Moreover, the potential usefulness of measuring *SOX17* expression in CCA as prognostic biomarker of patient outcome has been indicated. Genetic, molecular and/or pharmacological restoration of *SOX17* expression in CCA may represent a new therapeutic strategy that need to be evaluated in the next future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Anova	analysis of variance
BMIQ	beta mixture interquartile
BMP4	bone morphogenic protein 4
CAFs	cancer-associated fibroblasts
CCA	cholangiocarcinoma
CDK4	cyclin-dependent kinase 4
CK7	cytokeratin 7
CK19	cytokeratin 19
cMYC	myc protoncogene protein
CTAT	transparent accurate and timely account
DAPI	4',6-diamidino-2-phenylindole
DE	definitive endoderm
DMEM	dulbecco's modified eagle's medium
DMPs	differentially methylated probes
DNMTs	DNA methyltransferases
FDA	food and drug administration
FGF2	fibroblast growth factor 2
FN	fibronectin
GAPDH	glyceraldehyde-3 phosphatase dehydrogenase
HDAC6	histone deacetylase 6
HP	hepatic progenitor
HS	hepatic specification
iDC	iPSC-derived cholangiocytes
IMF	immunofluorescence
iPSC	induced pluripotent stem cells
KLF4	kruppel-like factor 4

LMD	laser microdissection
MOI	multiplicity of infection
NHC	normal human cholangiocytes
OCT4	octamer-binding transcription factor 4
P/S	penicillin/streptomycin
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S100A4	S100 calcium-binding protein A4
SFRP1	secreted frizzled-related protein 1
SHH	sonic hedgehog
shRNA	small hairpin RNA
siRNA	small interference RNA
SOX2	SRY-related HMG-box 2
SOX17	SRY-related HMG-box 17
Sox17−/−	Sox17-deficient mice
Sox17+/−	Sox17-haploinsuficient mice
TCGA	the cancer genome atlas
TGFβ	transforming growth factor beta
TV	tumor volume
Wnt3a	wingless-type MMTV integration site family member 3A
ZO-1	zonula occluding 1

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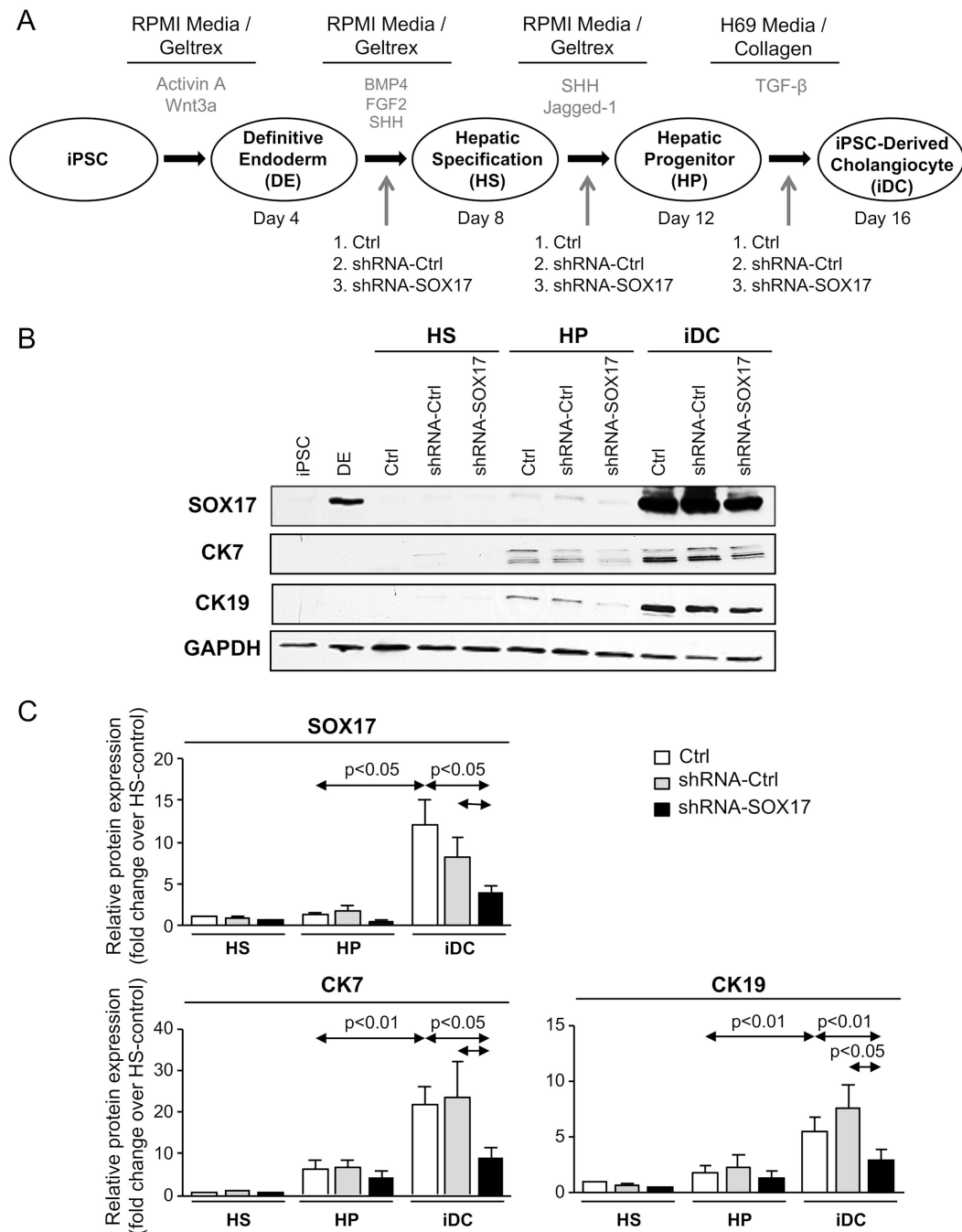
Figure 1. SOX17 regulates the differentiation of induced pluripotent stem cells into mature cholangiocytes

A) Chart summarizing the multistep process of differentiation from iPSC into iDC. Time-points of lentiviral administration are shown. B) Representative immunoblots and C) quantitative protein levels of SOX17, CK7 and CK19 in the differentiation steps from HS into iDC. Pooled data from 5 independent experiments (mean±SEM; p-value *vs* controls, Student's t-test).
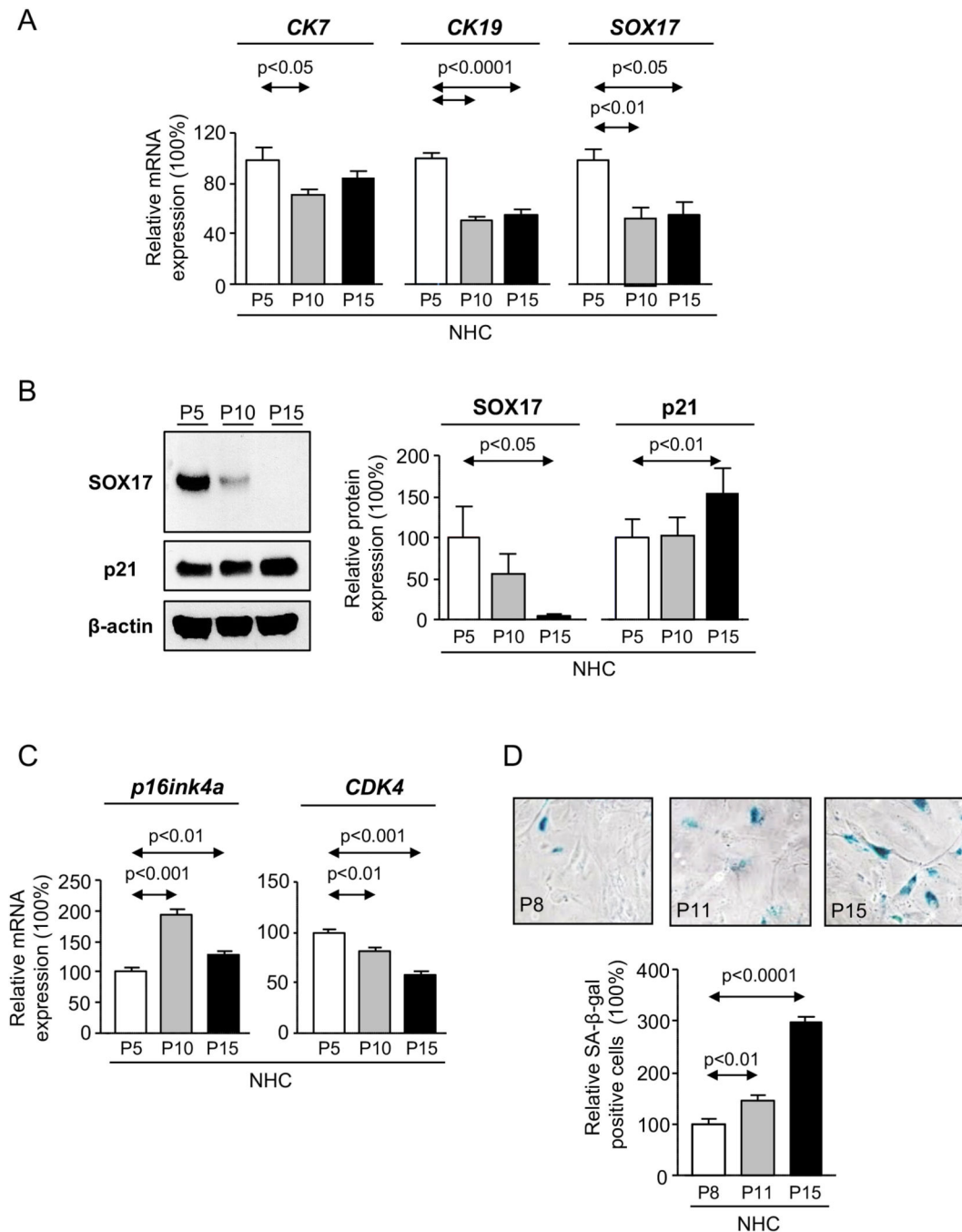
Figure 2. NHC dedifferentiate and enter into senescence over cell passages *in vitro*
A) Expression of the biliary markers *CK7, CK19* and *SOX17* (n=4–6). B) Immunoblots and quantification of SOX17 and p21 (senescence marker) levels (n=3). C) Expression of *p16ink4a* (senescence marker) and *CDK4* (cell cycle promoter) (n=4–5). D) Representative light microscopy images and relative quantification of β-galactosidase staining (n=6–8 wells from two independent experiments). Panels A-D: mean±SEM; p-value *vs* lowest cell passage (P5 or P8, Student's t-test).

A
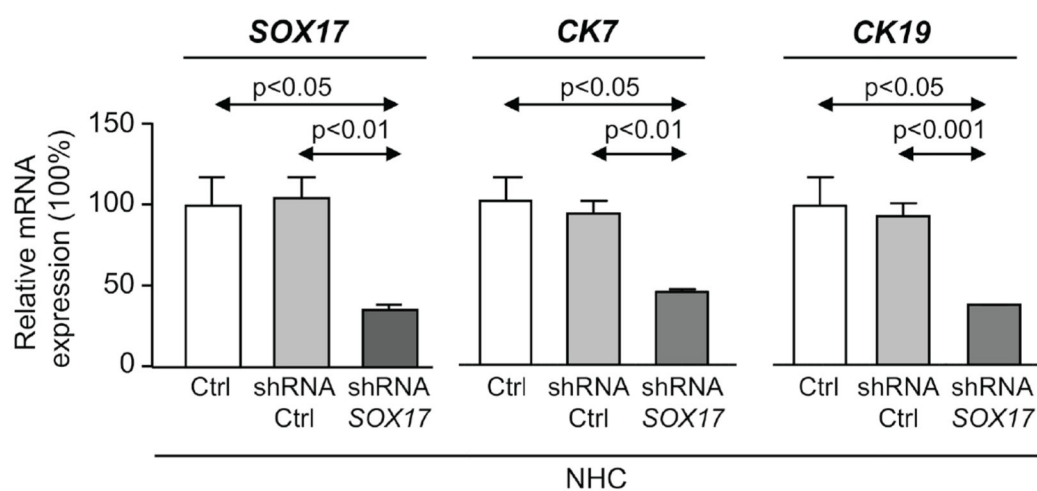
B
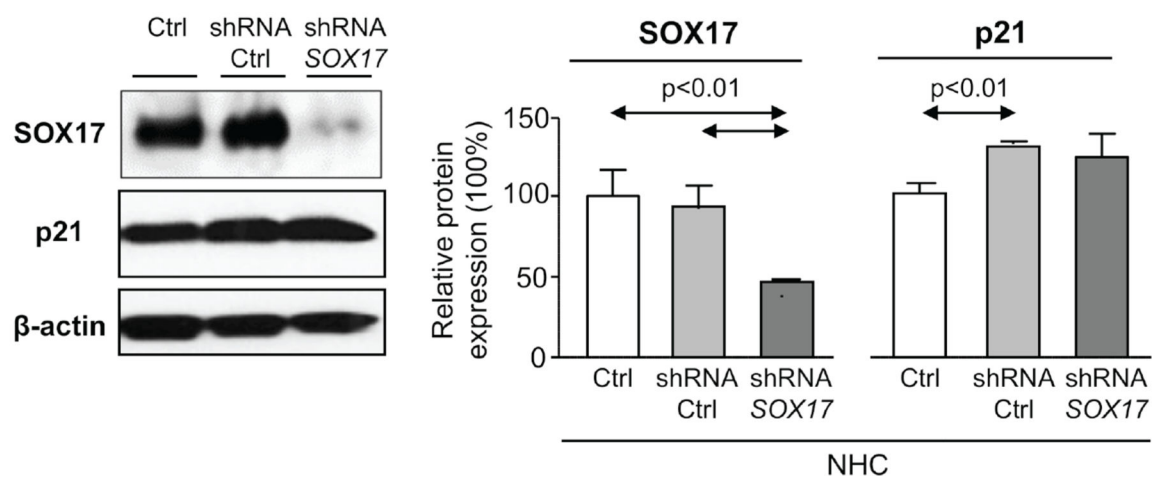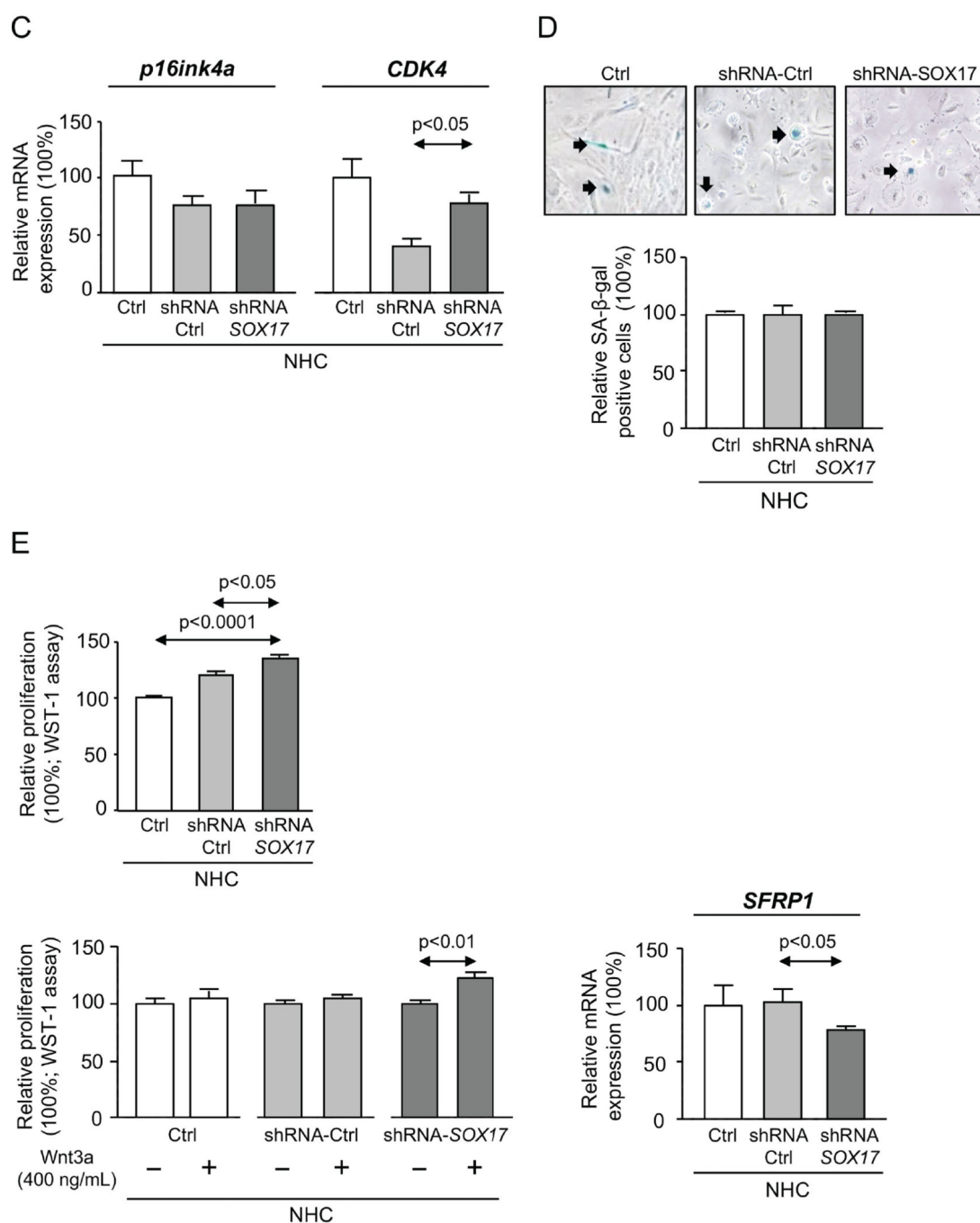
Figure 3. Experimental knock-down of SOX17 levels in NHC decreases the expression of biliary markers but does not affect senescence

A) Expression of *SOX17* and biliary markers (n=3–4). B) Representative immunoblots and quantification of SOX17 and p21 levels (n=3 independent experiments). C) Expression of *p16ink4a* and *CDK4* (n=5). D) Representative light microscopy images of β-galactosidase staining (n=7 wells from two independent experiments; NHC of passage 8). E) Basal and Wnt3a–dependent proliferation (n=8–9) and levels of the Wnt3a inhibitor *SFRP1* (n=4). Panels A-E: mean±SEM; p-value *vs* controls, Student's t-test.

A

B
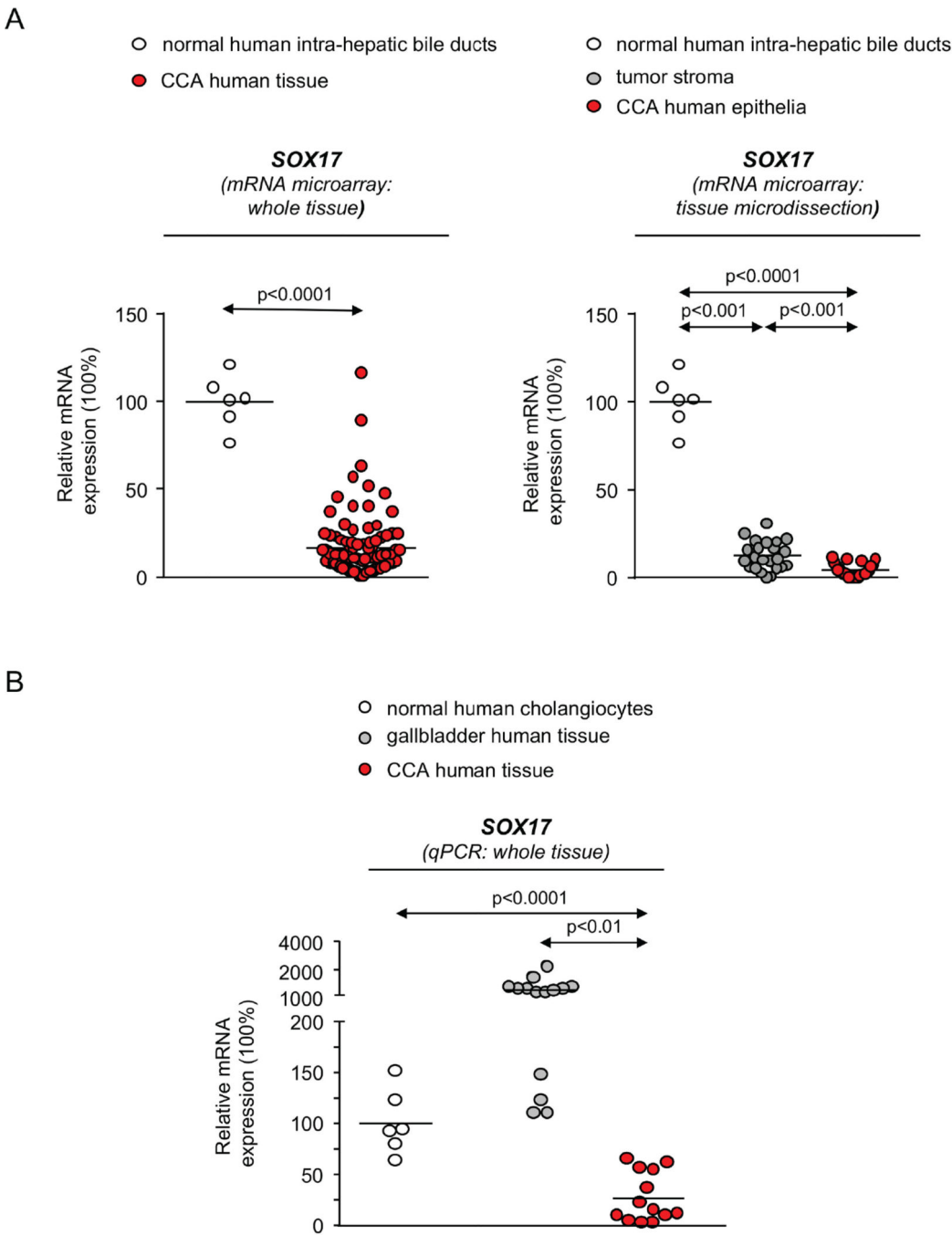
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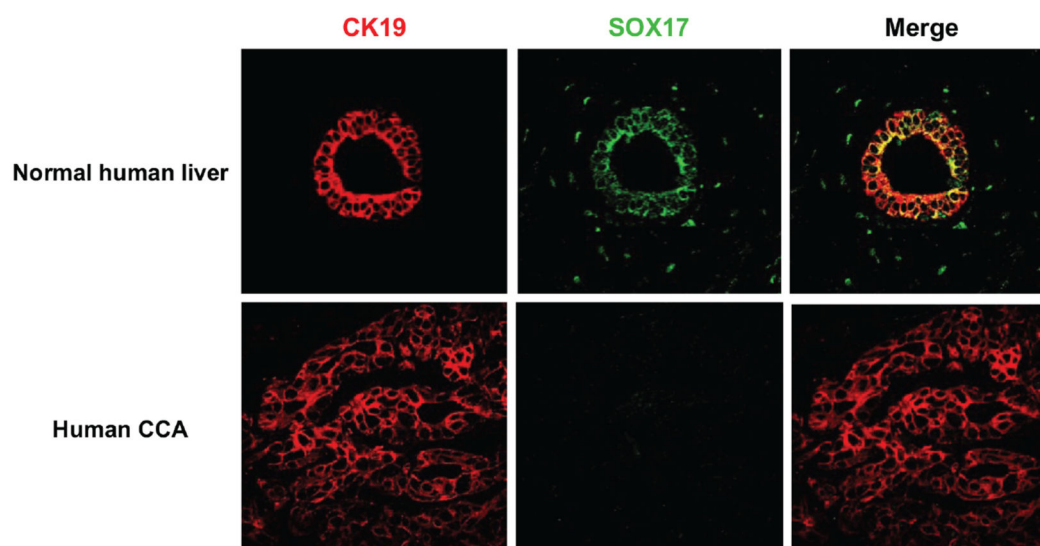
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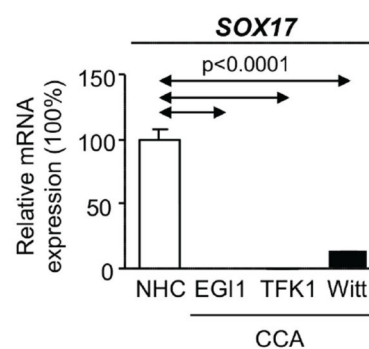
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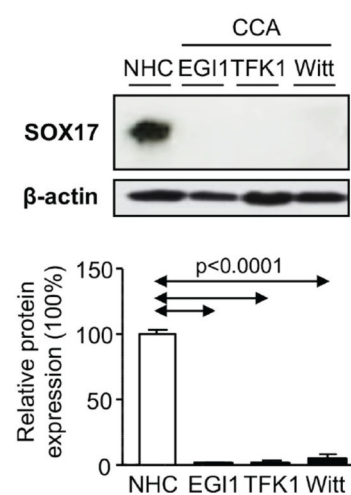
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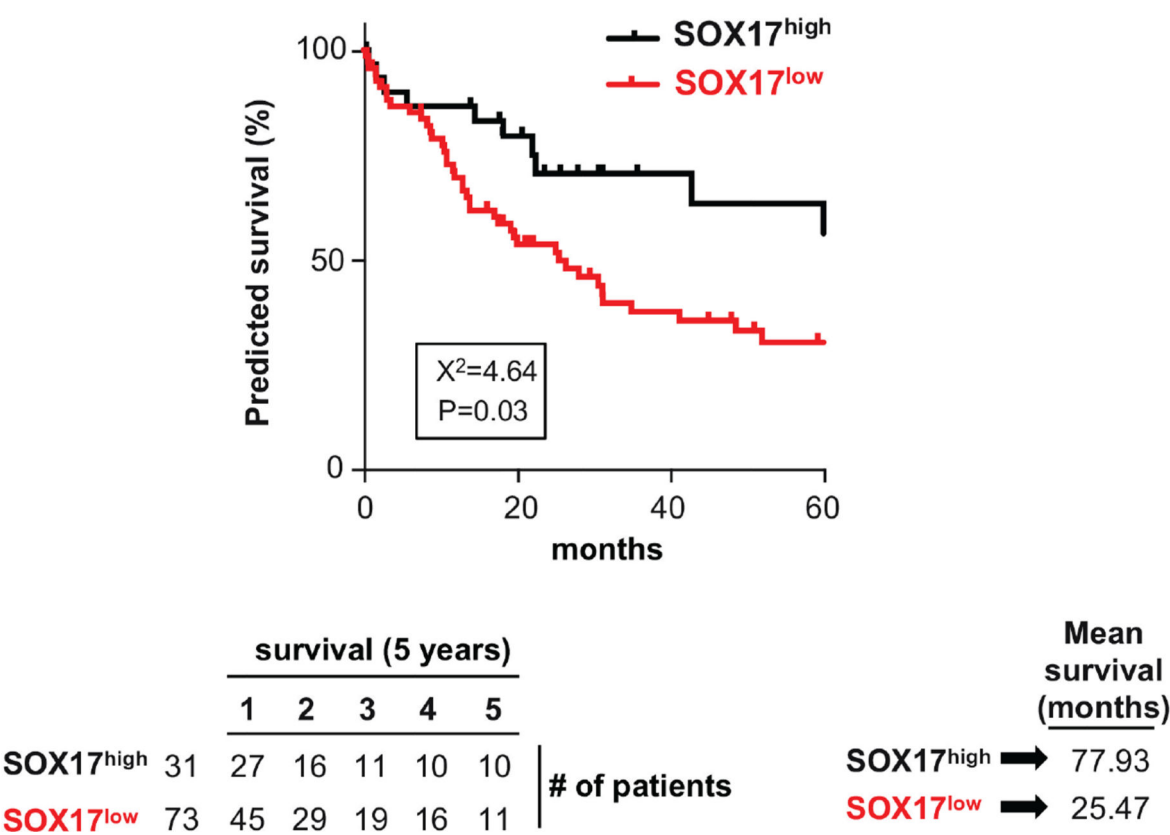
Figure 4. SOX17 expression is decreased in CCA tumors and cell lines and correlates with survival

A) *SOX17* expression (mRNA microarray) in CCA tumors (whole tissue) compared to normal intrahepatic bile ducts, and in CCA epithelia compared to stroma (mean±SEM; p-value *vs* controls, Mann-Whitney t-test and Wilcoxon matched-pairs signed rank test, respectively) (Copenhagen cohort). B) *SOX17* expression (qPCR) in CCA tumors compared to NHC and normal gallbladder tissue (San Sebastian cohort). Dots=number of patients. C) Representative IMFs of SOX17 (green) and CK19 (red) in normal human liver and CCA human tissue (n=5). SOX17 D) mRNA (n=4–6) and E) protein (n=4) levels in CCA cell lines and NHC. F) Kaplan-Meier analysis (Gehan-Breslow-Wilcoxon test) of *SOX17* mRNA expression in CCA tumors (Copenhagen cohort). Panels B,D,E: mean±SEM; p-value *vs* controls, Student's t-test.

A

B

C

D

Annexin V

Propidium Iodide (PI)

E

ROS **Caspase-3**

F
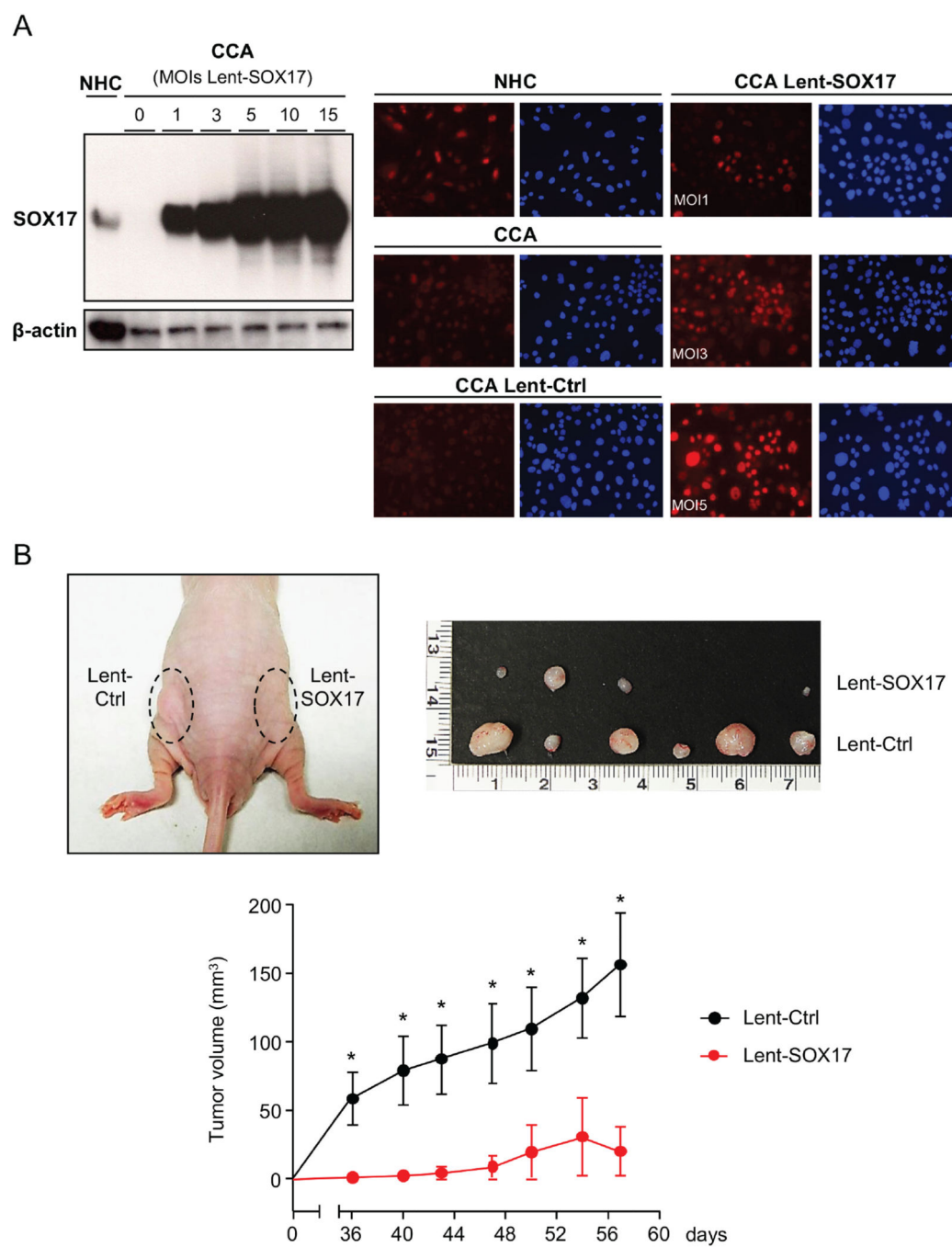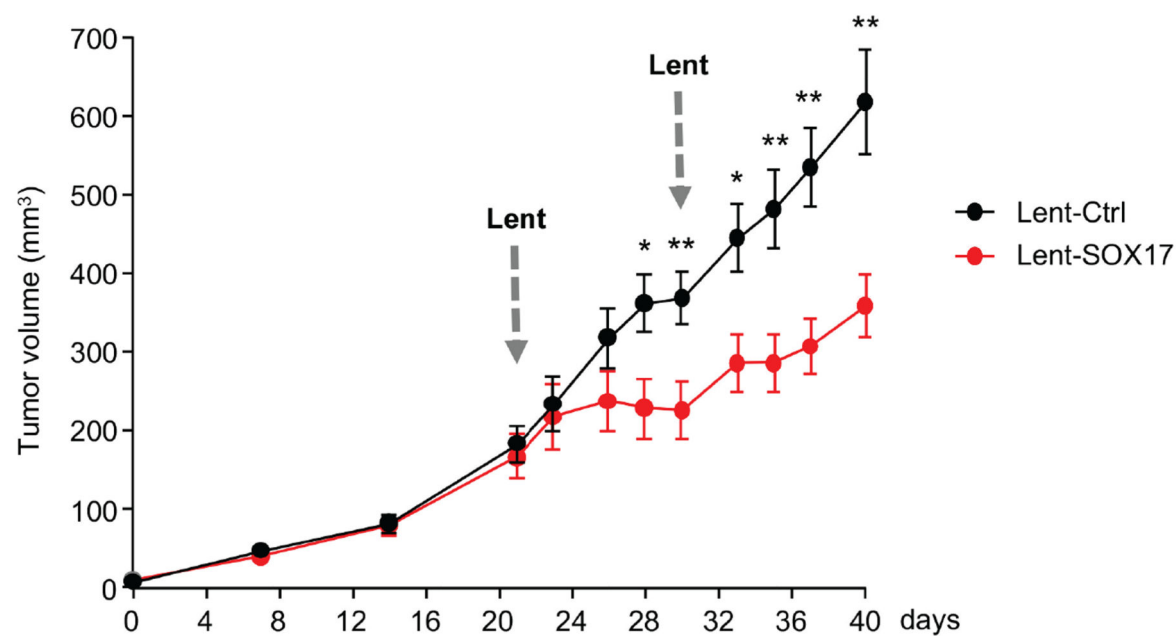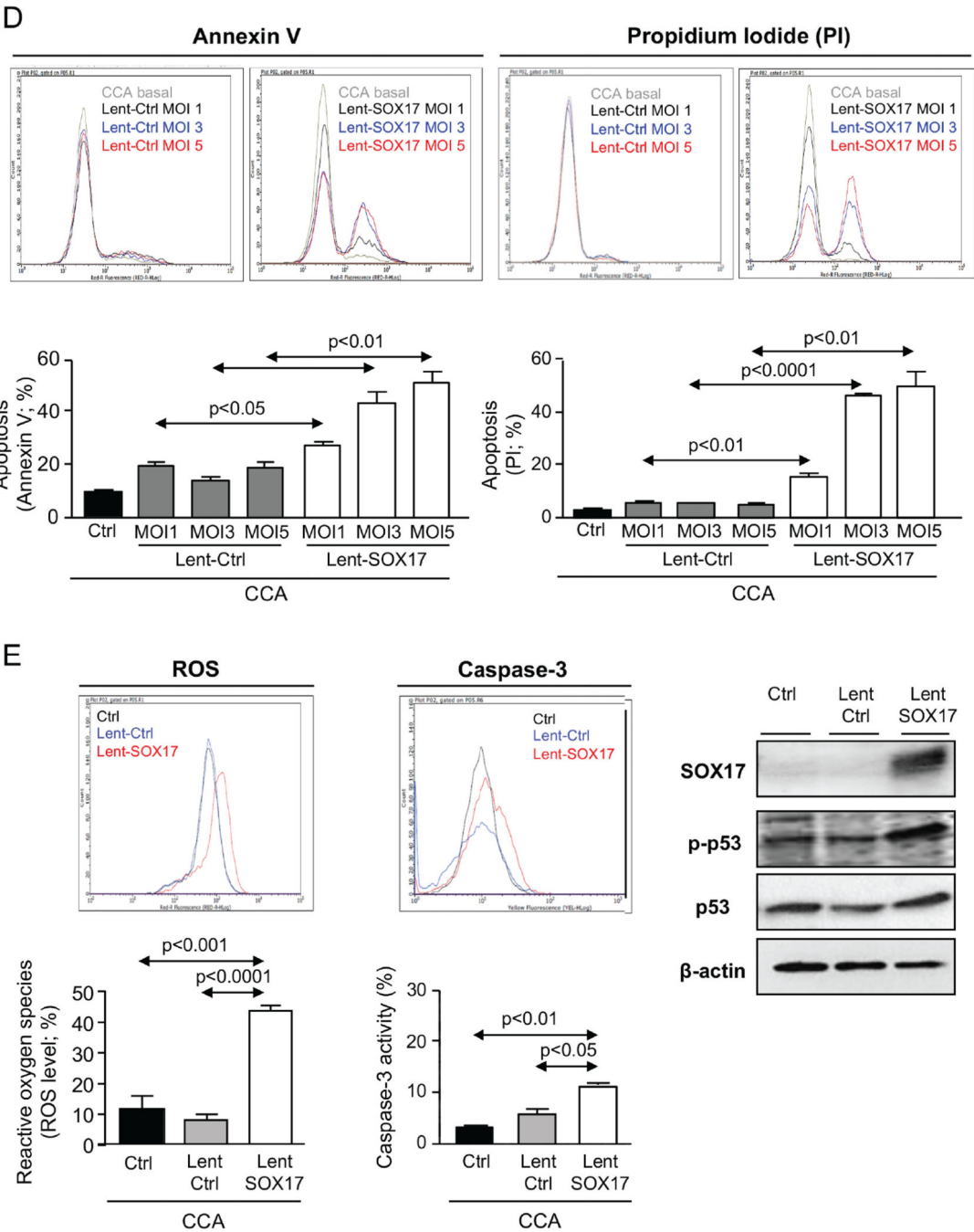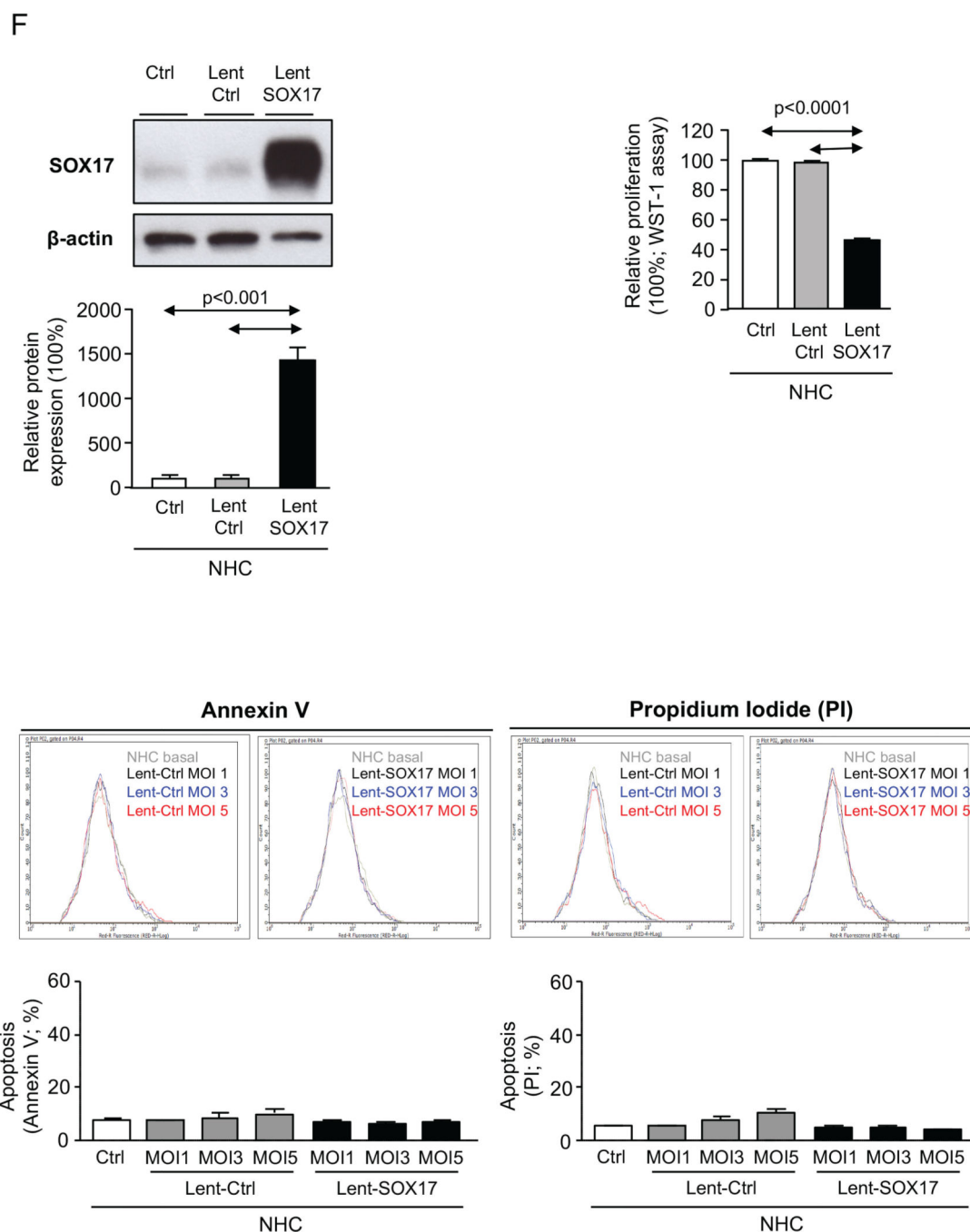
Figure 5. Experimental overexpression of SOX17 in CCA cells decreases their tumorigenic capacity by increasing apoptosis

A) Representative immunoblots and IMFs of SOX17 in CCA cells and NHC under experimental modulation of SOX17 expression (red: SOX17; blue: nucleus). B) SOX17-dependent subcutaneous CCA implantation and growth in immunodeficient mice. C) SOX17 experimental overexpression inhibits the growth of already developed subcutaneous CCA tumors in immunodeficient mice. Arrows: time-points of intratumoral lentiviral injection. Quantification (n=3, MOI 3) of flow cytometry-based D) apoptosis (n=3), E) oxidative stress and caspase-3 activity in CCA cells under experimental modulation of SOX17 expression.

Representative immunoblots of p-p53, p53, SOX17 and β-actin. F) Representative immunoblot and quantification of SOX17 expression in NHC under experimental modulation of SOX17 expression (n=3) and effects on proliferation (n=10) and apoptosis (n=3). Panels B-F: mean±SEM; p-value *vs* controls, Student's t-test. Data in D-F are representative of at least two independent experiments. (* and **:p-value <0.05 and <0.01, respectively).
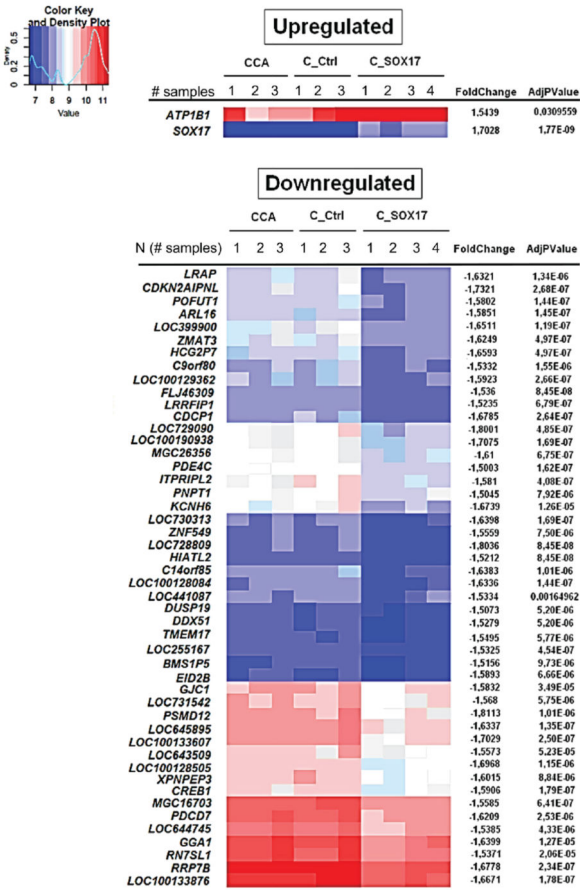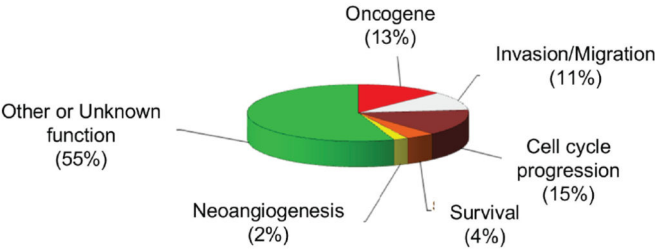
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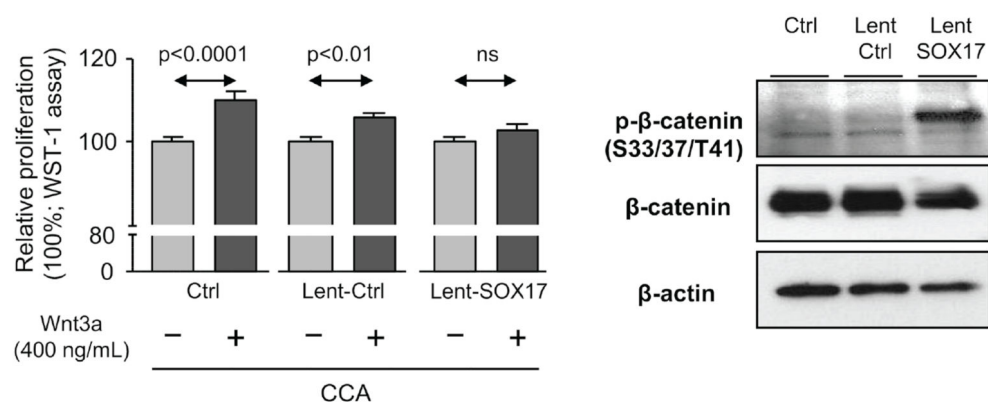
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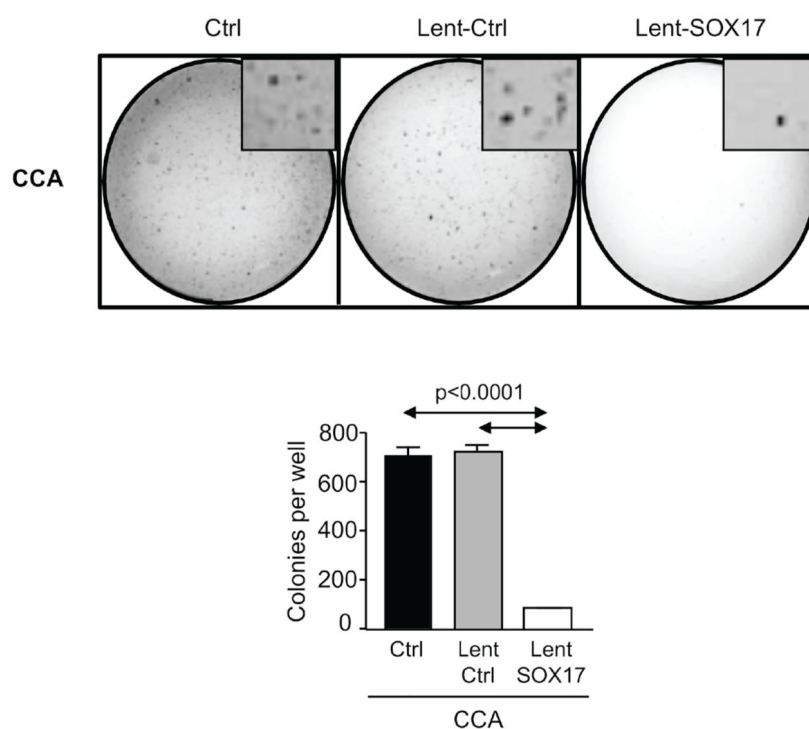
E

"CCA_SOX17" vs "CCA and CCA_Ctrl"
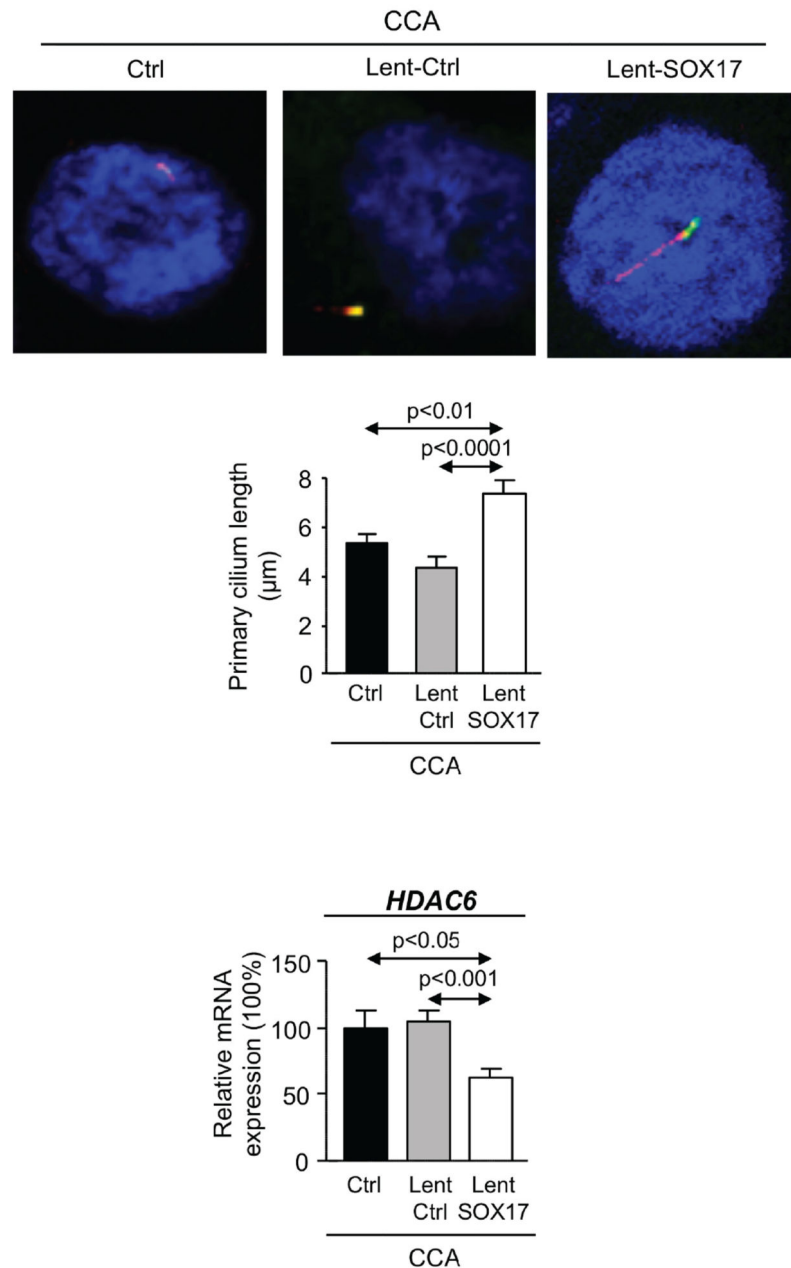
Downregulated genes

F

Figure 6. Experimental overexpression of SOX17 in CCA cells decreases their proliferation and migration capacity and increases their biliary differentiation features

A) Wnt3a–dependent proliferation (n=24) and immunoblots of p-β-catenin (S33/37/T41), β-catenin and β-actin. B) Anchorage-independent CCA cell growth in soft agar (n=6 from two independent experiments). C) Representative light microscopy images and quantification of CCA scratch-migration assays (n=6–8 from three independent experiments) and *S100A4* mRNA expression (n=4). D) Expression (mRNA) of biliary and epithelial markers (n=4). E) Heatmaps and clusters of dysregulated genes based on cellular function (n=3–4; Bayes moderated t-statistics). F) Representative IMF images and quantification of the primary

cilium length [acetylated α-tubulin (axoneme): red; γ-tubulin (centrioles): green; nucleus: blue)] (n=40–60) and *HDAC6* mRNA levels (n=4). Panels A–D,F: mean±SEM; p-value *vs* controls, Student's t-test.
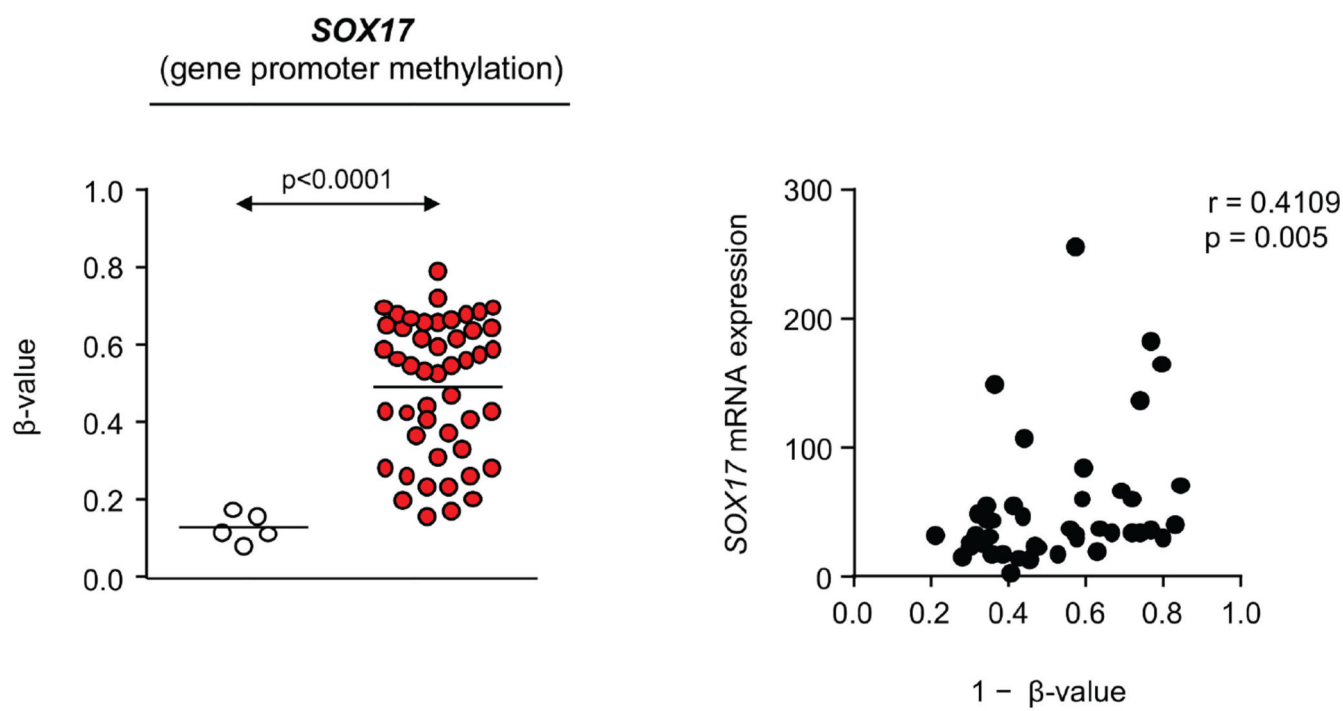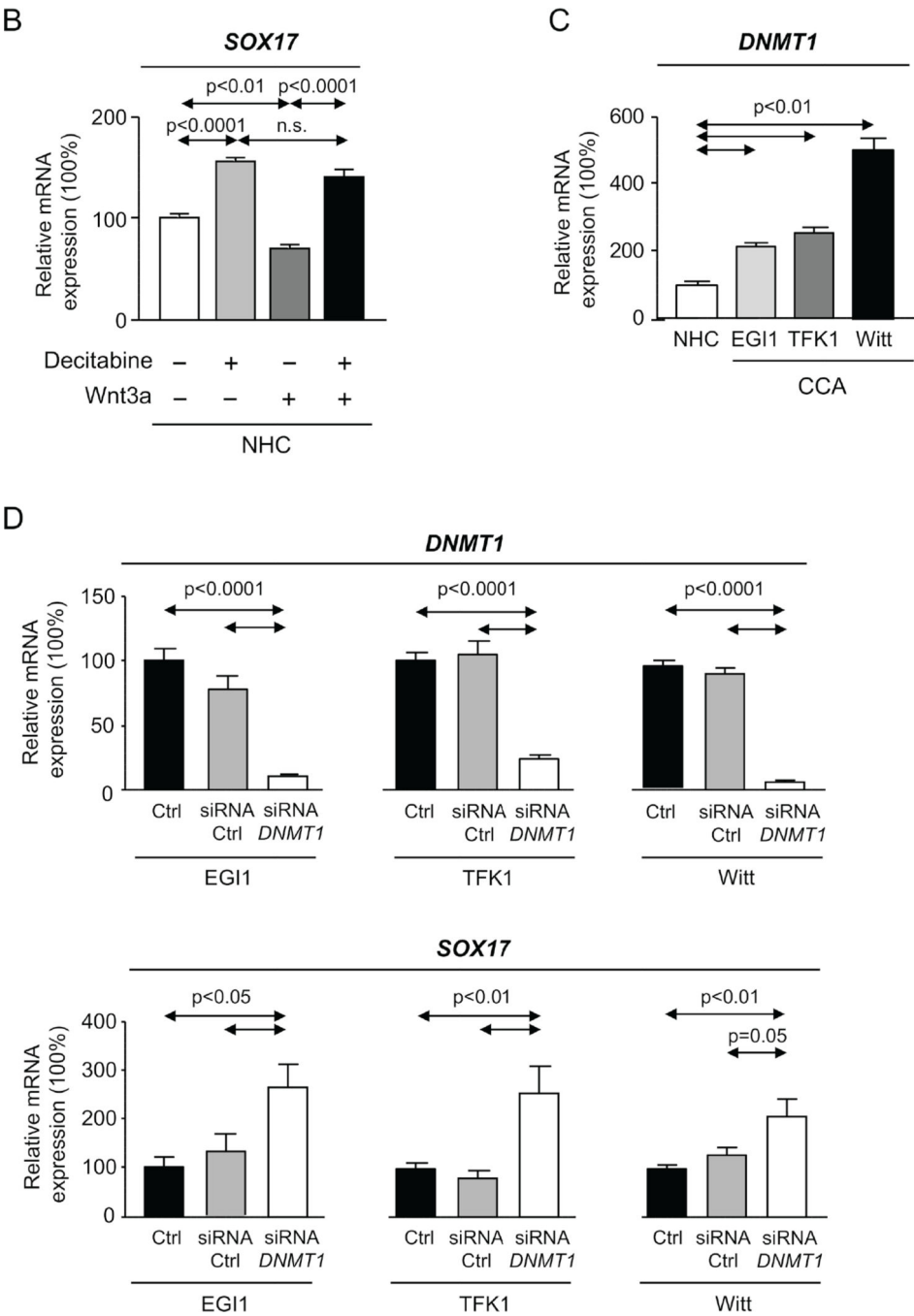
A

○ normal human intra-hepatic bile ducts
● CCA human tissue

SOX17
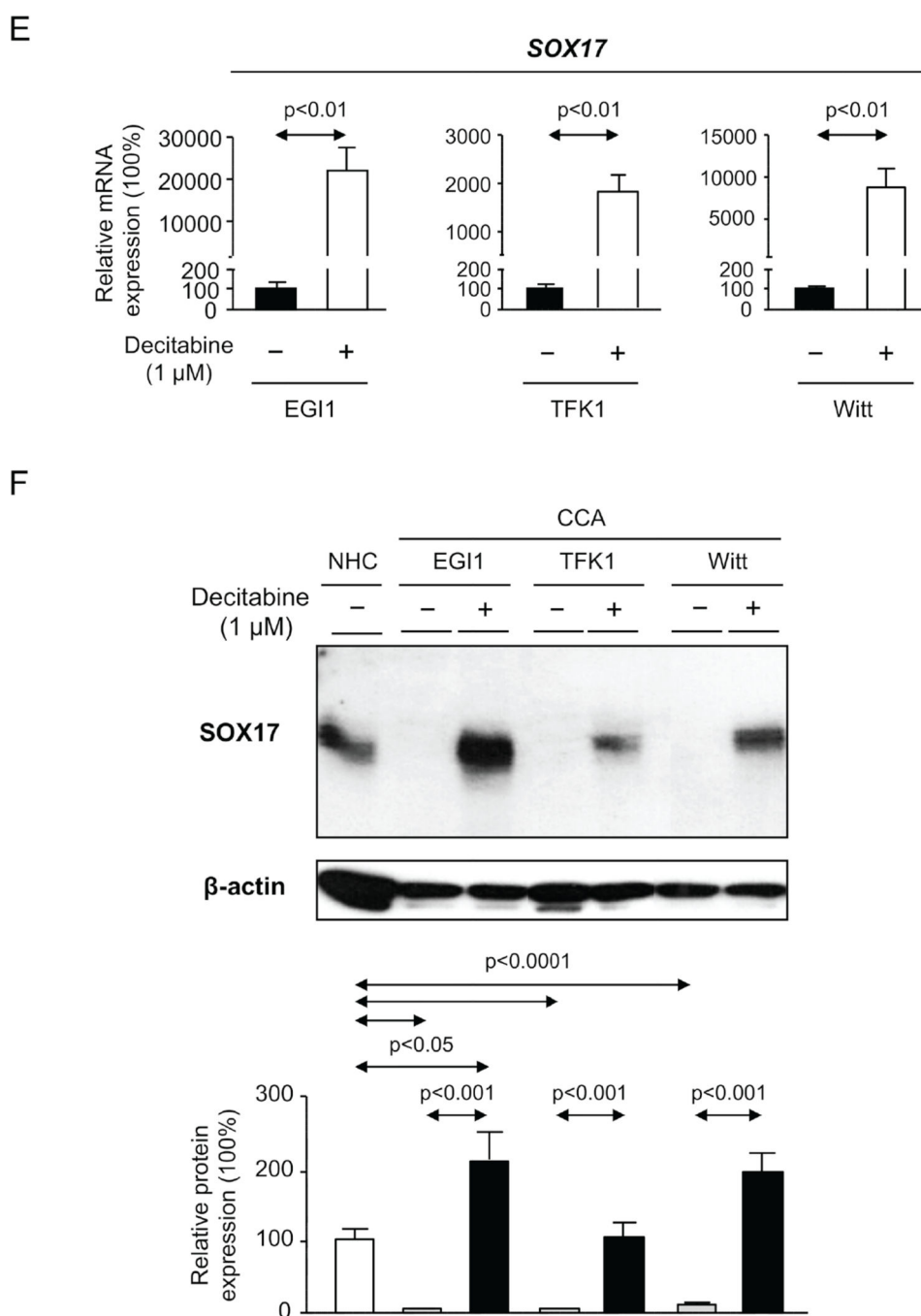(gene promoter methylation)

E

F

Figure 7. SOX17 expression is epigenetically regulated by DNMTs in NHC and CCA cells
A) Methylation levels of *SOX17* promoter in CCA human tissue compared to normal intrahepatic bile ducts (mean±SEM; p-value *vs* controls, Mann-Whitney t-test) and correlation (nonparametric Spearman test) with *SOX17* expression (Copenhagen cohort). Dots=number of patients. B) *SOX17* levels in NHC under the presence or absence of Wnt3a and with or without decitabine (n=4–5; mean±SEM; ANOVA+Tukey post hoc test; n.s.: non-significant). C) *DNMT1* levels in CCA cell lines and NHC (n=6). D) *DNMT1* and *SOX17* expression in CCA cells under experimental knock-down of *DNMT1* compared to

control conditions [n=7–10 for EGI1; n=12–13 for TFK1; and n=15–17 for Witt (pooled data from 2–4 independent experiments)]. SOX17 E) mRNA (n=6–9) and F) protein (n=6–7) levels in CCA cells under the presence or absence of decitabine. NHC were used as control. Panels C-F: mean±SEM; p-value *vs* controls, Student's t-test.