Title: Sirtuin-1 regulates acinar to ductal metaplasia and supports cancer cell viability in pancreatic cancer

Authors: Elke Wauters 2, Victor J. Sanchez-Arévalo Lobo* 4, Andreia V. Pinho* 1,4, Amanda Mawson 1, Jianmin Wu 1, Mark J. Cowley 1, Emily K. Colvin 1, Erna Ngawi Njicop 2, Rob L. Sutherland 1, Tao Liu 6, Manuel Serrano 5, Luc Bouwens 2, Francisco X. Real 4,7, Andrew V. Biankin 1, Ilse Rooman 1,2,3

Affiliations: 1Cancer Research Program, Garvan Institute of Medical Research, Sydney, Australia; 2Diabetes Research Center, VUB, Brussels, Belgium; 3St Vincent’s Clinical School, University New South Wales, Australia; 4Programa de Patología Molecular and 5Programa de Oncología Molecular, CNIO (Spanish National Cancer Research Center), Madrid, Spain; 6Children’s Cancer Institute Australia for Medical Research, Randwick, Australia; 7Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain

*These authors made equal contributions.

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**Corresponding author:** Ilse Rooman, Ph.D.

Cancer Research Program, The Garvan Institute of Medical Research

384, Victoria Street

Sydney NSW 2010, Australia

tel. +61 2 9355 5806  fax. 61 2 9355 5868  i.rooman@garvan.org.au

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**Abbreviations:** ADM: Acinar to Ductal Metaplasia; Amy2: Amylase 2; Cae-AP: Caerulein induced Acute Pancreatitis; Cttnb: beta-Catenin; Ctrb1: Chymotrypsin B1; Dbc1: Deleted in breast cancer 1; Ela1: Elastase 1; FC: fold change; Hprt: Hypoxanthine-guanine phosphoribosyltransferase; IF: immunofluorescence; IHC: Immunohistochemistry, Krt19: Keratin 19; LMB: Leptomycin B; NA: Nicotinamide; pCAF: P300/CBP-associated factor; PDAC: Pancreatic Ductal Adenocarcinoma; PDL: Pancreatic Duct Ligation; Ptfl1a: Pancreatic transcription factor 1a; Rbpjl: Recombinant signal binding protein for immunoglobulin kappa J-region-like; Tg: transgenic
Abstract

The exocrine pancreas can undergo acinar to ductal metaplasia (ADM), as in the case of pancreatitis where precursor lesions of pancreatic ductal adenocarcinoma (PDAC) can arise. The NAD⁺-dependent protein deacetylase Sirtuin-1 (Sirt1) has been implicated in carcinogenesis with dual roles depending on its subcellular localization.

In this study, we examined the expression and the role of Sirt1 in different stages of pancreatic carcinogenesis, i.e. ADM models and established PDAC. In addition, we analysed the expression of KIAA1967, a key mediator of Sirt1 function, along with other potential Sirt1 downstream targets.

Sirt1 was coexpressed with KIAA1967 in the nuclei of normal pancreatic acinar cells. In ADM, Sirt1 underwent a transient nuclear to cytoplasmic shuttling. Experiments where during ADM, we enforced repression of Sirt1 shuttling, inhibition of Sirt1 activity or modulation of its expression, all underscore that the temporary decrease of nuclear and increase of cytoplasmic Sirt1 stimulate ADM. Our results further underscore that important transcriptional regulators of acinar differentiation, i.e. pancreatic transcription factor-1a and β-catenin can be deacetylated by Sirt1. Inhibition of Sirt1 is effective in suppression of ADM and in reducing cell viability in established PDAC tumors. KIAA1967 expression is differentially down regulated in PDAC and impacts on the sensitivity of PDAC cells to the Sirt1/2 inhibitor Tenovin-6. In PDAC, acetylation of β-catenin is not affected, unlike p53, a well characterised Sirt1 regulated protein in tumor cells.

Our results reveal that Sirt1 is an important regulator and potential therapeutic target in pancreatic carcinogenesis.
INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States, an outcome that has not changed for 50 years (1). Understanding the molecular mechanisms of PDAC initiation and tumor maintenance is imperative to develop chemoprevention and therapeutic strategies. New insights in PDAC initiation show that adult exocrine acinar cells under stress can dedifferentiate and gain metaplastic ductal characteristics (referred to as acinar to duct metaplasia, ADM). There is compelling evidence from mouse models that ADM is a precursor lesion of PDAC (2, 3). ADM also occurs in pancreatitis, which may explain why pancreatitis is a major risk factor for PDAC (2, 3). Prevention of ADM and maintenance of acinar cell differentiation could suppress pancreatic carcinogenesis. Previously we observed that nicotinamide repressed acinar cell dedifferentiation and ADM in culture (4). Nicotinamide is an endproduct and feedback inhibitor of Sirtuin-mediated protein deacetylation (5).

The repertoire of Sirtuin functions is broader than the role in longevity for which they were originally identified (6) and Sirtuins target a range of nuclear, mitochondrial and cytoplasmic proteins. Sirtuins have multifaceted roles in cell death, differentiation, metabolism and senescence. Sirtuin 1 (Sirt1), the best studied of the family, plays also roles in cancer and has been reported to be an oncogene as well as a tumor suppressor (7). Related to pancreas, Sirt1 has been studied in islets and diabetes (8, 9) (10) but only limited evidence exists that Sirt1 is important in PDAC (11).
One of the best characterized regulators of Sirt1 is Deleted in Breast Cancer 1 (Dbc1, KIAA1967) (12). Dbc1 directly interacts with Sirt1 and inhibits Sirt1 activity (12, 13). Changes in either Dbc1 or Sirt1 expression result in altered Sirt1 driven effects.

Because there was preliminary evidence that a Sirtuin inhibitor impairs ADM and Sirt1 is currently seen as a promising target of therapeutic intervention in other cancers (14), we aimed to study the expression of Sirt1 and Dbc1 in normal exocrine pancreas and during ADM, to define the context specific target genes of Sirt1 and to reveal Sirt1 effects in PDAC.

**MATERIALS AND METHODS**

**Cell cultures** Primary acinar cell culture protocols were adapted from (15) and (16). The cell lines 266-6 and AR42J-B13, the PDAC cell lines and HEK293 cells were obtained from ATCC Cell Biology Collection and used within 6 months between resuscitation and experimentation. The ATCC’s authentication protocols include testing for mycoplasma, bacteria, fungi contamination, confirmation of species identity and detection of cellular contamination or misidentification using COI for interspecies identification and DNA profiling as well as cytogenetic analysis, flow cytometry and immunocytochemistry with consistent refinement of cell growth conditions as well as documentation systems, ensuring traceability. Cells were cultured with Leptomycin B (LMB, 0.25µg/ml, Sigma–Aldrich, St. Louis, MO, USA), Nicotinamide (20-40 mM, Sigma), Resveratrol (50mM, Sigma), Tenovin-6 (Cayman Chemical, Ann Arbor, MI, USA).
**Animals and in vivo experimentation** Pancreatitis and pancreatic duct ligation were performed as in (17, 18) and approved by the ethics committee of the Vrije Universiteit Brussel and the Garvan Animal Ethical Committee, and was performed in accordance with the Declaration of Helsinki as revised in 2000.

**Statistics** Results are presented as mean ± standard error of mean. Data were analyzed by Prism 5.0 (Student’s t-test or one sample t-test). Unless stated otherwise, the experiments were performed at least 3 times independently. $P$ values are indicated.

**RESULTS**

**Sirt1 and Dbc1 expression in normal exocrine tissue and ADM**

We assessed the expression of Sirt1 and Dbc1 in normal pancreas and mouse models of ADM. Staining of Sirt1 in cells with the digestive enzyme amylase or with insulin illustrates that Sirt1 is expressed in the exocrine and endocrine pancreas, with only the latter reported previously (8, 9) (Fig 1A). Sirt1 and Dbc1 were co-expressed in the nuclei of exocrine pancreas (Fig 1A). Expression was undetectable in about 30% of acinar cells (S Fig 1A).

In Caerulein induced acute pancreatitis (Cae-AP), acini undergo ADM and regenerate within one week (18). The ADM in our in vivo models corresponds to what has been described by Strobel et al. (19), i.e. tubular complexes and mucinous metaplastic lesions (MML), and occurs isolated in the absence of PanINs (20). The transient ADM is characterized by cytoplasmic retention of acinar enzymes, such as Carboxypeptidase A1 (CpA1) (Fig 1B), increased expression and cytoplasmic accumulation of beta-Catenin and induction of ductal markers as Keratin 19 (Krt19) (S Fig 1B,C)(18, 21). Nuclear Dbc1 staining did
not change (Fig 1B). In contrast, the Sirt1 staining observed in acini of untreated (Fig 1A) and control PBS-treated pancreata (Fig 1B), shifted from the nucleus to the cytoplasm within the first two days of Cae-AP (Fig 1B). Nuclear Sirt1 expression was re-established by day 8 (Fig 1B). We noted that one third of the mice with Cae-AP showed longer lasting ADM with more cytoplasmic beta-Catenin and Krt19^+ complexes, and more prominent cytoplasmic Sirt1 (S Fig 1B).

Pancreatic duct ligation (PDL) is another model of injury with acinar tissue replaced by Krt19^+ complexes resulting from permanent ADM and ductal proliferation (17) (Fig 1C, S Fig 2). Dbc1 expression remained unchanged (S fig 2B) but a nuclear to cytoplasmic shift of Sirt1 was again found (Fig 1C). This shift was early and transient with restoration of nuclear expression within one week. The percentage of mice in which we observed cytoplasmic Sirt1 matched the success rate of PDL.

In conclusion, in the two in vivo models of ADM, the Dbc1 expression pattern was unchanged but an obvious intracellular shift of Sirt1 was noted, confined to the exocrine cells, i.e. islet cells retained nuclear Sirt1 and Dbc1 (not shown). This observation suggests that in ADM, Sirt1 can have altered activity and effects on target proteins as a result of changed interaction with Dbc1 and changed intracellular localization.

**Functional significance of nuclear to cytoplasmic shuttling of Sirt1 in ADM**

We reported before on rodent and human exocrine cell cultures (4, 15, 16), the latter specifically providing insights into onset of ADM that cannot be obtained from in vivo (clinical) samples. Cells of non-acinar origin that also can
contribute to the *in vivo* ADM type lesions are likely not represented in the *in vitro* models.

Again we observed that, in contrast to the overall nuclear localization in normal tissue, the exocrine cells showed manifest cytoplasmic Sirt1 staining at the time of cell isolation (**Fig2A for human and 2B for mouse**). In human cultures, this was slightly more variable due to the variability in method and time of collection of donor tissue and the more elaborate isolation procedure, which allowed less control on the time point of analysis. Similar to the *in vivo* observations, nuclear Sirt1 expression became re-established during culture (**Fig 2A,B and S Fig 3B**) and nuclear localization of Dbc1 was unchanged (not shown).

We used the mouse ADM cultures for further functional analyses. No changes occur in levels of expression of Sirt1 or Dbc1 protein (**S Fig 3C**). Within 24 hours, acinar cells dedifferentiate and undergo ADM comparable to the changes of Cae-AP in a panel of relevant genes (**S Fig 3A**), induction of Krt19 and changed beta-catenin expression (**S Fig 3A,B**).

To address the impact of nuclear to cytoplasmic shuttling of Sirt1, we treated the acinar cells with Leptomycin B (LMB), an antibiotic that inhibits nuclear export of proteins with a nuclear export signal, including Sirt1 (22). This resulted in more cells presenting nuclear Sirt1 staining (co-localization with Mist1 was used to label the acinar cell nuclei) (**Fig 2C**). Western blotting confirmed higher nuclear Sirt1 expression with LMB (**S Fig 3D**). LMB restrained the induction of Krt19, a ductal marker that is strongly induced under control conditions, as seen in IF (**Fig 2C**). Consistent with this, a lesser induction of Krt19 mRNA was observed in LMB conditions (4.1±0.8-fold vs 10.9±2.2-fold in
controls, \(P<0.04\) (Fig 2D). No profound effect on the expression of acinar enzymes or transcription factors was found (not shown).

We explored further whether changes in Sirt1 expression impacted on acinar cell differentiation, starting with non-stress conditions. Pancreata from Pdx1-Cre; Sirt1\textsuperscript{ex4lox/lox} mice show a mutant Sirt1 protein band which migrates slightly faster in western blot analysis (S Fig 4A), in line with (23). This Sirt1 is an inactive mutant form and Sirt1 expression is also strongly suppressed (S Fig 4A,B). Whereas there is less Pancreatic transcription factor-1a (Ptf1a), no significant changes occur in the expression of the acinar genes Amylase 2 (Amy2), Elastase 1 (Ela1), Carboxypeptidase A1 (CpA1), Chymotrypsin B1 (Ctcb1) or the Recombinant signal binding protein for immunoglobulin kappa J-region-like (Rbpjl) (Fig 3B, control) suggesting that absence of Sirt1 in unstressed conditions does not affect acinar differentiation. However, upon acute caerulein pancreatitis (Fig 3A) the role of Sirt1 becomes apparent, with the pancreata from the Pdx1-Cre; Sirt1\textsuperscript{ex4lox/lox} mice showing a more profound ADM (Fig 3A,B), characterized by a higher suppression of acinar enzymes and of the transcription factors Ptf1a and Rbpjl. Similar results were obtained using the \textit{in vitro} ADM model (S Fig 4C). In the Pdx1-Cre; Sirt1\textsuperscript{ex4lox/lox} mice, the ADM was also more persistent with suppression of acinar markers 8 days post-caerulein. At this time point, the pancreatic tissue from control mice had almost completely recovered (with <10% of affected areas in tissue sections), while ADM lesions were still manifest in Pdx1-Cre; Sirt1\textsuperscript{ex4lox/lox} mice (3/5 animals with >50% of affected areas in tissue sections). We also noticed a reduction in pancreas volume in the Pdx1-Cre; Sirt1\textsuperscript{ex4lox/lox} (not shown). These results underscore that under stress conditions absence of Sirt1 enhances the ADM.
To evaluate whether increased Sirt1 expression also had effects on acinar gene expression and ADM, we analysed a Sirt1 transgenic (SirtTg) mouse strain (24), that expressed increased Sirt1 levels from its endogenous promoter ubiquitously - including in acinar cells (S Fig 5A), to evaluate whether increased Sirt1 expression had effects on the acinar gene expression. We did not detect significant differences (S Fig 5B) either in unstressed or upon pancreatitis/ADM conditions. We note that the Sirt1 overexpression in acinar cells was modest (~2-fold), in line with (24).

To further study Sirt1 overexpression and address the question whether Sirt1 accumulation in the cytoplasm of acinar cells has a contribution to ADM, we analysed whether acinar 266-6 cells with stable and robust overexpression of Sirt1 mostly confined to the cytoplasm (Fig 3C) had different features. We found consistent suppression of CpA1, Ptf1a and Rbpjl (Fig 3D).

Finally we wanted to explore whether pharmacological interference could affect ADM. We used a Sirtuin1 inhibitor Nicotinamide (NA) in the ADM in vitro model. NA treatment during the first 24h, when Sirt1 was predominantly cytoplasmic, resulted in a suppression of Krt19 induction, noted by IF and RT-qPCR (Fig 3E,F). Additionally, higher expression of the acinar cell specific markers Amy2, Ela1, Ctrb1, CpA1 and Rbpjl was maintained (Fig 3F). Similar ADM repression was observed in preliminary experiments with human acinar cells exposed to NA (S Fig 6A,B).

In conclusion, loss of nuclear Sirt1 and cytoplasmic accumulation both contribute to ADM. Inhibition of Sirt1’s shuttling or activity during ADM restrains the process.
**Sirt1 deacetylates beta-Catenin and Ptf1a**

Sirt1 can deacetylate beta-Catenin, a post-translational modification that impairs its transcriptional activity (25), and modulates Wnt/beta-Catenin signaling, as in colon cancer (26). Loss of beta-Catenin signaling is decisive in installing persistent ADM and impairs acinar cell regeneration (21).

In acini, beta-Catenin is associated with the plasma membrane (Fig 4A). A cytoplasmic localization of beta-Catenin has been reported in ADM (21, 27, 28) and correlates well with those in which we demonstrated a predominant cytoplasmic Sirt1. Therefore, we investigated a possible relation of beta-Catenin and Sirt1 in ADM.

First, we analyzed the cellular localization of beta-Catenin: in isolated acini, preferential membrane and occasional nuclear staining were seen. A cytoplasmic distribution predominated at 24h with a redistribution to the membrane by 5 days of culture (Fig 4A). This transient cytoplasmic staining was not observed in presence of NA (Fig 4B). In Western Blotting, two bands were detected: The higher band likely results from post-translational modifications (see below) and persisted upon treatment with NA whereas the lower band was induced in controls (Fig 4C). In Cae-AP in vivo, we found a similar pattern of beta-Catenin in immunostaining (S Fig 1B,C) and in western blot analysis (not shown).

We then used AR42J-B13 cells to further investigate the functional interaction of Sirt1 with beta-catenin in an acinar cell context. We chose these cells because Sirt1 was predominantly localized in the cytoplasm (S Fig 7A). The two reported isoforms of Sirt1 were detected (not shown). Sirt1 co-localized and interacted with beta-Catenin, as demonstrated by IF, the Duolink® assay and co-
IP (S Fig 7A,B). Using co-IP, we detected the Sirt1 isoform which has the highest deacetylase activity (29). As of beta-Catenin, two bands were detected (Fig 4E), consistent with findings by others (30). By co-IP, we showed that the upper beta-Catenin band corresponded to an acetylated form (Fig 4E). Exposure to Resveratrol to stimulate Sirt1 activity (31), resulted in a 3.2-fold increased density of the lower deacetylated beta-Catenin band (P<.05, n=4), specifically within the cytoplasm (S Fig 7C). Of note, the upper band was maintained when NA was applied to the primary culture model (Fig 4C).

We then examined whether our observations are related to Wnt/beta-Catenin signaling. Axin2, a Wnt/beta-Catenin target gene, was induced upon acinar cell isolation but was highly reduced at 24 h in control (PBS) ADM cultures (Fig 4D). This paralleled the IF data where membrane associated beta-Catenin and accumulation of beta-Catenin in the nucleus was only observed in isolated acini (Fig 4A). With NA, the expression of Axin2 was maintained at higher levels (Fig 4D). We confirmed this effect of NA on Wnt/beta-Catenin signaling by comparing a larger panel of targets and regulators of the pathway (S Fig 8A,B).

The nuclear co-localization of Sirt1 and Ptf1a in normal acini (Fig 5A), the changes in subcellular localization of Sirt1, and the changes in digestive enzyme levels in ADM suggested that Sirt1 might contribute to regulate the function of PTF1, the master regulator of the acinar programme. PTF1 is a transcription factor complex composed of Ptf1a, Rbpj1 and a class A bHLH protein (32). P300/CBP-associated factor (pCAF) is associated to it, acetylates Ptf1a and ensures high transcriptional activity of Ptf1a (33). We undertook ChIP experiments to verify if Sirt1 localized together with Ptf1a in the PTF1 binding
sites of acinar gene promoters. CpA1, CtrB1, Ela1, Ptf1a and Rbpj1 promoter areas were indeed 3-30 fold enriched in anti-Sirt1 ChIPs (Fig 5B). To assess whether Sirt1 and Ptf1a interact and impact on the acetylation state, Flag-pCAF, Flag-Ptf1a, Flag-Sirt1 and Flag-Sirt1* (a mutant Sirt1 in which a critical histidine in the deacetylase domain has been replaced by a tyrosine residue (H363Y)) were transfected in HEK293 cells and assayed by co-IP. The results confirmed that Ptf1a became acetylated upon co-transfection of pCAF (as reported in (33)), and there is loss of acetylation of Ptf1a when co-transfected with Sirt1, but not when co-transfected with the inactive Sirt1* (Fig 5C). There were no effects on Rbpj1 (not shown).

Together, our findings showed altered acetylation of the Sirt1 target beta-Catenin in the context of ADM and thereby interference with beta-Catenin/Wnt signaling. Furthermore, Sirt1 could deacetylate Ptf1a, the critical component of PTF1, and Sirt1 associated with PTF1 dependent gene promoters. These observations underpinned that Sirt1 can regulate acinar cell differentiation and have a role in ADM.

**Role of Sirt1 in advanced pancreatic tumors**

Subsequent to our investigations in metaplasia, we studied Sirt1 in human PDAC. Using Illumina expression microarray data from (34), we found that Sirt1 in PDAC tissue does not differ from normal pancreas ($P=.23$). IHC resulted in variable detection levels and patterns in normal and cancer tissue, again without apparent differences (not shown).

We studied the effect of genetic inhibition of Sirt1 expression in Panc1 and Panc10.05 cells with siRNAs. Knock-down of Sirt1 protein was confirmed up
to 120 hours after transfection (Fig 6A) and increased acetylation of p53 was detected, in accordance with p53 being the best described target of Sirt1(23). No difference was noted in beta-Catenin or Axin2 expression (Fig 6A, not shown). Up to 50% inhibition of cell viability was demonstrated (Fig 6B). In addition, we treated four PDAC cell lines with the Sirt1/2 inhibitor Tenovin-6. This reduced the percentage of viable HPAC, MiaPaca2 and Panc10.05 cells in a concentration dependent manner. However, Panc1 cells were much less sensitive (Fig 6C).

The observed differences in sensitivity to Tenovin-6, led us to hypothesize that the relative expression levels of Sirt1/Dbc1 determine the sensitivity. Sirt1/Dbc1 protein expression, as measured in western blots, did indeed correlate with the IC50 for Tenovin-6 (R=0.92, P<.05) (Fig 6D). In fact, Dbc1 expression is different among PDAC samples (Fig 6E,F). Compared to high Dbc1 intensity in normal tissue, the fraction of samples with low or medium intensity was significantly higher in PDAC, and about 10% were negative (S Fig 9A). The percentage of positive nuclei ranged between 3 and 100% in PDAC compared to 50 to 100% in controls (S Fig 9B). Dbc1 staining in chronic pancreatitis did not differ from controls (Fig 6A, S Fig 9A,B). In our Illumina expression microarray, we also found an overall decrease in Dbc1 expression in PDAC samples compared to samples of adjacent normal pancreas (fold change = 0.6, P=.0007).

In conclusion, three out of four PDAC cell lines that we tested are sensitive to Tenovin-6 and this highly correlated with their Sirt1/Dbc1 levels.

DISCUSSION

Our analyses show that Sirt1 is co-localized with Dbc1 in the nucleus of normal exocrine acinar cells. We find that this co-localization is disturbed in
ADM, i.e. Sirt1 shifts out of the nucleus into the cytoplasm. This was a general observation in four different experimental models of ADM, including mouse and human, regardless of the different types and origins of ADM (19). Dissociation of Sirt1 from its inhibitor activates Sirt1 (13, 35). Furthermore, altered intracellular localization likely changes the interactions with target proteins. Other studies have shown that intracellular Sirt1 localization changes in response to physiological and pathological stimuli resulting in altered cell differentiation and different roles during multistage carcinogenesis (22, 36, 37). The functional impact of these changes in Sirt1 in pancreas was explored in this study.

We manipulated the intracellular shuttling and the activity of Sirt1 in acini that undergo ADM. In the presence of LMB, when more nuclear Sirt1 is retained, we observed a lesser induction of Krt19, a prominent hallmark of ADM (2, 15). This indicated inhibition of ADM to a certain extent. We did not prevent the drop in acinar gene expression except when directly inhibiting Sirt1’s activity with Nicotinamide at the time of its cytoplasmic localization. This resulted in the partial preservation of typical acinar gene expression, an extension of our previous observations (4). Preliminary experiments with human cultured acinar cells showed similar findings reinforcing that our observations are relevant in human. It needs to be appreciated that the models used here (mouse models and human exocrine cultures) provide insights into initiating mechanisms that cannot be gathered from clinical samples usually collected at advanced disease, i.e. chronic pancreatitis or pancreatic cancer. Nicotinamide is used in clinical trials and more potent and Sirt1 specific drugs that mimic Dbc1 are under development. These may interfere with ADM in pancreatitis, and ultimately prevent development of PDAC.
Absence of functional Sirt1 in unstressed pancreas did not seem to impinge on acinar differentiation, likely because of the inhibited state of Sirt1 in normal pancreas or compensatory mechanisms. Under conditions of pancreatitis, however, it becomes clear that lack of nuclear Sirt1 aggravates the ADM. On the other hand, the increased cytoplasmic Sirt1 can also contribute to ADM. We indeed found repression of acinar genes in Sirt1 overexpressing 266-6 cells where expression was mainly in the cytoplasm. The effect was modest, perhaps because our experiment was performed in this cell line that has a less differentiated phenotype than mature acinar cells.

Next, we investigated the mechanisms through which Sirt1 could affect acinar cell differentiation in ADM. Beta-Catenin has an important role in embryonic acinar cell differentiation and proliferation (38, 39). Its abnormal expression in the cytoplasm in early stages of ADM (18, 21, 27) (and present results) coincident with altered Sirt1 localization suggested a functional link between both proteins. We hypothesized that beta-Catenin could be a target for deacetylation by Sirt1 in ADM as in colon cancer (26). Our experiments demonstrate that in an acinar cell context both proteins can co-localize, can interact, and that there is a relation between the acetylation of beta-Catenin and modulation of Sirt1 activity (Nicotinamide and Resveratrol). In addition, we discovered that in pancreatic acini Sirt1 is a novel regulator of Wnt/beta-Catenin signaling that is induced upon acinar cell isolation but rapidly lost in culture. Consequently, the acinar cells dedifferentiate permanently, in agreement with previous observations (21). We find that application of a sirtuin inhibitor resulted in maintenance of Wnt/beta-Catenin signaling and more membrane-bound beta-Catenin. However, effects of beta-Catenin separate from Wnt
signaling might also be involved (40). Ras and Notch signaling (21, 41), pathways that are activated in ADM and relevant to carcinogenesis (18, 21, 41, 42) can also regulate beta-Catenin. As such, it is worth investigating if/how Ras and Notch signaling impinge on Sirt1 activity and vice versa.

The effects on acinar cell differentiation also pointed to a putative effect on the PTF1 complex, its major regulator. Ptf1a is a critical component of PTF1 (43) whose acetylation by pCAF is essential for high digestive enzyme gene expression (33). The present study adds that Sirt1 and Ptf1a proteins co-localize in acinar cell nuclei at the PTF1 binding sites of acinar gene promoters and that Sirt1 can deacetylate Ptf1a. We propose that in the normal pancreas Dbc1 balances Sirt1 activity and acinar cells remain differentiated. Dbc1 does indeed inhibit Sirt1’s activity in the pancreas (35). Ptf1a can undergo cytoplasmic translocation in ADM (44, 45), similar to what we observe for Sirt1, making a deacetylation event of Ptf1a more likely. Of note, Ptf1a might also be affected by Sirt1’s action on pCAF that on its turn can be deacetylated by Sirt1 (46).

Finally, we analyzed Sirt1 in PDAC. We extended the observations of Zhao et al. (11) and showed that Tenovin6 or RNA interference for Sirt1 resulted in loss of PDAC cell viability. The IC50’s for Tenovin6 were in line with what has been reported in other tumor cells (47, 48). In contrast to Zhao et al., we did not detect differences in Sirt1 expression in PDAC compared to normal tissue. However, we found a decrease of Dbc1 in PDAC tissues, including a subset with undetectable expression. Loss of Dbc1 has been reported in breast, colon and lung cancer (12). We propose that variation in Dbc1 is relevant in PDAC as Dbc1 directly determines Sirt1 activity (12, 13, 49). The recent finding that c-myc activates Sirt1, and as such favour tumor growth, by sequestering Dbc1 from
Sirt1 supports this hypothesis (50). We applied an assay for quantifying Sirt1 activity (13) but failed to get Sirt1 specific results for our pancreatic tissue and cell extracts (not shown). In addition, we found that PDAC cell lines respond variably to Tenovin-6, and that this correlates with the levels of Sirt1/Dbc1. Our data therefore suggest that Dbc1 can be a biomarker for those pancreatic tumors that benefit from Sirt1 inhibitory drugs.

We did not investigate Sirt1 target genes in PDAC cells, but preliminary data point out that there is no effect on beta-Catenin (Fig6A). A role for p53 (Fig6A), the best characterised target of Sirt1, was beyond the scope of our study.

In conclusion, this is the first study that examines the role of Sirt1 on differentiation and tumor maintenance in the exocrine pancreas, contributing to our understanding of those mechanisms that could be harnessed in therapeutic control.
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References

Figure legends

**Fig 1: Sirt1 and Dbc1 expression in normal exocrine tissue and ADM. A)** IF detection of Sirt1 and Dbc1 in normal mouse pancreas; Co-localization of Dbc1 with Sirt1 (left panel), Sirt1 with the acinar cell marker Amylase (middle panel), and Sirt1 with the endocrine hormone Insulin (right panel). **B)** Sirt1 with Dbc1 or the acinar marker Carboxypeptidase A (CpA) in Caerulein induced acute pancreatitis (Cae-AP 0-8 days (d)). **C)** IF detection of Sirt1 and CpA in pancreatic duct ligation (PDL, 0-15 days(d)). Pictures in a sequence are taken with the same exposure times. Inset= higher magnification and arrow points to the nuclear to cytoplasmic shuttling.

**Fig 2: Sirt1 nuclear to cytoplasmic shuttling in ADM. A)** IF detection of Sirt1 and the ductal marker Krt19 in normal human pancreas and in the *in vitro* model of ADM. **B)** Same as A, here in mouse tissue and cells. The inset shows the nuclear to cytoplasmic shuttling of Sirt1 in detail. The arrow points at a Krt19 expressing duct in normal pancreas. **C)** IF detection of Sirt1 and Krt19 in acinar cells treated with the nuclear export inhibitor LMB (c,d) or its solvent Methanol (a,b). The arrow points to absence of Sirt1 in the nucleus of cells under control Methanol conditions. **D)** Fold change (FC) in mRNA expression of Krt19 in the conditions of panel C, i.e. cultured acini with LMB or its solvent relative to isolated cells (n=5-6, *P<.05).

**Fig 3: Modulation of Sirt1 expression and activity in ADM. A)** HE stainings of representative tissue sections from caerulein acute pancreatitis (2 days, 8 days and controls) in Pdx1-Cre; Sirt1<sup>ex4lox/lox</sup> mice and Pdx1-Cre only mice. **B)** Fold
Change (FC) in mRNA expression of acinar genes Amy 2, Ela 1, CtrB1, CpA1, Ptf1a and Rbpjl and ductal genes (Krt19 and Krt7) in the same conditions as A comparing Pdx1-Cre; Sirt1\textsuperscript{ex4/pox} mice to Pdx1-Cre only mice (n=5, *P<.05,**P<.01,***P<.001) C) Western Blot for Sirt1 using a total protein extract from 266-6 cells stably infected with a vector expressing human Sirt1 (84% homology with mouse) or a control with empty vector (-). Protein-input was assessed with Vinculin (Vcl) (left panels). Western Blot using fractionated samples of the Sirt1 overexpressing cells to assess Sirt1 and Ptf1a. Vcl and Histone H3 are used as controls for fractionation (right panels). D) FC in mRNA expression of acinar genes in 266-6 cells stably infected with Sirt1 relative to controls (n=3, *P<.05). E) IF detection of Krt19 in acinar cells treated with the Nicotinamide (NA) or its solvent PBS. F) FC in mRNA expression of acinar genes in cultured acini with NA, relative to controls (n=5-6, *P<.05,**P<.005). Dotted lines in B, D and F refer to control levels that equal 1.

**Fig 4: Targets of Sirt1 in acinar cells: beta-Catenin.** A) IF detection of beta-Catenin (Ctnnb) in normal mouse pancreas, and in different stages of ADM cultures (isolated acini, 24h and d5). The white arrow points at nuclear beta-Catenin staining. B) IF detection of beta-Catenin comparing isolated acini, and acini cultured with NA or its solvent PBS. C) Western blot for beta-Catenin (Ctnnb1) and beta-Actin (Actb) in isolated acini or acinar cells cultured for 24h with NA or PBS. D) mRNA expression levels of Axin2 (a direct target of Wnt/beta-Catenin signaling) in normal pancreas, isolated acini and acinar cells cultured with NA or PBS (n=5-12, *P<.05). E) Co-IP of acetyl lysine (AcLys) and
beta-Catenin (Ctnnb) in acinar AR42J-B13 cells. Results are shown for one representative experiment.

**Fig 5: Targets of Sirt1 in acinar cells: Ptf1a.** **A)** IF detection of Sirt1 and Ptf1a in normal mouse pancreas. Both channels are shown separately in the insets. **B)** Sirt1-ChIP analysis in 266-6 acinar cells. Fold enrichment of PTF1 containing promoter areas of acinar genes over controls (dotted line refers to controls that equal 1) (n=3, *P<.05). **C)** Co-IP in HEK293 cells transfected with Flag-pCAF, Flag-Ptf1a, Flag-Sirt1 and/or mutant Flag-Sirt1*, and immunoblotted for acetyl-lysine (AcLys) and Flag. Protein-input was assessed with Vinculin (Vcl).

**Fig 6: Sirt1 expression and function in PDAC.** **A)** Western Blot for Sirt1, beta-Catenin (Ctnnb), acetylated (ac) and total p53 in PDAC cells treated with siRNA for Sirt1 or a scrambled sequence, at different time points after transfection. A representative result is shown for Panc1. **B)** Viable cell number as a function of time and relative to day 0 in Panc1 and Panc10.05 cells transfected with siRNA for Sirt1 (n=6, ***P<.0001 in Panc1 cells, ###P<.0001 in Panc10.05 cells versus the control). **C)** Percentage of viable cells in function of the concentration of Tenovin-6 (plotted on a logarithmic scale), represented for four PDAC cell lines (n=12). **D)** Correlation plot for Sirt1/Dbc1 protein band density measured in Western Blots (not shown) versus IC50 for Tenovin-6 in the four PDAC cell lines used in C. **E)** IHC for Dbc1 in a tissue microarrays of human pancreas. A normal pancreas and two different PDAC tumors are shown. We distinguish epithelium (epit) and mesenchyme (mes). **F)** Dbc1 IHC scoring based on combined intensity
and percentage positive nuclei in the epithelium. Results are displayed for normal pancreas, chronic pancreatitis and PDAC (n=14-77, *P<.05; **P<.001).