Targeting developmental regulators of zebrafish exocrine pancreas as a therapeutic approach in human pancreatic cancer

Nelson S. Yee[1,2,*], Weiqiang Zhou[2,‡,§], Stephen G. Chun[2,‡,¶], I-Chau Liang[2,‡,] and Rosemary K. Yee[3]**

[1]Division of Hematology-Oncology, Department of Medicine, Penn State College of Medicine; Penn State Hershey Cancer Institute; Penn State Milton S. Hershey Medical Center; The Pennsylvania State University, Hershey, PA 17033, USA
[2]Division of Hematology, Oncology, and Blood & Marrow Transplantation, Department of Internal Medicine, Carver College of Medicine; Program of Cancer Signaling and Experimental Therapeutics, Holden Comprehensive Cancer Center; The University of Iowa, Iowa City, IA 52242, USA
[3]Penn State Harrisburg School of Humanities, The Pennsylvania State University, Middletown, PA 17057, USA

*Author for correspondence (nyee@hmc.psu.edu)
‡These authors contributed equally to this project
§Present address: Department of Pathogen Biology, Shenyang Medical College, Shenyang City, Liaoning Province, 110034, People's Republic of China
¶Present address: Department of Radiation Oncology, Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA
**Present address: College of Pharmacy, University of Iowa, Iowa City, IA 52242, USA

Biology Open 1, 295–307
doi: 10.1242/bio.2012539

Summary

Histone deacetylases (HDACs) and RNA polymerase III (POLR3) play vital roles in fundamental cellular processes, and deregulation of these enzymes has been implicated in malignant transformation. Hdacs and Polr3 are required for exocrine pancreatic epithelial proliferation during morphogenesis in zebrafish. We aim to test the hypothesis that Hdacs and Polr3 cooperatively control exocrine pancreatic growth, and combined inhibition of HDACs and POLR3 produces enhanced growth suppression in pancreatic cancer. In zebrafish larvae, combination of a Hdac inhibitor (Trichostatin A) and an inhibitor of Polr3 (ML-60218) synergistically prohibited the expansion of exocrine pancreas. In human pancreatic adenocarcinoma cells, combination of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and ML-60218 produced augmented suppression of colony formation and proliferation, and induction of cell cycle arrest and apoptotic cell death. The enhanced cytotoxicity was associated with supra-additive upregulation of the pro-apoptotic regulator BAX and the cyclin-dependent kinase inhibitor $p21^{CDKN1A}$. tRNAs have been shown to have pro-proliferative and anti-apoptotic roles, and SAHA-stimulated expression of tRNAs was reversed by ML-60218. These findings demonstrate that chemically targeting developmental regulators of exocrine pancreas can be translated into an approach with potential impact on therapeutic response in pancreatic cancer, and suggest that counteracting the pro-malignant side effect of HDAC inhibitors can enhance their anti-tumor activity.

© 2012. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Key words: RNA polymerase III, Histone deacetylases, Zebrafish, Exocrine pancreas, Pancreatic cancer, Suberoylanilide hydroxamic acid, Trichostatin A, Targeted therapy

Introduction

Pancreatic adenocarcinoma, the most common form of cancer in exocrine pancreas, is among the most lethal human malignancies (Siegel et al., 2011). Accumulating evidences indicate that the signaling pathways governing organogenesis are often deregulated in tumors, such as those of the pancreas (Yee and Pack, 2005; Koorstra et al., 2008). Zebrafish has been proven as a powerful model to study genetic regulation of vertebrate development and oncogenesis, and it is expected to facilitate drug discovery for treatment of various human diseases, including pancreatic cancer (Yee, 2010). The established models including wild-type (WT), germ-line mutant and transgenic zebrafish have been utilized to identify the genetic pathways and their interactions that control exocrine pancreatic development and cancer (Yee, 2010; Yee et al., 2011). Genetic or chemical modulation of the developmental regulators of exocrine pancreas, such as histone deacetylases (HDACs) and RNA polymerase III (POLR3), may offer new opportunities for improving therapeutic response in this generally incurable disease.

HDACs and histone acetyltransferases regulate gene transcription by controlling the acetylation status of nucleosomal histones as well as non-histone proteins (Haberland et al., 2009). HDACs play regulatory roles in various cellular processes that are fundamental to embryological development (Haberland et al., 2009; Brunmeir et al., 2009). Hdac1 is required for epithelial proliferation and normal growth of exocrine pancreas during morphogenesis in zebrafish (Noel et al., 2008; Zhou et al., 2011). HDACs play contributory roles in cellular proliferation and survival, as inhibition of HDACs induces cell cycle arrest, differentiation, and death in various cancers including pancreatic adenocarcinoma (Lane and Chabner, 2009). However, loss-of-function in HDACs exerts both stimulatory and inhibitory effects on gene transcription, and some of them may be opposing to their anti-tumor actions (Zupkovitz et al., 2006). Thus, the therapeutic potential of HDAC inhibitors in various malignancies including

that of exocrine pancreas may be enhanced when used in combination with small molecules that counteract their pro-malignant side effects such as an increase in POLR3-mediated transcripts.

POLR3 mediates transcription of non-coding RNAs, which are involved in a variety of cellular functions including protein biosynthesis and gene regulation (White, 2008). Deregulation of the POLR3 transcription complex plays a central role in mediating the actions of tumor suppressors, oncogenes, and transforming viruses (Marshall and White, 2008). In spite of the fundamental role of POLR3 in cellular functions, the zebrafish *polr3b*^{slimjim} mutation, which affects the second largest subunit of Polr3, selectively disrupts development of exocrine pancreas and intestine with impaired transcription of *tRNA* genes (Yee et al., 2005; Yee et al., 2007; Yee, 2010). These findings suggest that inhibition of POLR3 may preferentially perturb cell cycle progression of rapidly proliferating cells in cancers, given that POLR3 transcripts are elevated in malignant cells and over-expression of tRNA has been implicated in malignant transformation (Marshall and White, 2008). The small molecule ML-60218 was developed as a potent and selective inhibitor of Polr3-mediated transcription in eukaryotes (Wu et al., 2003). It will be enticing to test if ML-60218 used in combination with HDAC inhibitors can augment the growth-suppressive effect of HDAC inhibitors in tumors including that of exocrine pancreas, by counteracting their pro-malignant side effect of stimulating POLR3-mediated transcription.

The objective of this study is to test our hypothesis that combined inhibition of HDACs and POLR3 cooperatively suppresses the growth of exocrine pancreas during morphogenesis and in cancer. We present evidence that the HDAC inhibitor, trichostatin A (TSA) that reversibly inhibits classes I and II HDACs (Yoshida et al., 1995; Marks et al., 2001), in combination with ML-60218, synergistically arrested the growth of exocrine pancreas in zebrafish larvae by blocking cell cycle progression and up-regulating expression of the cyclin-dependent kinase (cdk) inhibitors. These effects are recapitulated in human pancreatic adenocarcinoma cells, in which combination of the clinical HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), and ML-60218 produced supra-additive suppression of cellular proliferation and induction of apoptotic cell death. These enhanced cytotoxic effects are associated with ML-60218-augmented SAHA-upregulated expression of BAX and p21^{CDKN1A} as well as ML-60218- repressed SAHA-stimulated expression of tRNAs. Results of this study indicate that chemical targeting of the epigenetic and transcriptional regulators of development in zebrafish exocrine pancreas can be potentially translated into a therapeutic approach in human pancreatic cancer.

Results

Hdacs are required for growth and morphogenesis in zebrafish exocrine pancreas

Our recent study indicates a crucial role of Hdac1 in exocrine pancreatic epithelial proliferation (Zhou et al., 2011). Here, we determined the role of Hdacs in the developing exocrine pancreas by treating WT zebrafish larvae with TSA between 48 and 72 hours post-fertilization (h.p.f.) when the pancreatic epithelia maximally proliferate during this period (Yee et al., 2007). First, TSA at various concentrations was added at 48 h.p.f., and acetylation of histones H3 and H4 was analyzed at 72 h.p.f. At a concentration of 165 nM, TSA induced maximal level of acetylated histone H3 and near-maximal level of acetylated histone H4 (Fig. 1). The effect of TSA on exocrine pancreas was then determined by incubating WT zebrafish larvae with 165 nM TSA for 24 hours. The TSA-treated larvae appeared grossly normal. They developed exocrine pancreas of reduced size, and acinar morphogenesis was disrupted (Fig. 2A). While TSA significantly reduced the number of pancreatic epithelia (4′6-diamidino-2-phenylindole or DAPI containing nuclei) by 34%, the proliferative rate as determined by the proportion of epithelia in S-phase (5-bromo-2′-deoxyuridine or BrdU containing nuclei) was not significantly decreased (Fig. 2B). The effect of TSA on exocrine pancreas was associated with increased levels of acetylated histones H3 and H4 (Fig. 2C). Therefore, Hdacs are required for normal growth and morphogenesis of exocrine pancreas through regulating the acetylation status of histones in zebrafish.

Combination of inhibitors of Hdacs and Polr3 arrests expansion of exocrine pancreas during morphogenesis

Considering the growth requirement of Hdacs (Figs 1, 2) and our previous findings of Polr3 in the proliferation of pancreatic epithelia (Yee et al., 2007), we hypothesize that Hdacs and Polr3 cooperatively regulate the growth of exocrine pancreas. To test this hypothesis, we examined the effects of a combination of TSA

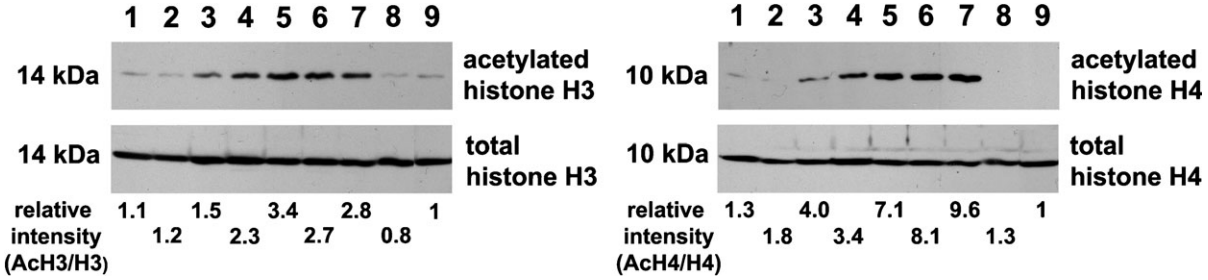


Fig. 1. TSA at 165 nM induces maximal acetylation of histone H3 and near-maximal acetylation of histone H4. Immunoblot analysis of acetylated histones H3 and H4. WT zebrafish larvae at 48 h.p.f. were incubated with TSA at various concentrations, DMSO, or no treatment, for 24 hours. Lane 1 (8.25 nM TSA), 2 (16.5 nM TSA), 3 (41.25 nM TSA), 4 (82.5 nM TSA), 5 (165 nM TSA), 6 (330 nM TSA), 7 (825 nM TSA), 8 (0.5% DMSO), and 9 (no treatment). Total protein was extracted from each group of larvae at 72 h.p.f., and analyzed by immunoblotting using the indicated antibodies. The intensity and area of each protein band was quantified by densitometric analysis. Each value represents the ratio of acetylated histone H3 (AcH3) to total histone H3, and acetylated histone H4 (AcH4) to H4, relative to that of control (no treatment).

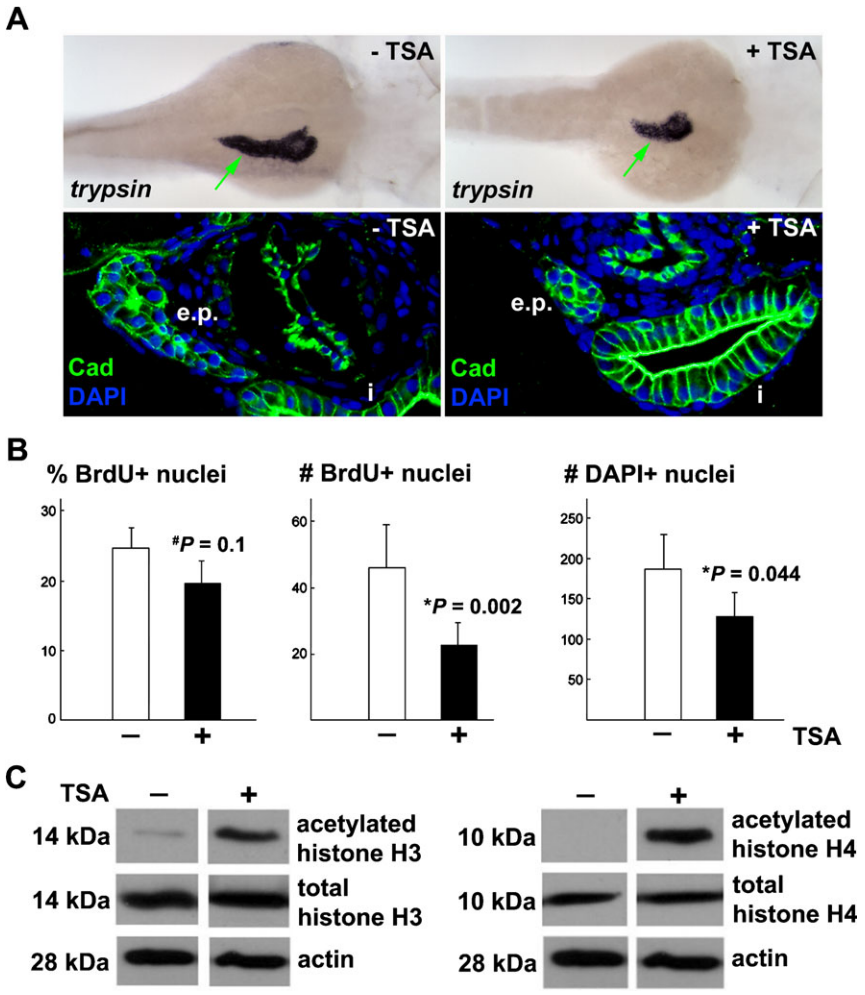


Fig. 2. TSA impairs growth and disrupts morphogenesis of exocrine pancreas in zebrafish larvae with hyperacetylation of nucleosomal histones. WT zebrafish larvae were incubated in the absence or presence of 165 nM TSA (added at 48 h.p.f.) for 24 hours and then analyzed. **(A)** Exocrine pancreas (arrows) was analyzed by in situ hybridization using *trypsin* anti-sense riboprobes. Pancreatic acinar morphology by immunohistochemistry using anti-cadherin (Cad) antibodies, followed by transverse histological sectioning. e.p. exocrine pancreas; i, intestine. **(B)** The larvae were pulse-labeled with BrdU and analyzed by immunohistochemistry using anti-BrdU antibodies, followed by transverse histological sectioning. The number (#) of DAPI+ nuclei, the number (#) of BrdU+ nuclei, and the proportion of cells in S phase (% BrdU+ nuclei) were determined. Result is presented as the mean + s.d., and * indicates statistical significance, # trend of statistical significance. **(C)** Total protein was extracted and analyzed for acetylated and total histones H3 and H4 by immunoblotting. Anti-total histones H3 and H4 antibodies and anti-actin antibodies were used as internal controls.

and the Polr3 inhibitor ML-60218 on the growth of exocrine pancreas during morphogenesis. First, we determined the optimal concentrations of TSA or ML-60218 by incubating WT zebrafish larvae at various concentrations of each small molecule and observing for signs of toxicity and lethality. The zebrafish larvae incubated in the medium containing TSA at a maximal concentration of 330 nM appeared grossly normal. The larvae treated with ML-60218 at a concentration of 110 µM or below appeared normal without precipitation of ML-60218 (supplementary material Table S1). Thus, TSA at 165 nM and ML-60218 at 110 µM were used for the subsequent experiments.

Addition of TSA and ML-60218 at 48 h.p.f. to WT zebrafish larvae for 24 hours completely arrested expansion of the exocrine pancreas, such that the size of the exocrine pancreas remained the same as that in untreated WT larvae at 48 h.p.f. (Fig. 3A; Yee et al., 2005). Neither TSA nor ML-60218 at the concentrations being used alone caused any apparent reduction of organ size. No apparent effect was observed by treatment with either TSA + dimethyl sulfoxide (DMSO, the solvent used to dissolve TSA and ML-60218) or DMSO alone (N. S. Yee, unpublished). The same effects of these small molecules were observed in WT zebrafish larvae of various strains including AB, TLF, and WIK (N. S. Yee, unpublished).

To determine the cellular basis of the growth-suppressive effect of the combination of inhibitors on exocrine pancreas, the rate of epithelial proliferation was assessed. The combination of TSA and ML-60218 caused a significant reduction in the proportion of S-phase (BrdU+) nuclei by 23% of that in control (Fig. 3B). Neither TSA nor ML-60218 significantly reduced the proportion of BrdU+ nuclei. Moreover, TSA + ML-60218 supra-additively reduced the number of DAPI positive nuclei by 48% ($P<0.01$). Using morphometric analysis, the combination of TSA + ML-60218 significantly reduced cell growth by 22%, whereas neither TSA nor ML-60218 alone caused any significant impairment of cell growth (Fig. 3B). No increase in apoptotic cell death was observed in the exocrine pancreas of the larvae treated with TSA + ML-60218 using Apoptag® (N. S. Yee, unpublished). These results indicate that the combination of TSA and ML-60218 produces enhanced suppression of exocrine pancreatic growth by inhibiting epithelial proliferation with impaired progression from G_1 to S phase of the cell cycle and reduced cell growth.

To explore the mechanism by which the combination of TSA and ML-60218 perturbed cellular proliferation, the acetylation status of histones H3 and H4 and expression of $p21^{cdkn1a}$ and $p27^{cdkn1b}$ as molecular markers of progression of cell cycle from G_1 to S phase were assessed by immunoblotting and real-time polymerase chain reaction (PCR), respectively. TSA caused a slight accumulation of acetylated histones H3 and H4, and combination with ML-60218 enhanced this effect by 2-fold and

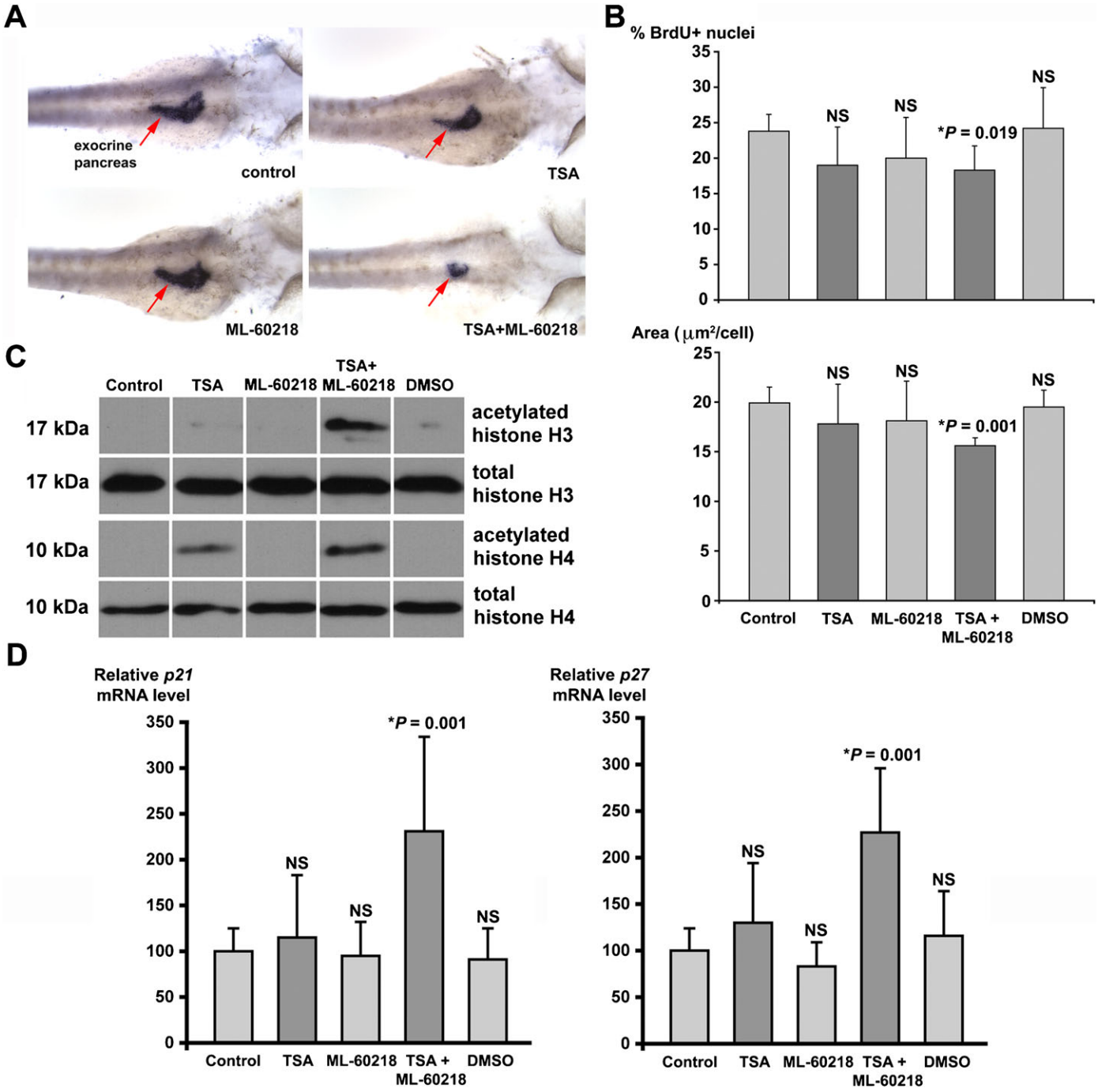


Fig. 3. TSA and ML-60218 synergistically inhibit expansion of exocrine pancreas in zebrafish by impeding cell cycle progression, with enhanced induction of histone acetylation and cyclin-dependent kinase inhibitors. Starting at 48 h.p.f., WT larvae were incubated in the presence of TSA, ML-60218, TSA + ML-60218, or control (DMSO or untreated) for 24 hours and then analyzed. **(A)** Dorsal view of larvae with the exocrine pancreas (arrows) analyzed by whole mount in situ hybridization using anti-sense *trypsin* riboprobes. **(B)** Exocrine pancreatic epithelial cells in the S phase (upper panel) and morphometric analysis of cell growth (lower panel). The larvae were pulse-labeled with BrdU, processed for immunohistochemistry with anti-BrdU or anti-cadherin antibodies, followed by histological analysis. Each column indicates the mean proportion of BrdU+ nuclei or cell growth (area in μm^2 per cell) in the exocrine pancreatic epithelia. **(C)** Total protein was extracted from the larvae and analyzed by immunoblotting using anti-acetylated histones H3 and H4 antibodies, and anti-total histones H3 and H4 antibodies. **(D)** Total RNA was extracted from the larvae and quantified for $p21^{cdkn1a}$ and $p27^{cdkn1b}$ mRNA using real-time PCR. Each column represents the mean $p21^{cdkn1a}$ or $p27^{cdkn1b}$ mRNA level normalized to *gapdh* mRNA and expressed as percentage of control from three independent experiments, with each real-time PCR conducted in triplicate samples. Bars represent s.e.m.; $*P<0.05$ considered statistically significant; NS, not statistically significant.

70%, respectively (Fig. 3C). Consistent with the effects on acetylated histones, the combination of TSA and ML-60218 significantly up-regulated the levels of both $p21^{cdkn1a}$ and $p27^{cdkn1b}$ mRNA by about 1.3-fold over control, and either TSA or ML-60218 alone caused relatively little changes (Fig. 3D). No significant alteration of total tRNA and 5s rRNA levels was detected among the experimental groups and controls as revealed by electrophoretic separation of total RNA extracted

from whole larvae in each group (N. S. Yee, unpublished). Taken together, the combination of TSA and ML-60218 causes growth arrest of exocrine pancreas by suppressing pancreatic epithelial proliferation that is associated with enhanced accumulation of acetylated histones and up-regulated expression of *p21*^{cdkn1a} and *p27*^{cdkn1b}.

Combination of SAHA and ML-60218 produces enhanced suppression of proliferation in human pancreatic adenocarcinoma by impairing cell cycle progression and inducing apoptosis

To translate the findings of the zebrafish studies, we examined the effects of the clinically used HDAC inhibitor SAHA in combination with ML-60218 in the human pancreatic adenocarcinoma cell lines PANC-1 and BxPC-3. These cells have been shown to carry mutations in *K-RAS* and *p53* (Berrozpe et al., 1994; Moore et al., 2001), and they are resistant to the cytotoxic effects of the chemotherapeutic agent gemcitabine at clinically used concentrations (1 μM and 10 μM) and even up to 50 μM (supplementary material Table S2A). SAHA is a synthetic inhibitor of classes I and II HDACs. It was used at 5 μM in this study based on the dose–response in cellular proliferation of PANC-1 and BxPC-3 (Chun et al., 2009). ML-60218 was used at 100 μM, which is approximately the same concentration used in the experiments with zebrafish (Fig. 3). In a soft agar assay that mimics in vivo conditions of tumor growth, the combination of SAHA and ML-60218 produced enhanced reduction of anchorage-independent colony formation (Fig. 4A). Neither SAHA nor ML-60218 alone significantly inhibited PANC-1 colony formation; but when combined, they reduced PANC-1 colony formation by 47%. In BxPC-3, SAHA reduced colony formation by 35%, ML-60218 had no effect, and SAHA + ML-60218 reduced colony formation by 70%, which was significantly different compared to SAHA alone. DMSO did not significantly affect colony formation either alone or in combination with SAHA (N. S. Yee, unpublished).

To gain insights into the effects on colony formation, cellular morphology was analyzed by phase contrast microscopy (Fig. 4B). In both PANC-1 and BxPC-3, the combination of SAHA and ML-60218 produced morphologic changes including cell enlargement and flattening, irregular shapes with cytoplasmic projections, accumulation of cytoplasmic vacuoles, and nuclear fragmentation. These morphologic changes suggest that the enhanced suppression of colony formation might involve induction of cytodifferentiation, apoptotic cell death, and senescence-like cytostasis.

To investigate the cellular mechanisms underlying the suppression of colony formation, the effects of SAHA and ML-60218 on cellular proliferation were analyzed using the MTS assay. The combination of SAHA and ML-60218 produced enhanced inhibition of cellular proliferation (Fig. 5A). In PANC-1, neither SAHA nor ML-60218 had any effect, but the combination significantly reduced proliferation by 23%. In BxPC-3, SAHA reduced proliferation by 49%, ML-60218 had no effect, and the combination of SAHA and ML-60218 reduced proliferation by 53%. To determine the cytotoxicity of SAHA and ML60218 in normal pancreatic epithelia, the immortalized human pancreatic ductal epithelia H6c7 were treated with the inhibitors and analyzed for proliferation. Similar to their effects on PANC-1 and BxPC-3, the combination of SAHA and ML-60218 produced enhanced cytotoxicity in H6c7 cells (supplementary material Table S2B). However, the proliferation-suppressing effect of the combination of SAHA and ML-60218 in H6c7 cells (reduced by 54%) (supplementary material Table S2B) is less than that of gemcitabine at 1 μM and 10 μM (reduced by 64% and 82%, respectively) (supplementary material Table S2A).

Next, the effect on cell cycle progression was analyzed by flow cytometric determination of DNA content (Fig. 5B). In PANC-1 and BxPC-3, SAHA increased the proportion of cells in G_0/G_1 (28%), and decreased the proportion of cells in the S phase (28% and 24%, respectively). ML-60218 alone produced no or little effect. The combination of SAHA and ML-60218 caused a further accumulation of cells in G_0/G_1 (additional 3% and 5% in PANC-1 and BxPC-3, respectively) as compared to SAHA alone. The effect on cell death was then determined by flow cytometric detection of Annexin-V binding. Compared to control PANC-1, SAHA caused 13% more cells to undergo apoptosis, ML-60218 had no effect, and SAHA + ML-60218 induced apoptosis in an additional 21% of cells (Fig. 5C). In H6c7 cells, the combination of SAHA and ML-60218 produced a smaller proportion of apoptotic cells than gemcitabine (3-fold and 4-fold, respectively) relative to control (supplementary material Table S2C). Moreover, the combination of SAHA and ML-60218 produced fewer dead cells than gemcitabine (no increase and 4-fold, respectively) as compared with no treatment (supplementary material Table S2C).

To further explore the mechanisms by which SAHA and ML-60218 induced apoptosis, expression of the anti-apoptotic protein survivin was evaluated by confocal microscopy. In the untreated or DMSO-treated BxPC-3 cells, survivin was present diffusely throughout the cells. Either SAHA or the combination of SAHA and ML-60218 caused survivin to be expressed almost exclusively in the nuclei (Fig. 5D), and this effect presumably abolishes the anti-apoptotic action of survivin by accelerating its degradation in the nucleus (Connell et al., 2008a,b).

These results show that SAHA induces cytotoxicity in pancreatic adenocarcinoma by causing cell cycle arrest and apoptotic cell death, and these effects are enhanced by ML-60218. The combination of SAHA and ML-60218 also produces enhanced cytotoxicity in the pancreatic ductal epithelia, but the extent of of cytotoxicity is less than that produced by gemcitabine.

SAHA and ML-60218 cooperatively up-regulate expression of BAX and p21^{CDKN1A} protein

In an attempt to gain insights into the mechanism underlying the enhanced actions of the combination of SAHA and ML-60218, we analyzed histone acetylation by immunoblotting (Fig. 6A). SAHA alone increased the levels of acetylated histones H3 and H4 in PANC-1 by 40% and 110%, respectively. In BxPC-3, SAHA increased acetylated histones H3 and H4 by 20% and 4.3-fold, respectively. The combination of SAHA and ML-60218 produced similar effects as SAHA alone in both cell lines. The increased level of acetylated histones is consistent with the established action of SAHA and expected to modulate gene expression.

To further characterize the growth-suppressive actions of SAHA and ML-60218, the level of the pro-apoptotic regulator BAX was analyzed by immunoblotting (Fig. 6B). Consistent with the results of the flow cytometric detection of apoptosis, the combination of SAHA and ML-60218 produced enhanced expression of BAX by 2.1-fold and 6.2-fold over control in PANC-1 and BxPC-3, respectively. Either SAHA or ML-60218

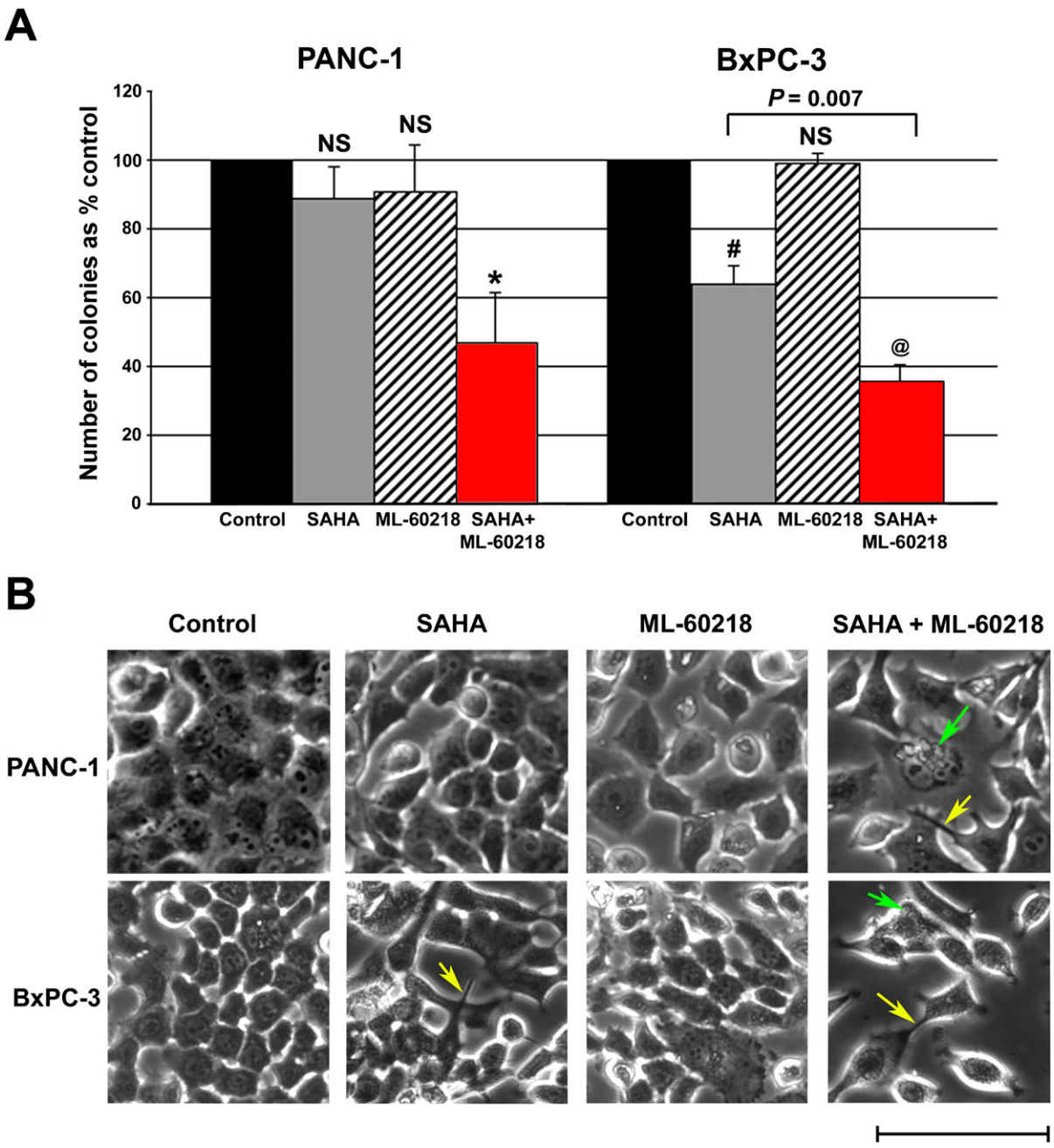


Fig. 4. SAHA and ML-60218 inhibit anchorage-independent colony formation and induce cellular morphology consistent with cellular senescence and cell death. (A) PANC-1 and BxPC-3 were treated with 5 μM SAHA, 100 μM ML-60218, 5 μM SAHA + 100 μM ML-60218, or untreated (control), and grown in soft agar for 14 days. Each column represents the mean number of colonies in each treatment group from 3 independent experiments, with each treatment group in triplicate; bars represent s.e.m. Statistical analysis was performed using Student's *t*-test to compare between each treatment and control except where indicated. *, $P<0.05$; $^{\#}$, $P<0.005$; $^{@}$, $P<0.0001$. NS, not statistically significant. **(B)** Bright field images of the cells treated as described in (A) for 24 hours were captured under a phase contrast microscope. Green arrows are pointing at nuclear membrane blebbing; yellow arrows, cytoplasmic projections. Scale bar, 100 μm.

alone produced relatively little or no increase in the level of BAX. Expression of the cyclin-dependent kinase inhibitor $p21^{CDKN1A}$ was then examined. In PANC-1 and BxPC-3, SAHA caused elevation of the $p21^{CDKN1A}$ protein levels by 60% and 80%, respectively, and these were further increased by 1.6-fold and 5-fold over control, respectively, when SAHA was combined with ML-60218.

These results indicate that the combination of SAHA and ML-60218 suppresses cellular proliferation by impairing cell cycle progression from G_1 to S phase with up-regulation of $p21^{CDKN1A}$ and by inducing apoptosis through the BAX-mediated mitochondrial pathway with nuclear localization of survivin.

ML-60218 reverses SAHA-stimulated tRNA expression

We attempted to understand the mechanism that mediates the enhanced cytotoxicty of the combination of SAHA and ML-60218 by examining their effects on tRNA levels. It has been shown that histone acetylation influences chromatin structure and

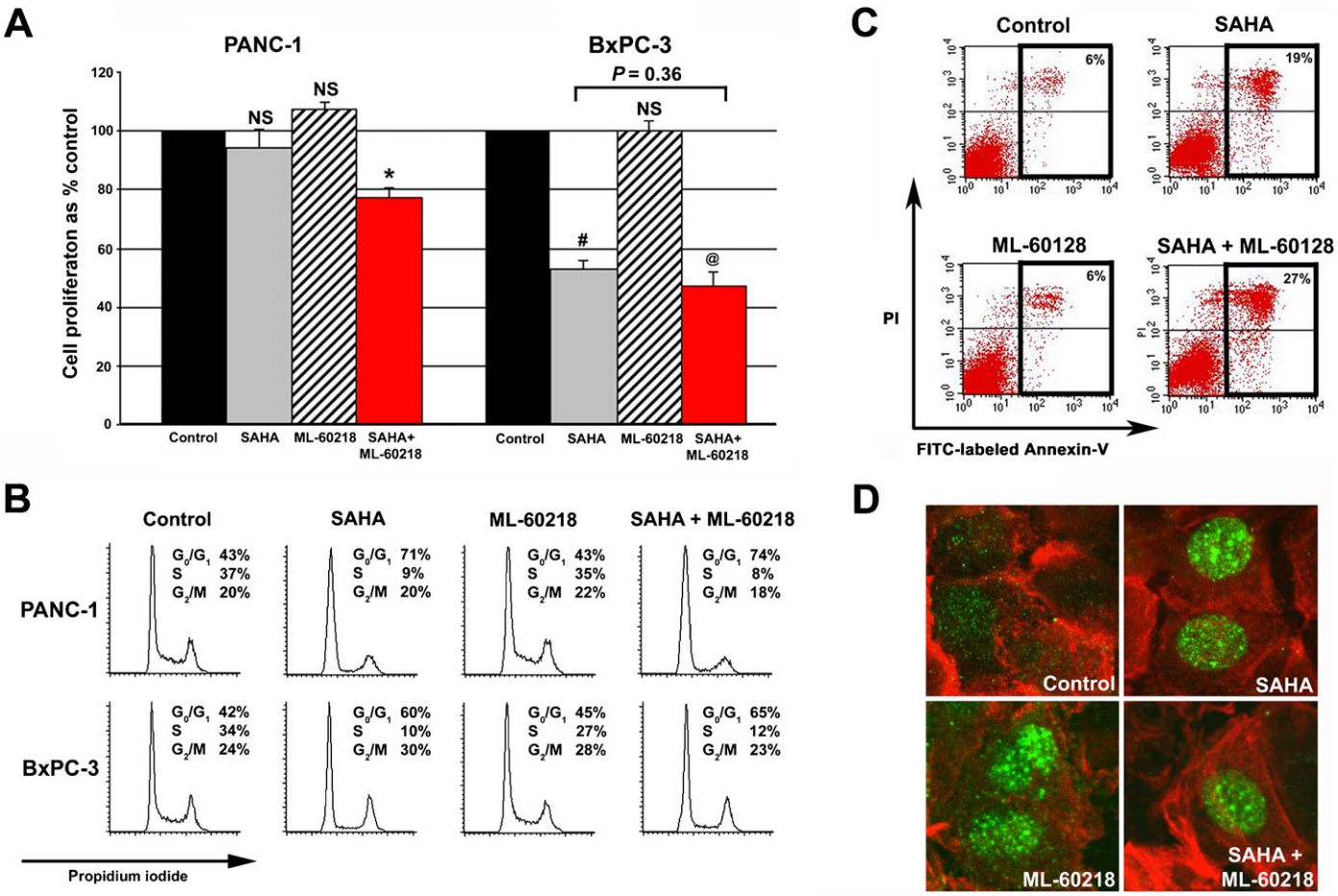


Fig. 5. Combination of SAHA and ML-60218 produces augmented suppression of cellular proliferation with impaired cell cycle progression, enhanced apoptotic cell death; SAHA either alone or in combination with ML-60218 induces nuclear localization of survivin. PANC-1 and BxPC-3 were treated with 5 μM SAHA, 100 μM ML-60218, 5 μM SAHA + 100 μM ML-60218, or untreated (control) for 48 hours and analyzed as follows. **(A)** Cellular proliferation by MTS assay. Each column represents the mean absorbance of three independent experiments, with each treatment in triplicate. Statistical analysis was performed using Student's *t*-test to compare between each treatment and control except where indicated. Bars represent s.e.m.; *, $P<0.005$; $^{\#}$, $P<0.005$; $^{@}$, $P<0.0005$. NS, not statistically significant. **(B)** Flow cytometric analysis of cell cycle. The data shown are representative of three independent experiments with similar results. Non-specific sub-G_0/G_1 events were gated out for accurate analysis of cell cycle distribution curves in the viable cells. **(C)** Flow cytometric analysis of apoptosis in PANC-1. The proportion of apoptotic cells is indicated within the black border. The data shown are representative of three experiments with similar results. PI, propidium iodide. **(D)** Confocal microscopic analysis of survivin expression in BxPC-3 following immunocytochemistry. Survivin (green); cytoplasmic actin (red).

organization that play a role in protein–DNA interactions and regulate expression of POLR3-transcribed genes (Lee et al., 1993; Boyd et al., 2000; Noma et al., 2006). The activity of POLR3 transcription complex is up-regulated in most of the examined pancreatic adenocarcinoma cell lines (Table 1), and over-expression of tRNA has been implicated in malignant transformation (Marshall and White, 2008). We hypothesize that inhibition of HDACs by SAHA leads to up-regulated expression of POLR3-mediated transcripts including tRNAs, which in turn may antagonize its anti-tumor activity. To test this hypothesis, we quantified tRNAs in the pancreatic cancer cells treated with SAHA alone or in combination with ML-60218 using real-time PCR. SAHA significantly increased the levels of $tRNA^{Met}$ in both PANC-1 and BxPC-3 by 71% and 85%, respectively, and increased $tRNA^{Ser}$ in PANC-1 and BxPC-3 by 2.2-fold and 1.9-fold, respectively (Fig. 7A). In control cells, ML-60218 alone had slight effect on the levels of $tRNA^{Met}$ and $tRNA^{Ser}$. When added to SAHA-treated cells, ML-60218 significantly reduced SAHA-stimulated expression of $tRNA^{Met}$ and $tRNA^{Ser}$ by 55% and 64%, respectively in PANC-1, and by 43% and 77%, respectively, in BxPC-3.

Therefore, SAHA inhibits HDACs and increases acetylation of histones H3 and H4, resulting in up-regulated expression of the pro-apoptotic regulator (BAX), the CDK inhibitor ($p21^{CDKN1A}$), and tRNAs ($tRNA^{Met}$ and $tRNA^{Ser}$). At a concentration that it does not significantly affect tRNA level, ML-60218 reverses SAHA-induced up-regulation of tRNAs, and this may contribute to the enhanced cell cycle arrest and apoptosis in pancreatic adenocarcinoma by the combination of SAHA and ML-60218, as illustrated in Fig. 7B.

Discussion

HDACs and POLR3 play critical roles in the signaling mechanisms that control embryonic and neoplastic development. It was unclear if HDACs and POLR3 coordinately regulate growth of exocrine pancreas. Whether POLR3 could be utilized as a therapeutic target or to enhance the anti-tumor activities of HDAC inhibitors was unexplored. In this

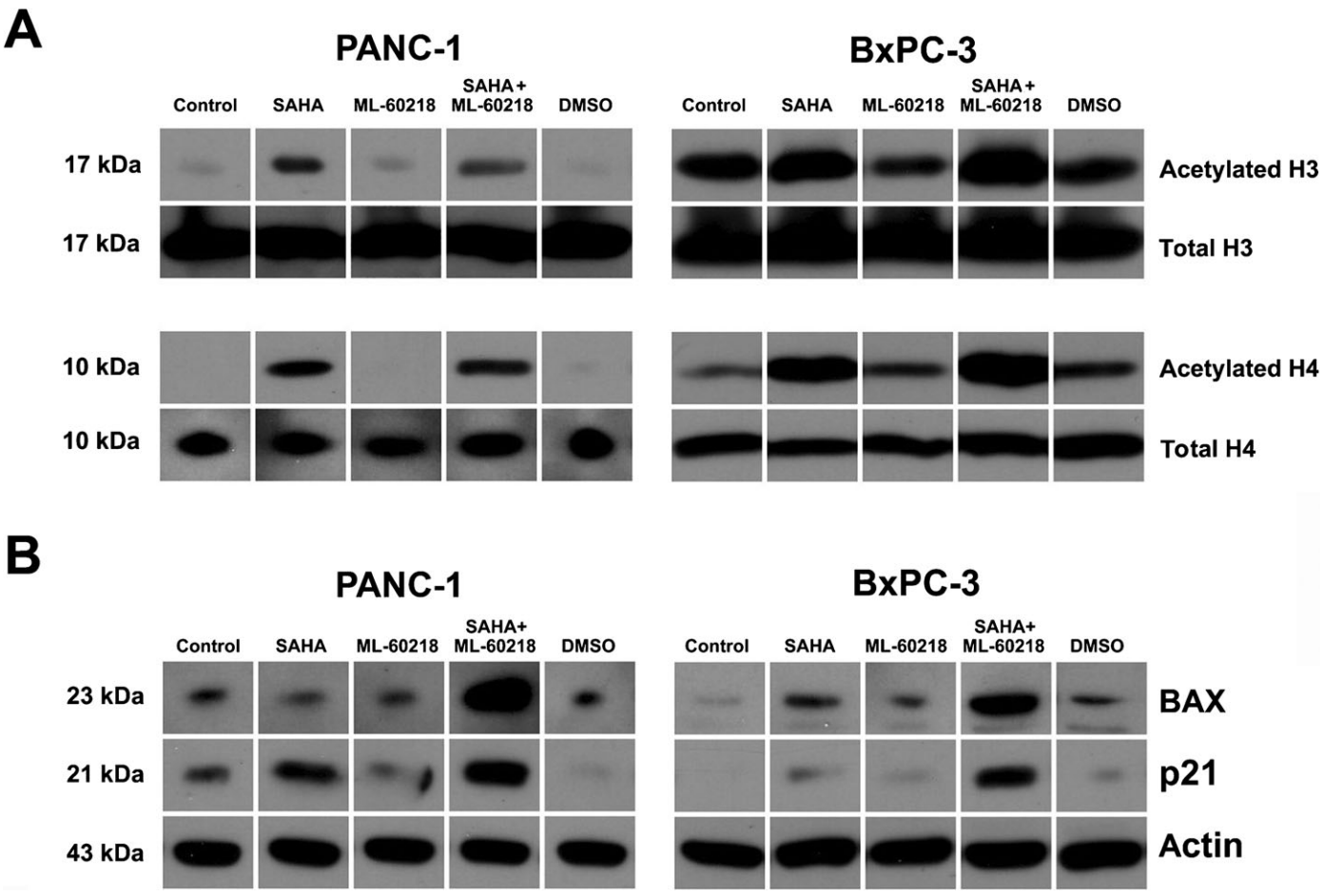
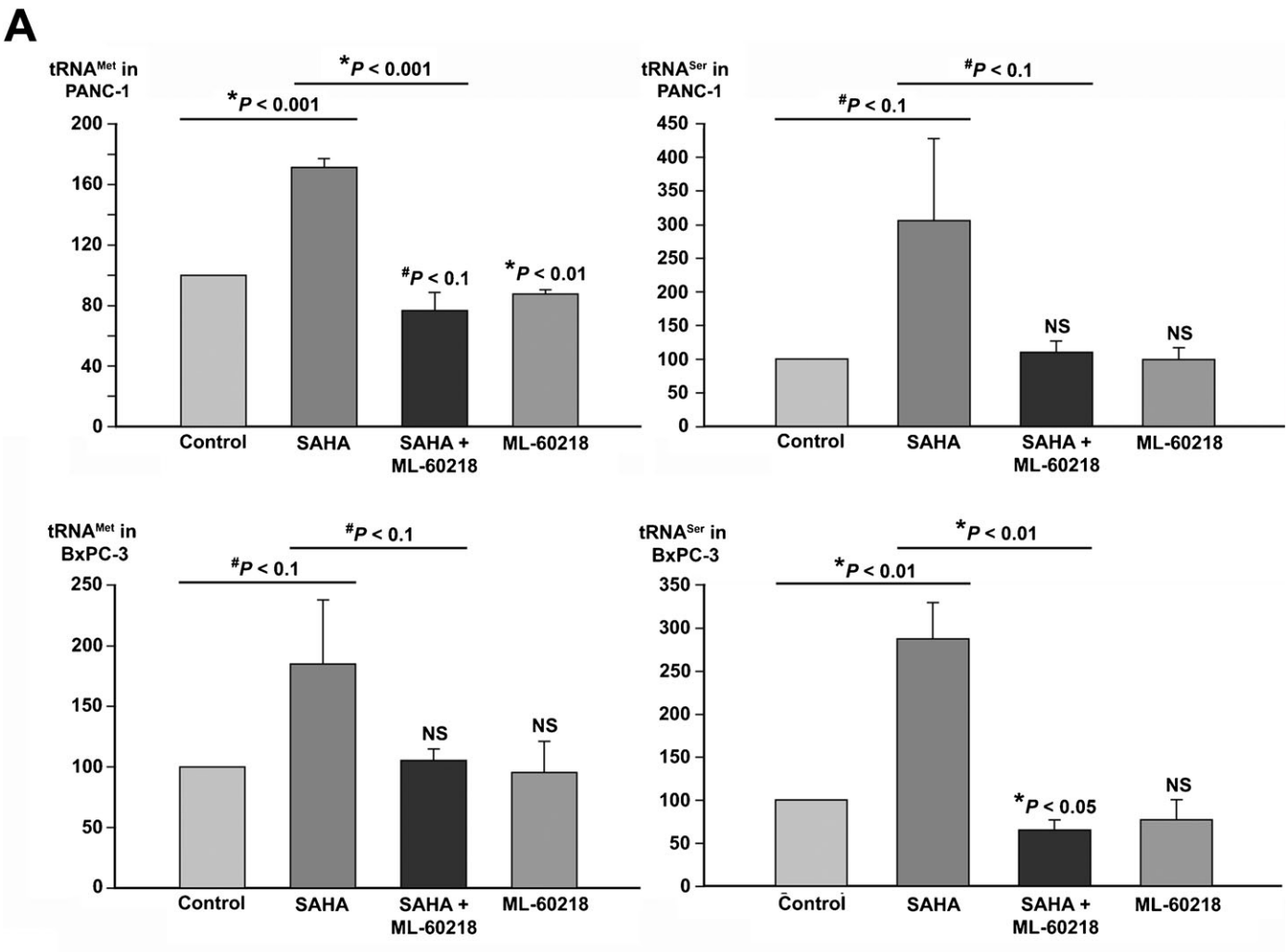


Fig. 6. Effect of SAHA and ML-60218 on histone acetylation, BAX, and p21^{CDKN1A} expression. PANC-1 and BxPC-3 cells were incubated for 48 hours with 5 μM SAHA, 100 μM ML-60218, 5 μM SAHA + 100 μM ML-60218, 0.5% DMSO, or untreated (control) and analyzed by immunoblotting. **(A)** Expression of acetylated histones H3 and H4 was assessed using anti-acetylated histones H3 and H4 antibodies. Anti-total histones H3 and H4 antibodies were used as control. **(B)** Expression of BAX and p21^{CDKN1A} protein was assessed using anti-BAX and anti-p21^{CDKN1A} antibodies. Anti-actin antibodies were used to indicate equal amount of protein loaded. The data shown are representative of three independent experiments with similar results.

study, we show that the combination of HDAC inhibitors and the POLR3 inhibitor ML-60218 produces supra-additive suppression of epithelial proliferation with cell cycle arrest in the developing exocrine pancreas of zebrafish as well as in the gemcitabine-resistant human pancreatic adenocarcinoma cell lines. Our data provide a proof of principle for enhancement of the anti-tumor activity of HDAC inhibitors potentially by counteracting their "pro-oncogenic" side effects, as ML-60218 represses SAHA-induced up-regulated expression of tRNAs. The precise mechanisms underlying the enhanced growth-suppressive activity of either SAHA or TSA in combination with ML-60218 remain to be determined. Results of this study demonstrate direct translation of the zebrafish studies into a therapeutic approach in human pancreatic adenocarcinoma by combinatorial

Table 1. Relative levels of tRNA^{Met} and tRNA^{Ser} in human pancreatic adenocarcinoma cell lines. Quantification of tRNA^{Met} and tRNA^{Ser} in pancreatic adenocarcinoma cell lines and the pancreatic ductal epithelia H6c7 by real-time PCR. Each value represents the mean relative tRNA level ± s.e.m. in the cell line as compared with that in the H6c7 cells. This experiment was repeated 3 times. Each sample of real-time PCR was performed in triplicates. Note that POLR3-mediated transcription has been shown to be cell cycle-dependent (White et al., 1995; Scott et al., 2001), and each tRNA level represents the outcome of an asynchronous population of cells. *P<0.05, statistically significant. $^{\#}P$<0.1, trend of statistical significance. NS, not statistically significant.

	BxPC-3	Capan-1	HPAF-II	MIA PaCa-2	Panc 02.03	PANC-1	PL45	H6c7
tRNA^{Met}	2.13	6.16	4.34	1.02	4.92	3.06	1.69	1.00
	±1.00	±0.66	±3.63	±0.48	±0.35	±0.32	±0.86	
	$^{\#}P$<0.1	*P<0.001	NS	NS	*P<0.001	*P<0.001	NS	
tRNA^{Ser}	1.81	1.87	0.64	1.44	0.88	1.47	1.15	1.00
	±0.50	±0.34	±0.31	±0.80	±0.31	±0.13	±0.86	
	$^{\#}P$<0.1	*P<0.05	NS	NS	NS	*P<0.01	NS	



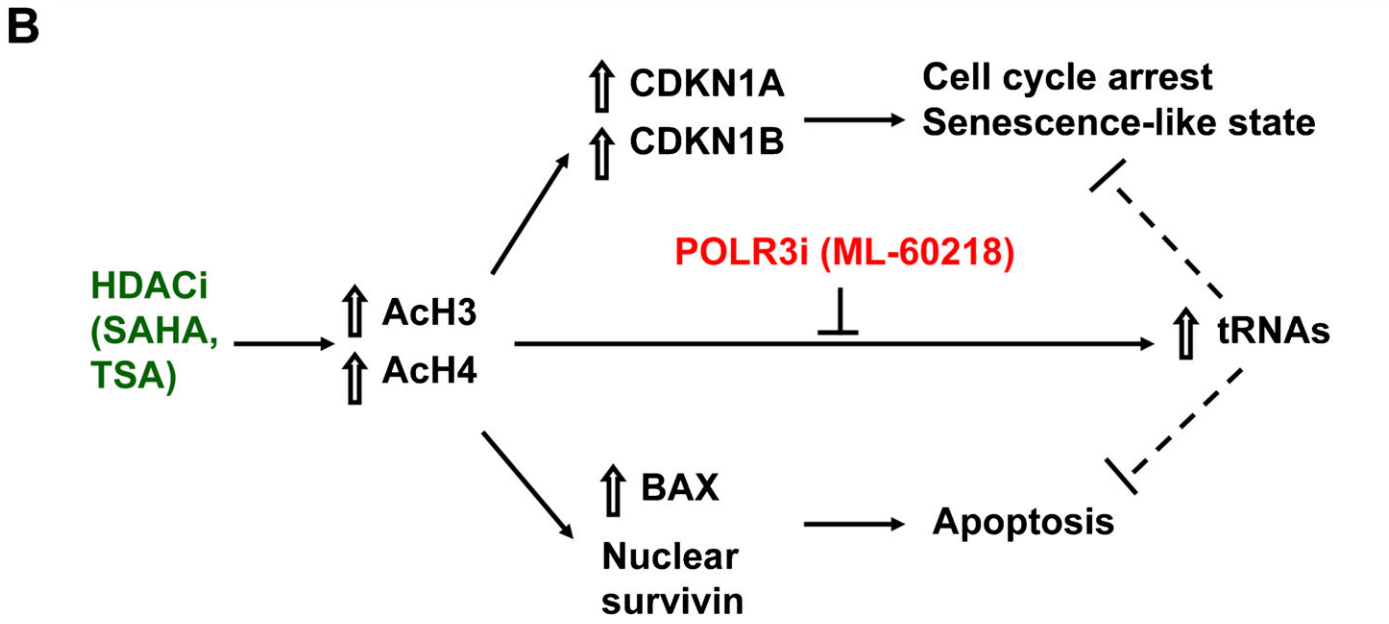


Fig. 7. ML-60218-mediated suppression of SAHA-induced up-regulation of tRNA as a potential mechanism that contributes to the enhanced anti-cancer actions in pancreatic adenocarcinoma. (A) The $tRNA^{Met}$ and $tRNA^{Ser}$ levels in PANC-1 and BxPC-3 cells treated for 48 hours as indicated are represented as % of control (untreated or DMSO). Each value is the mean + s.e.m. of three independent experiments, triplicate samples for each PCR, each PCR performed twice, and expressed as % relative to controls. Statistical analysis was performed comparing each experimental group with control, except where indicated. $^{*}P<0.05$, statistically significant. $^{\#}P<0.1$, trend of statistical significance. NS, not statistically significant. **(B)** A working model that summarizes the enhanced cytotoxic actions of the combination of ML-60218 and either TSA or SAHA. i, inhibitor; AcH3, acetylated histone H3; AcH4, acetylated histone H4.

targeting of an epigenetic regulator and a transcriptional mediator of exocrine pancreatic development.

Mechanisms underlying enhanced cytotoxicity of the combination of inhibitors of HDACs and POLR3 in exocrine pancreas

HDAC inhibitors exert their anti-tumor activities by inducing apoptosis, cell cycle arrest, differentiation, senescence, as well as by modulating immune responses and altering angiogenesis (Frew et al., 2009). Our data indicate that ML-60218 enhances the ability of HDAC inhibitors to induce apoptosis and cell cycle arrest. These cellular effects correlate with the ability of ML-60218 to augment SAHA-induced up-regulated expression of the pro-apoptotic protein BAX, and the CDK inhibitor $p21^{CDKN1A}$. The mechanisms that underlie the enhanced growth-suppressive action of HDAC inhibitors and ML-60218 may at least partially involve reversal of HDAC inhibitors-stimulated, up-regulated expression of tRNA as illustrated in the working model (Fig. 7B). Of note, ML-60218 was used at a relatively non-toxic concentration, such that it did not produce any gross abnormality in the zebrafish larvae, and it had no appreciable effect on tRNA levels in the pancreatic cancer cells.

Inhibition of HDACs is expected to stimulate transcription of a variety of genes including those encoding tRNAs, as shown in this study. In agreement with these findings, HDAC1 has been shown to repress POLR3-mediated transcripts such as 5S rRNA (Lee et al., 1993). Moreover, transcriptional profiling of zebrafish larvae with germ-line loss-of-function mutation in *hdac1* at 48 h.p.f. reveals increased mRNA levels of *tRNA synthetases* and *polr3* subunits (Zhou et al., 2011). We have previously shown that the zebrafish loss-of-function mutation, $polr3b^{slimjim}$, caused impaired activity of Polr3 and reduced levels of tRNAs, and led to diminished epithelial proliferation in exocrine pancreas (Yee et al., 2005; Yee et al., 2007; Yee, 2010). This is in agreement with the established role of POLR3-mediated transcripts such as tRNAs in protein translation required for fundamental processes including cell division and growth (White, 2008). Consistent with the fact that cancer cells exhibit uncontrolled proliferation, the POLR3 transcriptional complex level and/or activity are increased in various human cancers (White, 2008) and pancreatic adenocarcinoma cells (this study). We hypothesize that ML-60218-enhanced cytotoxicity of SAHA or TSA occurs at least in part by counteracting the HDAC inhibitors-induced up-regulation of POLR3 activity resulting from histone hyperacetylation. Our finding that ML-60218 abolishes SAHA-stimulated expression of Polr3-mediated transcripts including $tRNA^{Met}$ and $tRNA^{Ser}$ provides support for this mechanism. Our hypothesis is further supported by recent reports that over-expression of tRNA can lead to malignant transformation (Marshall et al., 2008; Johnson et al., 2008) and suppression of apoptosis (Mei et al., 2010). Whether other Polr3-transcribed RNAs, such as 5S rRNA, 7SL RNA and microRNAs, are involved in the enhanced cytotoxicity of ML-60128 and HDAC inhibitors remains to be determined. In future studies, we aim to test this hypothesis by directly modulating tRNAs and other POLR3-transcribed RNAs and determining the effect on SAHA-induced cytotoxicity and its enhancement by ML-60218.

Overlapping mechanisms that regulate HDACs and POLR3 may also contribute to the enhanced growth-suppressive effects of the combination of inhibitors of HDACs and POLR3. Several lines of evidence indicate that both HDAC1 and the POLR3 transcription complex share common regulatory pathways that involve retinoblastoma (RB) and p53 (Robertson et al., 2000; Rayman et al., 2002; Lagger et al., 2003; White et al., 1996; Cairns and White, 1998). Both RB and p53 exert their inhibitory actions through specific interaction with POLR3-specific transcription factor IIIB (TFIIIB) that is required for recruiting TFIIIC and transcribing *tRNA*s (Larminie et al., 1997; Chu et al., 1997; Sutcliffe et al., 1999; Crighton et al., 2003; reviewed by Marshall and White, 2008). Moreover, the complex containing HDAC1, DNMT1, and RB has been shown to interact with TFIIIB (Robertson et al., 2000). We hypothesize that functional interactions between HDACs and the POLR3-containing transcription complex coordinately regulate cellular proliferation and survival. Further investigation of the molecular nature of such interactions is expected to help elucidate the underlying mechanisms underlying the enhanced cytotoxicity of the inhibitors of HDACs and POLR3. In addition, modulation of the established pathways of proliferation and survival such as RAS/MEK/ERK and PI3K/AKT signaling as well as functional analysis of the transcriptomic and proteomic profiles of the cells treated with SAHA, ML-60218, or the combination of SAHA and ML-60218, will help understand the mechanisms underlying their enhanced cytotoxic actions.

Conclusion and Prospective

We present evidence supporting the use of zebrafish as a translational platform for therapy in human pancreatic cancer by chemically targeting developmental regulators of exocrine pancreas. Our results demonstrate that the combination of small molecule inhibitors of HDACs and POLR3 produces synergistic suppression of exocrine pancreatic growth in the developing zebrafish and enhanced impairment of proliferation in human pancreatic adenocarcinoma cells. The augmented anti-proliferative and pro-apoptotic effects may be partially attributed to ML-60218 reversal of SAHA-induced increase in tRNA levels, though the precise mechanisms remain to be determined. SAHA is well-tolerated clinically, yet the safety of using ML-60218 alone and in combination with SAHA in humans is unknown. Ongoing studies using the zebrafish and mouse models of pancreatic adenocarcinoma aim to determine the in vivo efficacy and potential toxicity of the combination of SAHA and ML-60218 and generate insights into the mechanisms for overcoming therapeutic resistance by modulating HDACs and RNA metabolism. However, our studies provide further support for utility of zebrafish as a biological system to identify the pathways and mechanisms that regulate the growth of exocrine pancreas with the goal of improving treatment response in patients with pancreatic adenocarcinoma and producing a beneficial impact on their survival.

Materials and Methods

Zebrafish stock

WT zebrafish of AB, TLF, or WIK strains were obtained from the Zebrafish International Resource Center (Portland, Oregon, USA). Zebrafish husbandry was conducted as described (Westerfield, 2007). To facilitate visualization of exocrine pancreas in the larvae, 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich ®, St. Louis, Missouri, U.S.A.) was added to embryo medium (E3) at 0.003% to inhibit skin pigmentation as indicated.

Small molecules and drugs

TSA (Upstate Biotechnology/Millipore™, Waltham, Massachusetts, U.S.A.) was dissolved in DMSO (Sigma-Aldrich ®) to prepare a stock solution of 3.3 mM (1 mg/ml), and stored in aliquots at 4℃. A working solution of TSA was made by diluting the stock solution with E3 medium to 33 μM (10 μg/ml) and stored at 4℃.

The diluted TSA solutions were added to E3 medium for treatment of larvae. ML-60218 (Calbiochem®/EMD Chemicals Inc./Merck KGaA, Darmstadt, Germany) and SAHA (BioMol® International/Enzo Life Sciences, Farmingdale, New York, U.S.A.) were dissolved in DMSO at 22 mM (10 mg/ml) and 50 mM respectively. Both SAHA and ML-60218 were divided into aliquots and stored at –20℃.

For small molecules treatment of zebrafish, 20 to 25 WT larvae were incubated in 4 ml of E3 medium (or in E3 medium with PTU for analysis by in situ hybridization) in each well of a 6 well cell culture cluster (costar®, Corning Incorporated, Corning, New York, U.S.A). At 48 h.p.f., TSA or ML-60218 at various concentrations, or the combination of TSA and ML-60218, were added to the medium, and incubation was continued at 28.5℃ for an additional 24 hours, followed by analysis. For controls conducted in parallel, 0.5% DMSO or no drug was added to the medium.

In situ hybridization, immunohistochemistry, and histological analysis

These procedures were conducted as previously described (Yee et al., 2001; Yee and Pack, 2005; Yee et al., 2005, Yee et al., 2007). For in situ hybridization, trypsin anti-sense riboprobes were generated as described (Yee and Pack, 2005; Yee et al., 2005). Zebrafish larvae were examined under a stereo-dissecting microscope (Leica MZ16F, Plymouth, Minnesota, USA) with bright light. Images were captured using a color digital camera and QCapture software (QImaging), and constructed using Adobe® Photoshop® 7.0. For immunohistochemistry, rabbit anti-carboxypeptidase A antibodies (1:100) (Rockland Immunochemicals, Inc., Gilbertsville, Pennsylvania, U.S.A.), mouse anti-BrdU antibodies (1:100) (Roche Ltd., Branford, Connecticut, U.S.A.), and rabbit anti-pan cadherin antibodies (1:100) (Sigma-Aldrich®) were used. For histological analysis, zebrafish larvae were embedded in JB-4® Plus solution (Polysciences Inc., Warrington, Pennsylvania, U.S.A.), and sectioned at 5-µm intervals using a microtome (Leica RM2255). The sections were examined under a compound microscope (Olympus BX51, Tokyo, Japan) using bright light or fluorescence, and the images were acquired using a DP71 digital camera (Olympus), analyzed using DP Manager software (Olympus Corp.) and constructed using Adobe® Photoshop® 7.0.

Exocrine pancreatic epithelia in S phase and morphometric analysis of cell growth

The procedures of analysis of S-phase cells using BrdU (Sigma-Aldrich®) are described in detail previously (Yee and Pack, 2005; Yee et al., 2007). In this study, WT larvae at 48 h.p.f. were incubated in E3 medium containing 165 nM TSA, 110 µM ML-60218, 165 nM TSA + 110 µM ML-60218, 0.5% DMSO or no treatment, for additional 24 hours. The larvae were fixed at 72 h.p.f. and processed for immunohistochemistry using anti-BrdU antibodies. The histological sections were examined and the images were acquired as described above. Five larvae in each group were evaluated, and for each larva three sections containing exocrine pancreas posterior to the last section of islet were analyzed. For each larva, the number of DAPI-positive nuclei, number of BrdU-positive nuclei, and proportion of DAPI-positive nuclei being BrdU-positive that represents the percentage of cells in the S phase were determined.

Morphometric analysis of cell growth was performed using cadherin as a marker of intercellular junctions (Yee et al., 2011). Briefly, the larvae as treated above were fixed at 72 h.p.f., immunoassayed using rabbit anti-pan-cadherin antibodies (Sigma-Aldrich®), histologically sectioned, examined, and imaged. Five larvae in each group were evaluated, and for each larva three sections containing exocrine pancreas posterior to the last section of islet were analyzed. The surface area of exocrine pancreas on each of three sections posterior to last section of islet, the number of DAPI-positive nuclei, and the surface area per cell that indicates cell growth were determined.

Cell lines and culture

The human pancreatic adenocarcinoma cell lines, BxPC-3, Capan-1, HPAF-II, MIA PaCa-2, Panc 02.03, PANC-1, and PL45 were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and grown at 37℃ in a humidified atmosphere containing 5% CO_2 according to ATCC's instructions. The immortalized human pancreatic ductal epithelia H6c7 was generously provided by Dr. Ming-Sound Tsao (University of Toronto) and cultured as described (Furukawa et al., 1996). All experiments were performed using culture media except indicated otherwise. The cells were used within 20 passages of the liquid nitrogen-frozen stocks from which the cells were periodically recovered.

Proliferation assay

Cellular proliferation was quantified using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, U.S.A.) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonylphenyl)-2H-tetrazolium (MTS) as described (Chun et al., 2009). In this study, PANC-1 and BxPC-3 were treated with 5 µM SAHA, 100 µM ML-60218, 5 µM SAHA + 100 µM ML-60218, 5 µM SAHA + 0.5% DMSO, 0.5% DMSO, or culture medium only. The experiment was performed three times with each treatment in triplicate samples.

Soft agar colony assay

A two-layer, soft agar gel was used to assess the ability of SAHA and ML-60218 to inhibit colony formation of PANC-1 and BxPC-3 in vitro as described (Chun et al., 2009). In this study, the cells were treated with SAHA, ML-60218, SAHA + ML-60218, SAHA + DMSO, or DMSO as described above. The experiment was performed three times with each treatment in triplicate samples.

Cell morphology

In each well of 6 well cell culture cluster (costar®, Corning Inc.), 5×10^5 PANC-1 or BxPC-3 cells / 4 ml medium were seeded and allowed to adhere overnight. The cells were then incubated with SAHA, ML-60218, SAHA + ML-60218, or control (DMSO or untreated) for 24 hours. Images were acquired under an inverted light microscope with phase contrast (Olympus IX81).

Flow cytometric analysis of cell cycle

PANC-1 and BxPC-3 cells were seeded at 5×10^5 cells in 4 ml medium per well of a 6 well cell culture cluster (costar®, Corning Inc.) and incubated overnight. The cells were starved from serum for 24 hours and then incubated in culture medium containing SAHA, ML-60218, SAHA + ML-60218, or control (DMSO or untreated) for 48 hours. As previously described (Chun et al., 2009), the cells were incubated with propidium iodide, and the DNA content of the cells was analyzed using a FACScan™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Non-specific sub-G_0/G_1 events were gated out for accurate analysis of cell cycle distribution in the viable cells.

Flow cytometric analysis of cell death

PANC-1 cells seeded at 5×10^5 in 3 ml medium per well of a 6 well cell culture cluster (costar®, Corning Inc.) were treated with SAHA, ML-60218, and SAHA + ML-60218, or control (DMSO or untreated) for 48 hours. The cells were incubated with (1:20) diluted recombinant human Annexin V conjugated with fluorescein isothiocyanate (FITC) (Invitrogen™/Life Technologies, Grand Island, New York, U.S.A.), and then propidium iodide (Invitrogen™/Life Technologies) at a final concentration of 1 µg/ml at room temperature. Analysis was conducted within 1 hour on the FACScan™ flow cytometer (Becton, Dickinson and Company) by obtaining 10^4 events per histogram. The data was analyzed using CellQuest Pro software.

Immunofluorescent detection of survivin

BxPC-3 cells were seeded at 2×10^4 in 1 ml medium per chamber of a 2-chambered glass slide (LAB TEK®, Nunc™, Thermo Fisher Scientific, Rochester, New York, U.S.A.), treated with SAHA, ML-60218, and SAHA + ML-60218 for 48 hours, fixed with 4% paraformaldehyde in phosphate buffered saline (pH 7.4), and incubated with 500 µl of (1:100) diluted monoclonal mouse anti-survivin antibodies (Cell Signaling Technology, Inc., Danvers, Massachusetts, U.S.A.), washed with Tris-buffered saline, and then incubated with 500 µl of (1:100) diluted goat anti-mouse Immunoglobulin G conjugated to Alexa Fluor® 488 nm (Invitrogen™/Life Technologies). Phalloidin conjugated to Alexa Fluor® 546 nm (Invitrogen™/Life Technologies) was used at 1:100 dilution to detect actin and visualize the cytoplasm. The expression pattern of survivin was examined and imaged under the MRC-1024 confocal laser scanning microscope (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.).

Immunoblot analysis

Total protein was extracted from zebrafish larvae and human cell lines and then analyzed by immuno-blotting. For zebrafish, the larvae at 72 h.p.f. were homogenized in a lysis buffer containing 63 mM Tris-HCl, pH 6.8, 10% glycerol, 5% ß-mercaptoethanol, 3.5% sodium dodecyl sulfate (SDS), and protease inhibitors (Roche Diagnostics, Indianapolis, Indiana, U.S.A.), rotated at 4℃ for 1 hour, and supernatant was collected after centrifugation. For PANC-1 and BxPC-3, the cells were grown to approximately 70% confluence in 100-mm tissue culture dishes, and then treated for 48 hours with SAHA, ML-60218, SAHA + ML-60218, or DMSO, and the cells were fixed with 1% para-formaldehyde, then treated with glycine, washed with phosphate-buffered saline, and lysed using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The lysates of either zebrafish or human cells were then sonicated on ice for 30 seconds, centrifuged at 2×10^4 g for 10 minutes at 4℃, and the protein concentration of the supernatant was quantified using the BCA Protein Assay Kit (Bio-Rad).

Immunoblot analysis was essentially performed as described (Yee et al., 2010). Primary antibodies include rabbit anti-human acetylated histone H3 (1:5,000), rabbit anti-human acetylated histone H4 (1:5,000), rabbit anti-human histone H3 (1:25,000), rabbit anti-human histone H4 (1:25,000) (Upstate Biotechnology/Millipore™), goat anti-human BAX (Santa Cruz Biotechnology Inc., Santa Cruz,

California, U.S.A., 1:1,000), rabbit anti-human p21^{CDKN1A} (Abcam®, Cambridge, Massachusetts, U.S.A., 1:25,000), and rabbit anti-human actin (Sigma®, 1:200). Secondary antibodies include horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, 1:5,000) or donkey anti-goat IgG (Santa Cruz Biotechnology, Inc., 1:5,000). The intensity and area of the protein bands were quantified using Adobe® Photoshop® 7. The levels of acetylated histones H3 and H4 were compared to those of total histones H3 and H4, respectively; those of BAX and p21^{CDKN1A} were compared with actin.

Quantitative real-time PCR

Total RNA was isolated from zebrafish larvae or human cell lines using TRIzol® (Invitrogen™/Life Technologies) RNeasy® Mini Kit (Qiagen, Inc., Valencia, California, U.S.A.) according to the manufacturer's instructions. To generate cDNA, the RNA was reverse-transcribed with SuperScript III Reverse Transcriptase and Random Primers (Invitrogen™/Life Technologies) according to the manufacturer's protocol. The cDNA was amplified with gene-specific primer pairs located on two adjacent exons to achieve a high level of specificity and to avoid detection of genomic DNA. Primers were designed to have similar GC contents and annealing temperature using the Primer3 Program (http://frodo.wi.mit.edu). The primers were synthesized by International DNA Technology, Inc. (Coralville, Iowa, U.S.A.). Transcript levels were determined by using TaqMan Universal PCR Master Mix (Applied Biosystems®, Foster City, California, U.S.A.) and the ABI Prism® 7500 real-time PCR system (Applied Biosystems®) essentially as described (Yee et al., 2010). The conditions were 2 minutes at 50℃, 10 minutes at 95℃ followed by 40 cycles of 15 seconds at 95℃, and 1 minute at 60℃ followed by a final step of 10 minutes at 72℃.

The sequences of the primers used for the analysis of zebrafish *p21^{cdkn1a}*, *p27^{cdkn1b}*, and *gapdh* expression were designed using the GenBank accession numbers (AL912410, BC056519, and BC095386, respectively) as follows:

p21^{cdkn1a}: 5′-ATGCAGCTCCAGACAGATGA-3′, 5′-CGCAAACAGACCA-ACATCAC-3′

p27^{cdkn1b}: 5′-TGAAGCCTGGAACTTCGACT-3′, 5′-TGTGAATATCGGAG-CCCTTC-3′

gapdh: 5′-GATACACGGAGCACCAGGTT-3′, 5′-CGTTGAGAGCAATAC-CAGCA-3′

The sequences of the primers used for the analysis of human *tRNA^{Met}* and *tRNA^{Ser}* expression were designed from Genomic tRNA Database of Homo sapiens (http://lowelab.ucsc.edu/GtRNAdb/Hsapi):

tRNA^{Met}: 5′-CCTCGTTAGCGCAGTAGGTAG-3′, 5′-TGCCCCGTGTGAGG-ATCGAAC-3′

tRNA^{Ser}: 5′-GCTGTGATGGCCGAGTGGTT-3′, 5′-CGCTGTGAGCAGGA-TTCGAA-3′

Each tRNA level was determined with a standard curve using known concentrations of tRNA^{Met} or tRNA^{Ser}.

Statistical analysis

Student's *t*-test was performed to determine the mean, standard deviation (s.d.), standard error of mean (s.e.m.), and *P*-values. A difference between the experimental group and control with a value of $P < 0.05$ is considered statistically significant. A *P*-value <0.1 indicates a trend of statistical significance.

Acknowledgements

N.S.Y. is supported by the Physician Scientist Stimulus Package from The Pennsylvania State University College of Medicine. S.G.C. was supported by the Clinical Research Fellowship of the Doris Duke Charitable Trust at the University of Iowa. This work was supported by Penn State Hershey Cancer Institute (N.S.Y.), Pilot Grant in Translational Research from Department of Internal Medicine of Carver College of Medicine at the University of Iowa (N.S.Y.), Fraternal Order of Eagles (N.S.Y.), and Cancer Center Support Grant (P30 CA 086862) by National Cancer Institute to Holden Comprehensive Cancer Center at the University of Iowa (N.S.Y.). The Zebrafish International Resource Center is supported by grant P40 RR12546 from the NIH-NCRR.

Competing Interests

The authors declare no competing financial interests.

References

Berrozpe, G., Schaeffer, J., Peinado, M. A., Real, F. X. and Perucho, M. (1994) Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. *Int. J. Cancer* **58**, 185-191.

Boyd, D. C., Greger, I. H. and Murphy, S. (2000). In vivo footprinting studies suggest a role for chromatin in transcription of the human 7SK gene. *Gene* **247**, 33-44.

Brunmeir, R., Lagger, S. and Seiser, C. (2009). Histone deacetylase 1 and 2-controlled embryonic development and cell differentiation. *Int. J. Dev. Biol.* **53**, 275-289.

Cairns, C. A. and White, R. J. (1998). p53 is a general repressor of RNA polymerase III transcription. *EMBO J.* **17**, 3112-3123.

Chu, W. M., Wang, Z., Roeder, R. G. and Schmid, C. W. (1997). RNA polymerase III transcription repressed by Rb through its interactions with TFIIIB and TFIIIC2. *J. Biol. Chem.* **272**, 14755-14761.

Chun, S. G., Zhou, W. and Yee, N. S. (2009). Combined targeting of histone deacetylases and hedgehog signaling produces enhanced cytotoxicity in pancreatic cancer. *Cancer Biol. Ther.* **8**, 1328-1339.

Connell, C. M., Colnaghi, R. and Wheatley, S. P. (2008a). Nuclear survivin has reduced stability and is not cytoprotective. *J. Biol. Chem.* **283**, 3289-3296.

Connell, C. M., Wheatley, S. P. and McNeish, I. A. (2008b). Nuclear survivin abrogates multiple cell cycle checkpoints and enhances viral oncolysis. *Cancer Res.* **68**, 7923-7931.

Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J. P., Warnock, L. J., Milner, J., White, R. J. and Johnson, D. L. (2003). p53 represses RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIIB. *EMBO J.* **22**, 2810-2820.

Frew, A. J., Johnstone, R. W. and Bolden, J. E. (2009). Enhancing the apoptotic and therapeutic effects of HDAC inhibitors. *Cancer Lett.* **280**, 125-133.

Furukawa, T., Duguid, W. P., Rosenberg, L., Viallet, J., Galloway, D. A. and Tsao, M.-S. (1996). Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am. J. Path.* **148**, 1763-1770.

Haberland, M., Montgomery, R. L. and Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* **10**, 32-42.

Johnson, S. A., Dubeau, L. and Johnson, D. L. (2008). Enhanced RNA polymerase III-dependent transcription is required for oncogenic transformation. *J. Biol. Chem.* **283**, 19184-19191.

Koorstra, J. B., Hustinx, S. R., Offerhaus, G. J. and Maitra, A. (2008). Pancreatic carcinogenesis. *Pancreatology* **8**, 110-125.

Lagger, G., Doetzlhofer, A., Schuettengruber, B., Haidweger, E., Simboeck, E., Tischler, J., Chiocca, S., Suske, G., Rotheneder, H., Wintersberger, E. et al. (2003). The tumor suppressor p53 and histone deacetylase 1 are antagonistic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene. *Mol. Cell. Biol.* **23**, 2669-2679.

Lane, A. A. and Chabner, B. A. (2009). Histone deacetylase inhibitors in cancer therapy. *J. Clin. Oncol.* **27**, 5459-5468.

Larminie, C. G., Cairns, C. A., Mital, R., Martin, K., Kouzarides, T., Jackson, S. P. and White, R. J. (1997). Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein. *EMBO J.* **16**, 2061-2071.

Lee, D. Y., Hayes, J. J., Pruss, D. and Wolffe, A. P. (1993). A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell* **72**, 73-84.

Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T. and Kelly, W. K. (2001). Histone deacetylases and cancer: Causes and therapies. *Nat. Rev. Cancer* **1**, 194-202.

Marshall, L. and White, R. J. (2008). Non-coding RNA production by RNA polymerase III is implicated in cancer. *Nat. Rev. Cancer* **8**, 911-914.

Marshall, L., Kenneth, N. S. and White, R. J. (2008). Elevated tRNA(iMet) synthesis can drive cell proliferation and oncogenic transformation. *Cell* **133**, 78-89.

Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G. and Yang, X. (2010). tRNA binds to cytochrome c and inhibits caspase activation. *Mol. Cell* **37**, 668-678.

Moore, P. S., Sipos, B., Orlandini, S., Sorio, C., Real, F. X., Lemoine, N. R., Gress, T., Bassi, C., Kloppel, G., Kalthoff, H. et al. (2001). Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-*ras*, *p53*, *p16* and *DPC4*/*Smad4*. *Virchows Arch.* **439**, 798-802.

Noel, E. S., Casal-Sueiro, A., Busch-Nentwich, E., Verkade, H., Dong, P. D. S., Stemple, D. L. and Ober, E. A. (2008). Organ-specific requirements for Hdac1 in liver and pancreas formation. *Dev. Biol.* **322**, 237-250.

Noma, K., Cam, H. P., Maraia, R. J. and Grewal, S. I. (2006). A role of TFIIIC transcription factor complex in genome organization. *Cell* **125**, 859-872.

Rayman, J. B., Takahashi, Y., Indjejian, V. B., Dannenberg, J. H., Catchpole, S., Watson, R. J., te Riele, H. and Dynlacht, B. D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev.* **16**, 933-947.

Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L. and Wolffe, A. P. (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat. Genet.* **25**, 338-342.

Scott, P. H., Cairns, C. A., Sutliffe, J. E., Alzuherri, H. M., McLees, A., Winter, A. G. and White, R. J. (2001). Regulation of RNA polymerase III transcription during cell cycle entry. *J. Biol. Chem.* **276**, 1005-1014.

Siegel, R., Ward, E., Brawley, O. and Jemal, A. (2011). Cancer Statistics, 2011. *CA Cancer J. Clin.* **61**, 212-236.

Sutcliffe, J. E., Cairns, C. A., McLees, A., Allison, S. J., Tosh, K. and White, R. J. (1999). RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. *Mol. Cell. Biol.* **19**, 4255-4261.

Westerfield, M. (2007). *The Zebrafish Book*: *A Guide For The Laboratory Use Of Zebrafish (Danio rerio)*, 5th edn.Eugene, Oregon: University of Oregon Press.

White, R. J. (2008). RNA polymerases I and III, non-coding RNAs and cancer. *Trends Genet.* **24**, 622-629.

White, R. J., Gottlieb, T. M., Downes, C. S. and Jackson, S. P. (1995). Cell cycle regulation of RNA polymerase III transcription. *Mol. Cell. Biol.* **15**, 6653-6662.

White, R. J., Trouche, D., Martin, K., Jackson, S. P. and Kouzarides, T. (1996). Repression of RNA polymerase III transcription by the retinoblastoma protein. *Nature* **382**, 88-90.

Wu, L., Pan, J., Thoroddsen, V., Wysong, D. R., Blackman, R. K., Bulawa, C. E., Gould, A. E., Ocain, T. D., Dick, L. R., Errada, P. et al. (2003). Novel small-molecule inhibitors of RNA polymerase III. *Eukaryot. Cell* **2**, 256-264.

Yee, N. S. (2010). Zebrafish as a biological system for identifying and evaluating therapeutic targets and compounds. In *Drug Discovery In Pancreatic Cancer* (H. Han and P. J. Grippo), 95-112.New York: Springer.

Yee, N. S. and Pack, M. (2005). Zebrafish as a model for pancreatic cancer research. *Meth. Mol. Med.* **103**, 273-298.

Yee, N. S., Yusuff, S. and Pack, M. (2001). Zebrafish pdx1 morphant displays defects in pancreas development and digestive organ chirality, and potentially identifies a multipotent pancreas progenitor cell. *Genesis* **30**, 137-140.

Yee, N. S., Lorent, K. and Pack, M. (2005). Exocrine pancreas development in zebrafish. *Dev. Biol.* **284**, 84-101.

Yee, N. S., Gong, W., Huang, Y., Lorent, K., Dolan, A. C., Maraia, R. J. and Pack, M. (2007). Mutation of RNA pol III subunit rpc2/polr3b leads to deficiency of subunit Rpc11 and disrupts zebrafish digestive development. *PLoS Biol.* **5**, 2484-2492.

Yee, N. S., Zhou, W. and Lee, M. (2010). Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. *Cancer Lett.* **297**, 49-55.

Yee, N. S., Zhou, W. and Liang, I.-C. (2011). Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg^{2+}-sensitive Socs3a signaling in development and cancer. *Dis. Model Mech.* **4**, 240-254.

Yoshida, M., Kijima, M., Akita, M. and Beppu, T. (1995). Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by Trichostatin A. *J. Biol. Chem.* **265**, 17174-17179.

Zhou, W., Liang, I.-C. and Yee, N. S. (2011) Histone deacetylase 1 is required for exocrine pancreatic epithelial proliferation in development and cancer. *Cancer Biol. Ther.* **11**, 659-670.

Zupkovitz, G., Tischler, J., Posch, M., Sadzak, I., Ramsauer, K., Egger, G., Grausenburger, R., Schweifer, N., Chiocca, S., Decker, T. et al. (2006) Negative and positive regulation of gene expression by mouse histone deacetylase 1. *Mol. Cell. Biol.* **26**, 7913-7928.

Biology Open