北海道公立大学法人
札幌医科大学
Sapporo Medical University
SAPPORO MEDICAL UNIVERSITY INFORMATION AND KNOWLEDGE REPOSITORY

Title 論文題目	FOXO3a activation by HDAC class IIa inhibition induces cell cycle arrest in pancreatic cancer cells （HDAC クラスIIa 阻害による FOXO3a の活性化は膵癌細胞において細胞周期停止を誘導する）
Author(s) 著　者	宇佐美，信
Degree number 学位記番号	甲第 3006 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2018-03-31
Original Article 原著論文	札幌医学雑誌　第 87 巻　第 1 号（平成 31 年 3 月）掲載予定
Doc URL	
DOI	
Resource Version	Author Edition

学位申請論文

FOXO3a activation by HDAC class Ⅱa inhibition induces

cell cycle arrest in pancreatic cancer cells

腫瘍内科学講座

宇佐美　信

Title:

FOXO3a activation by HDAC class Ⅱa inhibition induces cell cycle arrest in pancreatic cancer cells

Authors and affiliations:

Makoto Usami*, Shohei Kikuchi*, Kohichi Takada, Michihiro Ono, Yusuke Sugama, Yohei Arihara, Naotaka Hayasaka, Hajime Nakamura, Yuuki Ikeda, Masahiro Hirakawa, Makoto Yoshida, Koji Miyanishi, Masayoshi Kobune, Junji Kato

*These authors contributed equally to this work

Department of Medical Oncology, Sapporo Medical University School of Medicine, Sapporo, Japan.

Running title: FOXO3a activation by class Ⅱa HDACi in pancreatic cancer

Correspondence:

Masayoshi Kobune, M.D., Ph.D.

E-mail: mkobune@sapmed.ac.jp:

Junji Kato M.D., Ph.D.

E-mail: jkato@sapmed.ac.jp:

Address: S-1, W-16, Chuo-Ku, Sapporo, Japan. 060-8543

Telephone number: +81-11-611-2111 (ext. 3254);

Fax number: +81-11-612-7987

Disclosure of Potential Conflicts of Interest: No conflicts of interest were disclosed.

Authors' Contributions:

Conception and design: M. Usami, S. Kikuchi, K. Takada and M. Ono

Development of methodology: M. Usami, S. Kikuchi and K. Takada

Acquisition of data: M. Usami and S. Kikuchi

Analysis and interpretation of data: M. Usami, S. Kikuchi, K. Takada, M. Ono, Y. Sugama, Y. Arihara, N. Hayasaka, H. Nakamura, Y. Ikeda, M. Hirakawa and M. Yoshida

Writing, review, and/or revision of the manuscript: M. Usami and S. Kikuchi

Administrative, technical, or material support: M. Usami and S. Kikuchi

Study supervision: K. Miyanishi, M. Kobune and J. Kato

Abstract

Pancreatic cancer is a highly aggressive with multiple oncogenic mutations such as K-RAS and p53. The efficacy of current chemotherapy is poor and new therapeutic targets are needed.

The forkhead box (FOX) proteins are multidirectional transcriptional factors strongly implicated in malignancies. Although they are negative regulators of proliferation repressing of cyclin and inducing cell cycle inhibitors such as $p21^{Waf1/Cip1}$, their expression is consistently suppressed by several oncogenic pathways such as PI3K/AKT signaling activated in pancreatic cancer. Upregulating FOXO3a could therefore be a promising strategy to circumvent the impact of oncogenic mutations. A recent study showed class IIa histone deacetylases (HDAC) can act as a transcriptional suppressor. In this study, we hypothesized HDAC class IIa inhibition would upregulate FOXO3a expression, thereby inducing its transcription-dependent anti-tumor effects.

We observed selective HDAC class IIa inhibitor, TMP269, induced FOXO3a expression in a dose-dependent manner, and inhibited cell growth in AsPC-1 cells. G1/S arrest was observed; this was concomitant with $p21^{Waf1/Cip1}$ upregulation and downregulation of CDK2, 4, and 6, and cyclin D1 and D2. Importantly, AsPC-1 are p53-null, suggesting p53-independent induction of p21. These findings suggest HDAC class IIa inhibition activates FOXO3a-dependent transcription and cell cycle arrest. Because FOXO3a is subject to ubiquitylation-mediated proteasome degradation, we examined synergistic activation of FOXO3a by TMP269 combined with proteasome inhibitor carfilzomib. As expected, FOXO3a expression was further increased and cell growth inhibition was dramatically enhanced.

In conclusion, dual inhibition of class IIa HDACs and proteasome could be a promising new strategy for modifying FOXO3a activity against pancreatic cancer.

Keywords: FOXO3a, class IIa HDACs, cell cycle arrest, HDAC inhibitor, pancreatic cancer

1. Introduction

Pancreatic cancer, which commonly manifests as a ductal adenocarcinoma, is a highly aggressive malignancy that is the fourth leading cause of cancer-related deaths in the United States and Japan(1,2). Surgical resection is the only potentially curative treatment option; however, most patients are diagnosed when the cancer is locally advanced or metastatic and unresectable. Though systemic cytotoxic chemotherapy is given, its therapeutic efficacy is poor. The addition of the molecular targeting agents such as erlotinib and bevacizumab, which have clinical benefit against other malignancies such as lung cancer and colorectal cancer(3,4), has had little effect in pancreatic cancer, despite the importance of their cellular targets during disease progression (5,6). Pancreatic adenocarcinoma is accompanied by multiple genetic mutations, including those in KRAS and p53, which are associated with disease progression and chemo-resistance(7-9). The presence of multiple alterations is one of the main reasons that single molecular targeting agents do not improve clinical outcome. Thus, novel agents that can improve clinical outcome are urgently needed; ideally the effects of such agents will be sufficiently broad as to overcome the oncogenic effects of multiple genetic mutations.

The forkhead box O (FOXO) proteins are a subgroup of the forkhead box (FOX) family, and function as multidirectional transcriptional factors that control a wide spectrum of biological process(10,11), including cell growth, survival, metabolism and oxidative state (12). Though FOXO proteins, and particularly FOXO3a, normally act as tumor suppressors via their activation of cell cycle arrest and apoptosis (13,14), they can be constitutively inactivated by several major oncogenic signaling pathways, such as PI3K/AKT, MAPK, Ras-MEK-ERK and IKK pathways(13,15,16). In pancreatic cancer, the PI3K/AKT pathway is frequently activated (17), leading to inactivation of FOXO proteins, cancer cell survival, and chemo-resistance. Thus, upregulating FOXO3a activity could be a promising strategy for pancreatic cancer treatment

independently of the underlying oncogenic mutations. However, targeting transcription factors is a relatively novel approach and is challenging in terms of tractability.

Histone deacetylases (HDAC)s, can be divided into four groups (class I: HDAC1, 2, 3 and 8; class IIa: HDAC4, 5, 7 and 9; class IIb: 6 and 10; class III: SIRT 1-7; class IV: HDAC11)(18). Class IIa HDACs are quite distinct, as they have nucleus-cytoplasmic shuttling capability and minimal deacetylase activity when compared to nuclear class I HDCAs and cytoplasmic Class IIb HDACs (19). HDAC inhibitors (HDACi) have been extensively investigated and used clinically for cancer treatment, and their major mechanism of action is considered to be the modulation of histone modification, predominantly as a result of their activity against class I HDACs. The biological significance of class IIa HDACs in cancer is not fully understood. Recent studies have indicated that Class IIa HDACs repress the transcription factors. In a preclinical study, the selective HDAC class IIa inhibitor, TMP269, induced upregulated transcription factor ATF4 followed by induction of apoptosis in multiple myeloma cells(20). The expression and activity of FOXO proteins are mainly negatively regulated through their AKT-dependent phosphorylation, which facilitates their binding to 14-3-3 proteins and their subsequent export to the cytoplasm(12,21). Indeed, 14-3-3 proteins are pivotal interacting partners of class IIa HDACs(19,22). Thus, targeting class IIa HDACs has great potential as a strategy to restore the activity of FOXO proteins.

In this study, we investigated the expression of FOXO3a in pancreatic cancer cells, and tested our hypothesis that class IIa HDACs inhibition would upregulate FOXO3a expression and induce anti-tumor effects by restoring FOXO3a transcriptional activity. Our results provide the rationale for HDAC class IIa inhibitor-based therapeutic strategies designed to restore FOXO3a activity in pancreatic cancer.

2. Materials and Methods

2.1. Cell lines and culture conditions

The pancreatic cancer cell lines AsPC-1 and BxPC-3 were purchased from American Type Culture Collection. MIA PaCa-2 and PANC-1 were obtained from the Riken BRC Cell Bank (Japan). All cell lines were cultured with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 2μM glutamine, 100U/mL penicillin, and 100μg/mL streptomycin (Sigma-Aldrich).

2.2. Reagents

The selective HDAC class IIa inhibitor TMP269 was purchased from Selleck Chemicals (Houston, TX, USA) and the proteasome inhibitor carfilzomib (CFZ) from LC Laboratories (Woburn, MA, USA).

2.3. Immunoblotting

Cells were harvested, washed with PBS, and lysed using RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane (Merck Millipore, Germany), and immunoblotted with the following antibodies: anti-HDAC4, HDAC7, FOXO3a, PARP, caspase-3, CDK2, CDK4, CDK6, Cyclin E1, Cyclin E2, Cyclin D1, and Cyclin D3 (Cell Signaling Technology, Danvers, MA, USA); anti-HDAC5, HDAC9, p21, and actin (Santa Cruz Biotechnology, Dallas, TX, USA). Protein expression level was evaluated by standard chemiluminescence using ImageQuant LAS-4000 (GE Healthcare, USA) and quantified by ImageJ software (NIH).

2.4. Quantitative RT-PCR and PCR Arrays

AsPC-1 cells were cultured for 48 h with the designated dose of TMP269, or in control complete media, and harvested. Total RNA was purified with the RNeasy Plus Mini Kit

(QIAGEN, Germantown, USA) according to the manufacturer's instructions. Reverse transcription was carried out using SuperScript VILO Master MIX (Thermo Fisher Scientific). qRT-PCR was performed with an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Analysis of target genes was conducted in triplicate using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Transcript levels were normalized to *β-actin* expression. The PCR primers were designed: 5′ – CGGACAAACGGCTCACTCT – 3′ and 5′- GGACCCGCATGAATCGACTAT – 3′ for *FOXO3*; 5′ – GGCATCCTCACCCTGAAGTA – 3′ and 5′ – GAAGGTGTGGTGCCAGATTT – 3′ for *β-actin*. For PCR array, total RNA was reverse transcribed using RT^2 First Strand Kit (QIAGEN). PCR array was performed using RT^2 Profiler PCR Array Human PI3K-AKT Signaling Pathway according to the manufacturer's instruction.

2.5. Growth inhibition assay

The growth inhibitory effect of TMP269, alone or with CFZ, was assessed by measuring 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide (MTT, Sigma-Aldrich) dye absorbance. Briefly, 5000 AsPC-1 cells were cultured with 100 μL of complete medium in each well of 96-well culture plate. Twenty-four hours later, supernatants were removed and 100 μL of complete media containing the designated dose of TMP269 and/or CFZ was added into each well. Ten microliters of 5 mg/mL MTT were added to each well for the last four hours of the 48-h treatment. Absorbance was measured at 570 nm with absorbance at 650 nm subtracted as a reference using a multi-mode plate reader (Infinity M1000 PRO; TECAN, Switzerland). All experiments were performed in triplicate.

2.6. Cell cycle assay

AsPC-1 cells cultured with or without TMP269 for 48 h were harvested, washed with PBS and fixed with 70% of ethanol. Cells then were stained with 50 μg/mL propidium iodine

solution (Sigma-Aldrich). Following flow cytometry on a FACS Canto II instrument, data were analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate.

2.7. Statistical analysis

Statistical significance was determined by Student's t-test. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. FOXO3a expression is suppressed in pancreatic cancer.

We first investigated FOXO3a expression in pancreatic cancer patients using publicly available gene expression profiling data. FOXO3a expression was significantly lower in 118 resected pancreatic adenocarcinoma tumor tissues than in 13 normal pancreatic tissues (Figure 1A) (23). Furthermore, in paired analysis between cancer tissue and non-cancer tissue in the same pancreatic ductal adenocarcinoma patient, FOXO3a expression was significantly lower in the cancer tissues (Figure 1B)(24). To investigate the impact of FOXO3a expression on survival, we analyzed gene expression profiling data from a retrospective microarray study of 63 resected early stage pancreatic adenocarcinoma patients (25). We divided the 63 patients into three groups according to FOXO3a expression level (low expression group: FOXO3a expression under the 25^{th} percentile, middle expression group: 25^{th}–75^{th} percentile, high expression group: above 75^{th} percentile; Supplemental Figure S1). The median survival of the low expression group was 13 months less than that of the middle expression group (21.5 months) and high expression group (33 months) (Figure 1C), indicating that low FOXO3a expression is an indicator of poor survival. These findings indicate that FOXO3a upregulation might be a therapeutic strategy for pancreatic cancer treatment.

3.2. HDAC class Ⅱa inhibition induces FOXO3a upregulation.

Because class IIa HDACs have tissue-specific expression(19), we investigated their expression in pancreatic cancer cell lines (Figure 2A), and in publicly available gene expression profiling data of pancreatic adenocarcinoma cancer tissue and non-cancer tissue (Figure 2B)(26). Expression of all isozymes was confirmed in the cell lines we tested (Figure 2A), and in pancreas cancer and non-cancer tissue (Figure 2B).

We next used a potent cell permeable and selective class IIa HDAC inhibitor (TMP269; $C_{25}H_{21}F_3N_4O_3S$, (27)) to probe the role of this HDAC subfamily in pancreatic cancer. In the PCR array assay, FOXO3 was the fifth most upregulated gene among 84 tested (1.99-fold increase, Figure 2C and Table 1). Importantly, FASLG, a FOXO3a target was the most upregulated gene, strongly suggesting that FOXO3a transcriptional activity was increased upon treatment. We confirmed that FOXO3a expression at the mRNA and protein level was increased in a dose-dependent manner following treatment with TMP269 (Figure 2D and 2E). These findings confirm that HDAC class IIa inhibition induces FOXO3a upregulation.

3.3. TMP269 treatment induces modest cell growth inhibition effect via cell cycle arrest.

To investigate the anti-tumor effect of HDAC class IIa inhibition by TMP269, we performed an MTT-based cell growth assay. TMP269 treatment induced modest cell growth inhibition in AsPC-1 pancreatic cancer cells, with an IC50 of 57.5 μM; the IC50 was not achieved in other cell lines, even with the maximum dose of 100 μM for 48 h (Figure 3A). To elucidate the mechanism of cell growth inhibition, we evaluated induction of apoptosis with immunoblotting and examined changes in the cell cycle using flow cytometry. Because the PCR array assay revealed upregulated expression of *FASLG*, the gene encoding FAS ligand (Figure 2C, Table 1 and Supplemental Table S1), we suspected that the apoptosis pathway may have

been engaged. However, neither cleaved PARP nor caspase-3 was observed following immunoblotting (Figure 3B). A cell cycle assay revealed G1/S arrest was induced in a dose-dependent fashion (Figure 3C), showing that cell growth inhibition was due to cell cycle arrest rather than apoptosis. On further examination of the relationship between FOXO3a and proteins related to G1/S cell cycle transition, we observed downregulation of CDK2, -4, and -6, cyclin D1, D2, and upregulation of $p21^{Waf1/Cip1}$, consistent with induction of G1/S arrest and transcriptional activation of FOXO3a (Figure 4A, 4B and 4C). Importantly, $p21^{Waf1/Cip1}$ upregulation was observed in the AsPC-1 p53-null cell line, highlighting p53-independent upregulation. These findings suggest that upregulation and transcriptional activation of FOXO3a following treatment with HDAC class IIa inhibitors leads to inhibition of cell growth.

3.4. Combined proteasome and HDAC class Ⅱa inhibition enhances FOXO3a activation and cell growth inhibition.

Because FOXO3a is subject to ubiquitylation-mediated proteasome degradation, we examined the effects of the irreversible proteasome inhibitor carfilzomib (CFZ) when combined with TMP269. We hypothesized that CFZ-dependent stabilization of FOXO3a would synergize with TMP269-dependent activation of FOXO3a-dependent transcriptional activity. As expected, FOXO3a expression was further increased when TMP269 was combined with CFZ (Figure 5A). Importantly, treatment with CFZ alone did not increase FOXO3a expression, indicating that dual inhibition of HDAC class IIa and the proteasome required in order to enhance FOXO3a activity. Concomitant with the increased FOXO3a activity with dual inhibitor treatment, $p21^{Waf1/Cip1}$ expression was further upregulated (Figure 5B) and cell growth inhibition was dramatically enhanced (Figure 5C). These data suggest that dual inhibition of HDAC class IIa and the proteasome is a promising strategy for achieving antiproliferative effects in pancreatic cancer.

4. Discussion

In this study, we showed that cell cycle arrest is triggered by FOXO3a upregulation in pancreatic cancer cell lines. FOXO3a is a negative regulator of cell proliferation repressing the activity of cyclin D1 and D2 and inducing specific cell cycle inhibitors such as $p21^{Waf1/Cip1}$(10,28). Consistent with earlier reports, we showed that cyclins were downregulated whereas p21 was upregulated; these findings are compatible with transcriptionally activated FOXO3a. To date, the biological impact of FOXO3a expression on pancreatic cancer cell survival and/or chemo-resistance has remained unclear. Here, we demonstrated that FOXO3a is downregulated in pancreatic cancer tissues, and that there was a trend of poor survival in patients with low FOXO3a expression in tumors. FOXO3a expression is suppressed by several oncogenic pathways including PI3K/AKT signaling, which is constitutively activated in pancreatic cancer; FOXO3a expression is also lower in pancreatic cancer cell lines than in the normal human immortalized pancreatic ductal epithelial cell line, HPDE6(29). In bladder cancer, high expression of FOXO proteins, including FOXO3, is reportedly correlated with better prognosis(30). Together with our results, these findings strongly suggest that loss of FOXO3a activity is implicated in the progression and survival of pancreatic cancer. It follows that FOXO3a upregulation may provide promising opportunities for developing effective treatments to target pancreatic cancer.

Class IIa HDACs are considered to function as repressors of transcription factors(31). They shuttle between cytoplasm and nucleus and, because of minimal enzymatic activity, act by interacting with other proteins and/or by recruiting other enzymatically active HDACs such as HDAC3(32). These features seem to be essential to the function of Class IIa HDACs as transcriptional co-repressors. Prior examples include the regulation of FOX proteins by HDAC7

and -9 (33). In cancer cells, class IIa HDACs are known to interact with other transcription factors. For example, HDAC4 interacts with PLZF-RARα in acute promyelocytic leukemia cells(34), and HDAC4 interacts with HIF-1α in renal carcinoma cells(35). To upregulate FOXO3a expression, we used TMP269, the first selective class IIa HDACi with a new trifluoromethyloxazodale (TFMO) moiety and high selectivity without class I and IIb activity(27). As expected, we observed FOXO3a upregulation upon HDAC class IIa inhibition using TMP269; however, the cell growth inhibitory effects of TMP269 were modest. We inferred that this was because upregulation of FOXO3a was in itself insufficient. As the activity of FOX proteins is regulated by also post-translational modifications such as ubiquitylation(11), we hypothesized that a proteasome inhibitor such as CFZ would synergize with TMP269 with respect to upregulation FOXO3a levels and activity.

In pancreatic cancer treatment, the clinical outcome of systemic chemotherapy is poor, with 39% of subjects achieving an objective response rate and 11.1 months of median overall survival; this is true even with the intensive triplet regimen of fluorouracil (FU) plus oxaliplatin and irinotecan (FORFIRINOX)(36). To improve clinical outcome in pancreatic cancer, molecular targeted therapies that have demonstrated efficacy compared to chemotherapy alone in other malignancies such as lung cancer or colorectal cancer(3,4), have been tried, but all have fallen short of expectation. For example erlotinib, a small molecule tyrosine kinase inhibitor targeting the epidermal growth factor receptor (EGFR) that is often expressed in pancreatic cancer(37), was tried in a combination therapy with gemcitabine in a Phase III clinical trial. Although survival was improved compared with gemcitabine monotherapy, the increase in life expectancy was only two weeks (median survival ; 6.2 versus 5.9 months, $p = 0.038$)(5). Bevacizumab, which targets vascular endothelial growth factor (VEGF), is also often expressed in pancreatic cancer(38).

However, it conferred no additional survival benefit when combined with gemcitabine in a Phase Ⅲ trial(6). These disappointing results are partly due to the fact that pancreatic adenocarcinoma is accompanied by multiple genetic mutations such as KRAS and P53; these mutations are associated with more rapid tumor progression and chemo-resistance(7-9), and single molecular targeted agents are not likely to have an effect in such circumstances. From this point of view, modifying transcription factor activity downstream of oncogenic mutations may offer a novel and promising strategy for reversing the tumorigenic state. Indeed, we showed that p21 was upregulated concomitant with induction of cell cycle arrest following FOXO3a upregulation in p53-null AsPC-1 cells. This shows that some of the tumor suppressor functions of p53 (such as p21-dependent cell cycle arrest) can be recovered, even when p53 itself has been functionally inactivated.

In conclusion, we show here that HDAC class Ⅱa inhibition induced FOXO3a upregulation, resulting in cell cycle arrest in a pancreatic cancer cell line. These studies provide the framework for dual inhibition of the proteasome and class Ⅱa HDACs as a novel treatment strategy for activation of the FOXO3a transcription factor in pancreatic cancer.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Number JP16K09399.

Reference

1. Yee NS, Zhou W, Chun SG, Liang IC, Yee RK. Targeting developmental regulators of zebrafish exocrine pancreas as a therapeutic approach in human pancreatic cancer. Biology open **2012**;1(4):295-307.
2. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin **2017**;67(1):7-30.
3. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med **2004**;350(23):2335-2342.
4. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C, Sanchez JM, Porta R, Cobo M, Garrido P, Longo F, Moran T, Insa A, De Marinis F, Corre R, Bover I, Illiano A, Dansin E, de Castro J, Milella M, Reguart N, Altavilla G, Jimenez U, Provencio M, Moreno MA, Terrasa J, Munoz-Langa J, Valdivia J, Isla D, Domine M, Molinier O, Mazieres J, Baize N, Garcia-Campelo R, Robinet G, Rodriguez-Abreu D, Lopez-Vivanco G, Gebbia V, Ferrera-Delgado L, Bombaron P, Bernabe R, Bearz A, Artal A, Cortesi E, Rolfo C, Sanchez-Ronco M, Drozdowskyj A, Queralt C, de Aguirre I, Ramirez JL, Sanchez JJ, Molina MA, Taron M, Paz-Ares L, Spanish Lung Cancer Group in collaboration with Groupe Francais de P-C, Associazione Italiana Oncologia T. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol **2012**;13(3):239-246.

5. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W, National Cancer Institute of Canada Clinical Trials G. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol **2007**;25(15):1960-1966.

6. Kindler HL, Niedzwiecki D, Hollis D, Sutherland S, Schrag D, Hurwitz H, Innocenti F, Mulcahy MF, O'Reilly E, Wozniak TF, Picus J, Bhargava P, Mayer RJ, Schilsky RL, Goldberg RM. Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). J Clin Oncol **2010**;28(22):3617-3622.

7. Smit VT, Boot AJ, Smits AM, Fleuren GJ, Cornelisse CJ, Bos JL. KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. Nucleic Acids Res **1988**;16(16):7773-7782.

8. Sinn BV, Striefler JK, Rudl MA, Lehmann A, Bahra M, Denkert C, Sinn M, Stieler J, Klauschen F, Budczies J, Weichert W, Stenzinger A, Kamphues C, Dietel M, Riess H. KRAS mutations in codon 12 or 13 are associated with worse prognosis in pancreatic ductal adenocarcinoma. Pancreas **2014**;43(4):578-583.

9. Scarpa A, Capelli P, Mukai K, Zamboni G, Oda T, Iacono C, Hirohashi S. Pancreatic adenocarcinomas frequently show p53 gene mutations. Am J Pathol **1993**;142(5):1534-1543.

10. Lam EW, Brosens JJ, Gomes AR, Koo CY. Forkhead box proteins: tuning forks for transcriptional harmony. Nature reviews Cancer **2013**;13(7):482-495.

11. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. Nature reviews Cancer **2007**;7(11):847-859.
12. Coomans de Brachene A, Demoulin JB. FOXO transcription factors in cancer development and therapy. Cellular and molecular life sciences : CMLS **2016**;73(6):1159-1172.
13. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **1999**;96(6):857-868.
14. Kops GJ, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffer PJ, Lam EW, Burgering BM. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol **2002**;22(7):2025-2036.
15. Yang JY, Zong CS, Xia W, Yamaguchi H, Ding Q, Xie X, Lang JY, Lai CC, Chang CJ, Huang WC, Huang H, Kuo HP, Lee DF, Li LY, Lien HC, Cheng X, Chang KJ, Hsiao CD, Tsai FJ, Tsai CH, Sahin AA, Muller WJ, Mills GB, Yu D, Hortobagyi GN, Hung MC. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. Nat Cell Biol **2008**;10(2):138-148.
16. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell **2004**;117(2):225-237.
17. Cicenas J, Kvederaviciute K, Meskinyte I, Meskinyte-Kausiliene E, Skeberdyte A, Cicenas J. KRAS, TP53, CDKN2A, SMAD4, BRCA1, and BRCA2 Mutations in Pancreatic Cancer. Cancers **2017**;9(5).
18. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nature reviews Drug discovery **2006**;5(9):769-784.

19. Verdin E, Dequiedt F, Kasler HG. Class II histone deacetylases: versatile regulators. Trends in genetics : TIG **2003**;19(5):286-293.

20. Kikuchi S, Suzuki R, Ohguchi H, Yoshida Y, Lu D, Cottini F, Jakubikova J, Bianchi G, Harada T, Gorgun G, Tai YT, Richardson PG, Hideshima T, Anderson KC. Class IIa HDAC inhibition enhances ER stress-mediated cell death in multiple myeloma. Leukemia **2015**;29(9):1918-1927.

21. Van Der Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J **2004**;380(Pt 2):297-309.

22. Wang AH, Kruhlak MJ, Wu J, Bertos NR, Vezmar M, Posner BI, Bazett-Jones DP, Yang XJ. Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. Mol Cell Biol **2000**;20(18):6904-6912.

23. Janky R, Binda MM, Allemeersch J, Van den Broeck A, Govaere O, Swinnen JV, Roskams T, Aerts S, Topal B. Prognostic relevance of molecular subtypes and master regulators in pancreatic ductal adenocarcinoma. BMC Cancer **2016**;16:632.

24. Zhang G, Schetter A, He P, Funamizu N, Gaedcke J, Ghadimi BM, Ried T, Hassan R, Yfantis HG, Lee DH, Lacy C, Maitra A, Hanna N, Alexander HR, Hussain SP. DPEP1 inhibits tumor cell invasiveness, enhances chemosensitivity and predicts clinical outcome in pancreatic ductal adenocarcinoma. PLoS One **2012**;7(2):e31507.

25. Chen DT, Davis-Yadley AH, Huang PY, Husain K, Centeno BA, Permuth-Wey J, Pimiento JM, Malafa M. Prognostic Fifteen-Gene Signature for Early Stage Pancreatic Ductal Adenocarcinoma. PLoS One **2015**;10(8):e0133562.

26. Badea L, Herlea V, Dima SO, Dumitrascu T, Popescu I. Combined gene expression analysis of whole-tissue and microdissected pancreatic ductal adenocarcinoma identifies

genes specifically overexpressed in tumor epithelia. Hepatogastroenterology **2008**;55(88):2016-2027.

27. Lobera M, Madauss KP, Pohlhaus DT, Wright QG, Trocha M, Schmidt DR, Baloglu E, Trump RP, Head MS, Hofmann GA, Murray-Thompson M, Schwartz B, Chakravorty S, Wu Z, Mander PK, Kruidenier L, Reid RA, Burkhart W, Turunen BJ, Rong JX, Wagner C, Moyer MB, Wells C, Hong X, Moore JT, Williams JD, Soler D, Ghosh S, Nolan MA. Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. Nature chemical biology **2013**;9(5):319-325.

28. de Keizer PL, Packer LM, Szypowska AA, Riedl-Polderman PE, van den Broek NJ, de Bruin A, Dansen TB, Marais R, Brenkman AB, Burgering BM. Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer Res **2010**;70(21):8526-8536.

29. Yan H, Li Q, Wu J, Hu W, Jiang J, Shi L, Yang X, Zhu D, Ji M, Wu C. MiR-629 promotes human pancreatic cancer progression by targeting FOXO3. Cell death & disease **2017**;8(10):e3154.

30. Zhang Y, Jia L, Zhang Y, Ji W, Li H. Higher expression of FOXOs correlates to better prognosis of bladder cancer. Oncotarget **2017**;8(56):96313-96322.

31. Martin M, Kettmann R, Dequiedt F. Class IIa histone deacetylases: regulating the regulators. Oncogene **2007**;26(37):5450-5467.

32. Fischle W, Dequiedt F, Fillion M, Hendzel MJ, Voelter W, Verdin E. Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. The Journal of biological chemistry **2001**;276(38):35826-35835.

33. Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, Basu S, Riley JL, Hancock WW, Shen Y, Saouaf SJ, Greene MI. FOXP3 interactions with histone acetyltransferase

and class II histone deacetylases are required for repression. Proc Natl Acad Sci U S A **2007**;104(11):4571-4576.

34. Chauchereau A, Mathieu M, de Saintignon J, Ferreira R, Pritchard LL, Mishal Z, Dejean A, Harel-Bellan A. HDAC4 mediates transcriptional repression by the acute promyelocytic leukaemia-associated protein PLZF. Oncogene **2004**;23(54):8777-8784.

35. Qian DZ, Kachhap SK, Collis SJ, Verheul HM, Carducci MA, Atadja P, Pili R. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. Cancer Res **2006**;66(17):8814-8821.

36. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M, Groupe Tumeurs Digestives of U, Intergroup P. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med **2011**;364(19):1817-1825.

37. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res **2000**;60(11):2926-2935.

38. Itakura J, Ishiwata T, Shen B, Kornmann M, Korc M. Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer. Int J Cancer **2000**;85(1):27-34.

Figure legends

Figure 1. FOXO3a expression in pancreatic cancer

Publicly available gene expression profiling data GSE 62125 (A) and GSE28735 (B) were analyzed. FOXO3a expression was significantly lower in 118 resected pancreatic adenocarcinoma tumor tissue samples than in 13 normal pancreatic tissues $P < 0.0001$ (A). FOXO3a expression was significantly lower in cancer tissue compared with adjacent non-cancer normal tissue of resected pancreas in 45 pancreatic ductal adenocarcinoma patients $P = 0.0015$ (B). Differences in overall survival among the low expression group (FOXO3a level under 25th percentile of 63 patients, red line), middle expression group (25th–75th percentile, black line) and high expression level (above 75th percentile, blue line). Median survival times of the low, middle and high expression groups were 13, 21.5, and 33 months, respectively (C).

Figure 2. HDAC class Ⅱa inhibition induces FOXO3a upregulation

Cell lysates were obtained from AsPC-1, MIA PaCa-2, Panc-1, and BxPC-3 pancreatic cancer cell lines. Cell lysates were immunoblotted with indicated antibodies. β-actin was used as a loading control (A). Gene expression levels of HDAC4, HDAC5, HDAC7, and HDAC9 as obtained from the publicly available gene expression profiling dataset, GSE 15471 (B). Heatmap displaying the differentially expressed genes of the PI3K-AKT pathway in the AsPC-1 pancreatic cancer cell line. Data of the indicated genes were collected from the PCR array performed with AsPC-1 in the presence or absence of TMP269. FOXO3 (position: C02) was the fifth most upregulated gene among 84 examined genes, with a 1.99-fold increase (C). FOXO3a mRNA and protein expression was increased in a dose-dependent fashion (D and E).

Figure 3. TMP269 induces cell growth inhibition effect

AsPC-1, MIA PaCa-2, BxPC-3, and Panc-1 were treated with the indicated doses of TMP269 (0–100 μM) for 48 h, and cell growth was assessed using the MTT assay. Data represent mean ± s.d. in triplicate (A). AsPC-1 cells were treated with TMP269 (0–50 μM) for 48 h. Cell lysates were immunoblotted with the indicated antibodies. β-actin was used as loading control (B). Cell cycle distribution was analyzed using flow cytometric analysis (C).

Figure 4. TMP269 inhibits cell growth

AsPC-1 cells were treated with TMP269 (0–50 μM) for 48 h. Cell lysates were immunoblotted with the indicated antibodies. β-actin was used as loading control (A, B and C).

Figure 5. TMP269 combined with CFZ enhances FOXO3a upregulation

AsPC-1 cells were treated with TMP269, CFZ or both for 48 h. Cell lysates were immunoblotted with FOXO3a (A) and p21 (B). β-actin was used as loading control. Cell growth was assessed using the MTT assay (C). Data represent mean ± s.d. in triplicate.

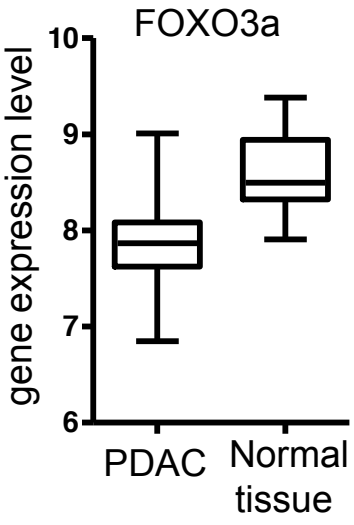
Supplemental Figure Legend

Supplemental Figure S1. FOXO3a gene expression level in 63 pancreatic cancer patients

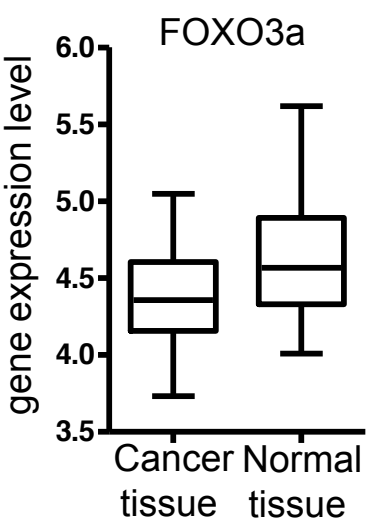
Publicly available gene expression profiling data GSE57495 was analyzed. FOXO3a expression levels in 63 resectable early stage pancreatic adenocarcinoma patient samples are shown as boxplots. The median level was 11.29, minimum and maximum levels were 10.65 and 12.17, respectively, and 25^{th} and 75^{th} percentile levels were 11.09 and 11.46.

Figure 1.

A.


FOXO3a
gene expression level
10
9
8
7
6
PDAC
Normal tissue

B.


FOXO3a
gene expression level
6.0
5.5
5.0
4.5
4.0
3.5
Cancer tissue
Normal tissue

C.

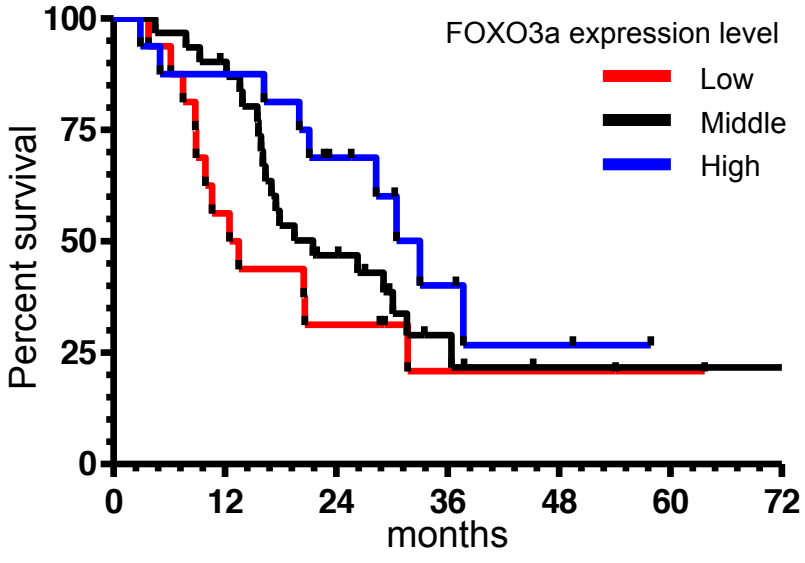

FOXO3a expression level
Low
Middle
High
Percent survival
100
75
50
25
0
0
12
24
36
48
60
72
months

Figure 2.

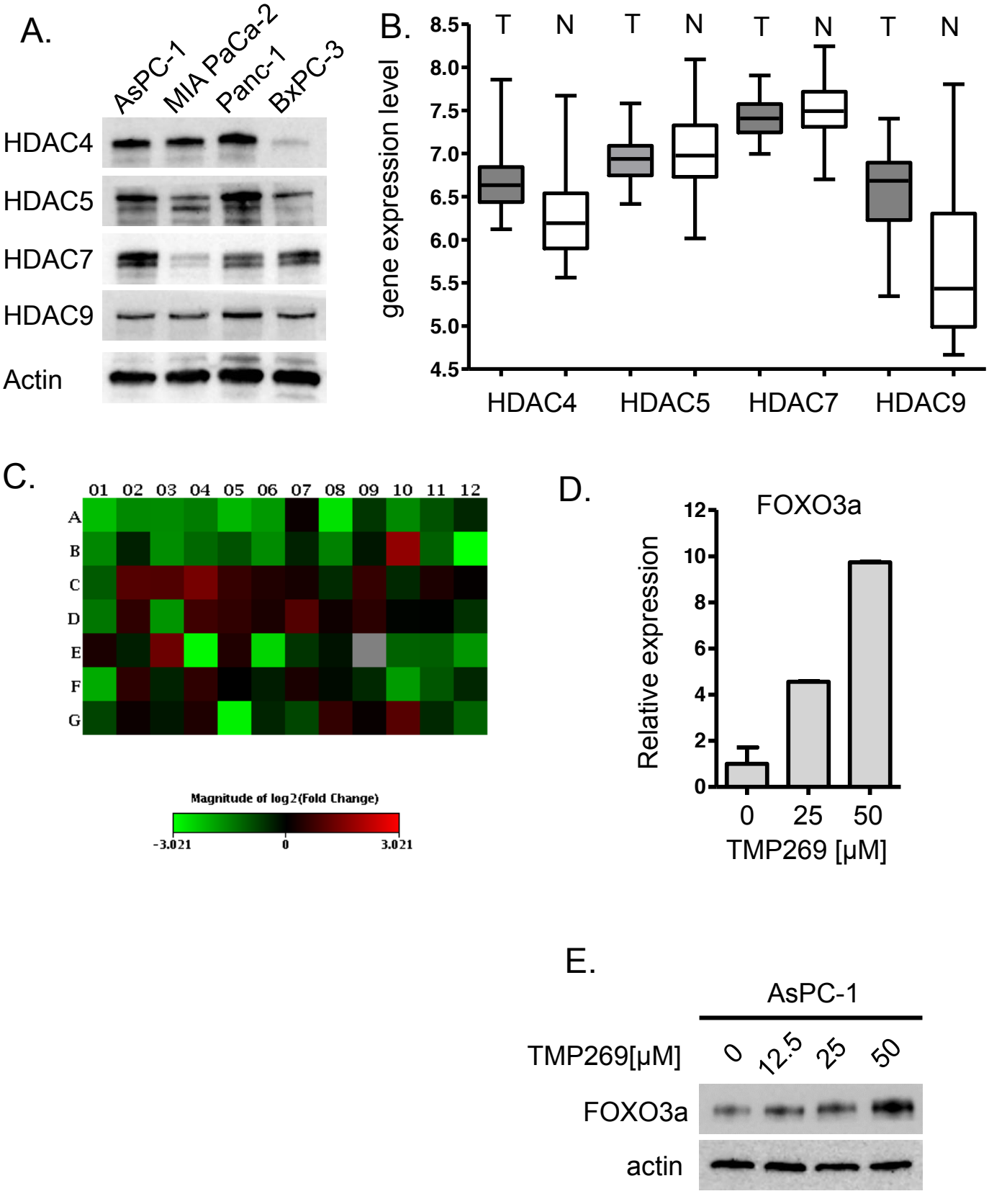

A.
AsPC-1
MIA PaCa-2
Panc-1
BxPC-3
HDAC4
HDAC5
HDAC7
HDAC9
Actin
B.
gene expression level
T N T N T N T N
HDAC4
HDAC5
HDAC7
HDAC9
C.
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-3.021
0
3.021
D.
FOXO3a
Relative expression
TMP269 [μM]
E.
AsPC-1
TMP269[μM]
0
12.5
25
50
FOXO3a
actin

Figure 3.

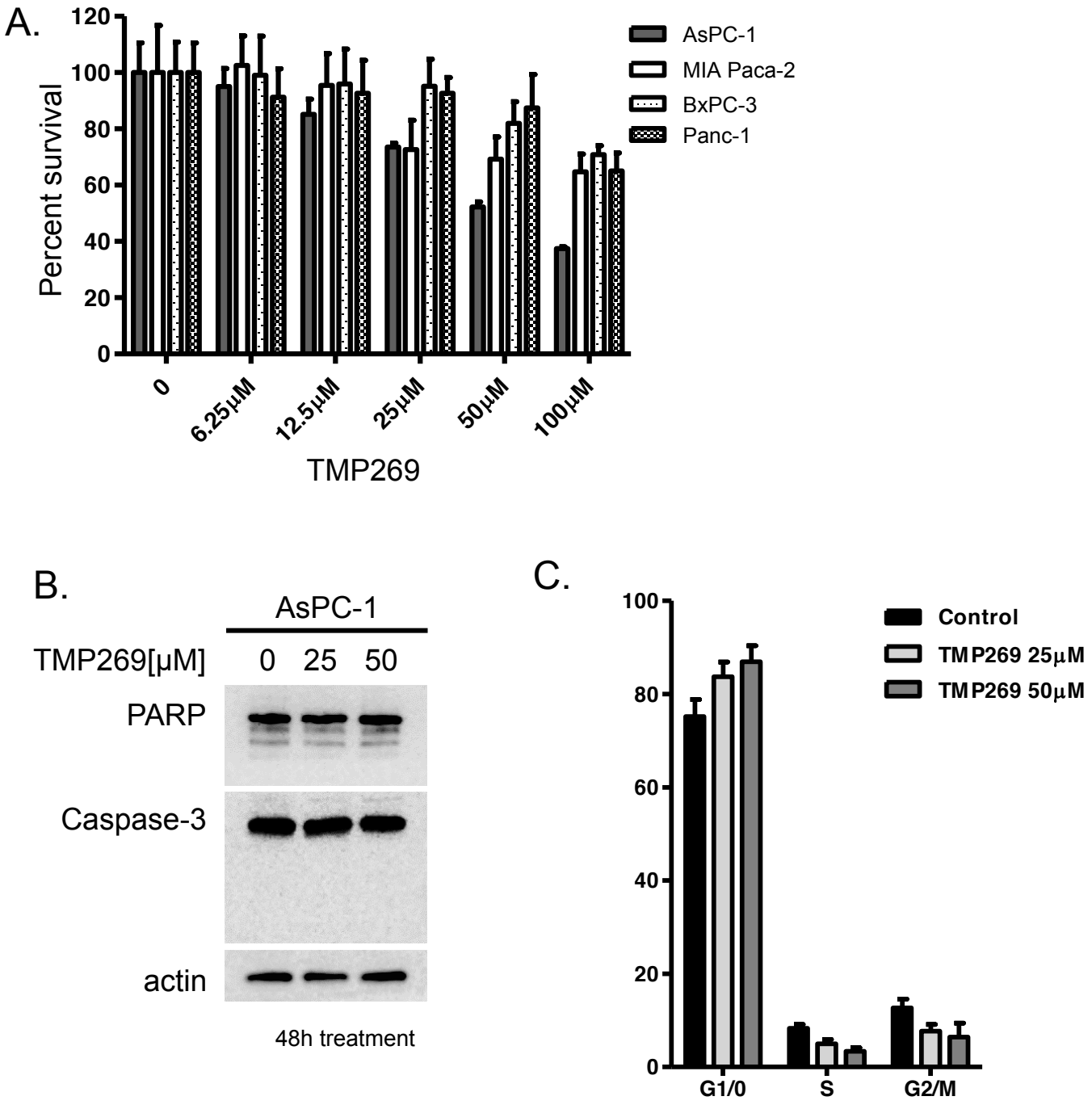
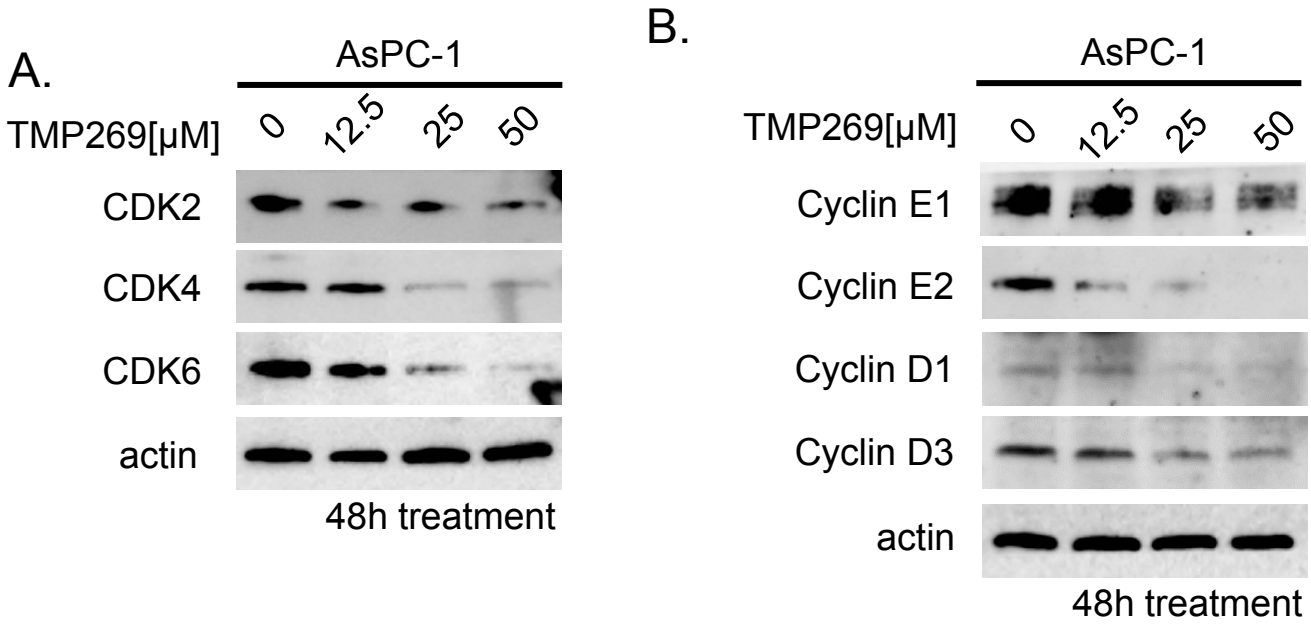

A.
Percent survival
120
100
80
60
40
20
0
0
6.25μM
12.5μM
25μM
50μM
100μM
TMP269
AsPC-1
MIA Paca-2
BxPC-3
Panc-1
B.
AsPC-1
TMP269[μM]
0 25 50
PARP
Caspase-3
actin
48h treatment
C.
100
80
60
40
20
0
G1/0
S
G2/M
Control
TMP269 25μM
TMP269 50μM

Figure 4.


A.
AsPC-1
TMP269[μM]
0
12.5
25
50
CDK2
CDK4
CDK6
actin
48h treatment
B.
AsPC-1
TMP269[μM]
0
12.5
25
50
Cyclin E1
Cyclin E2
Cyclin D1
Cyclin D3
actin
48h treatment

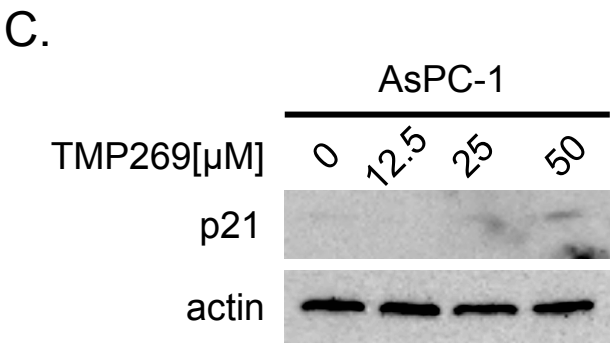

C.
AsPC-1
TMP269[μM]
0
12.5
25
50
p21
actin

Figure 5.

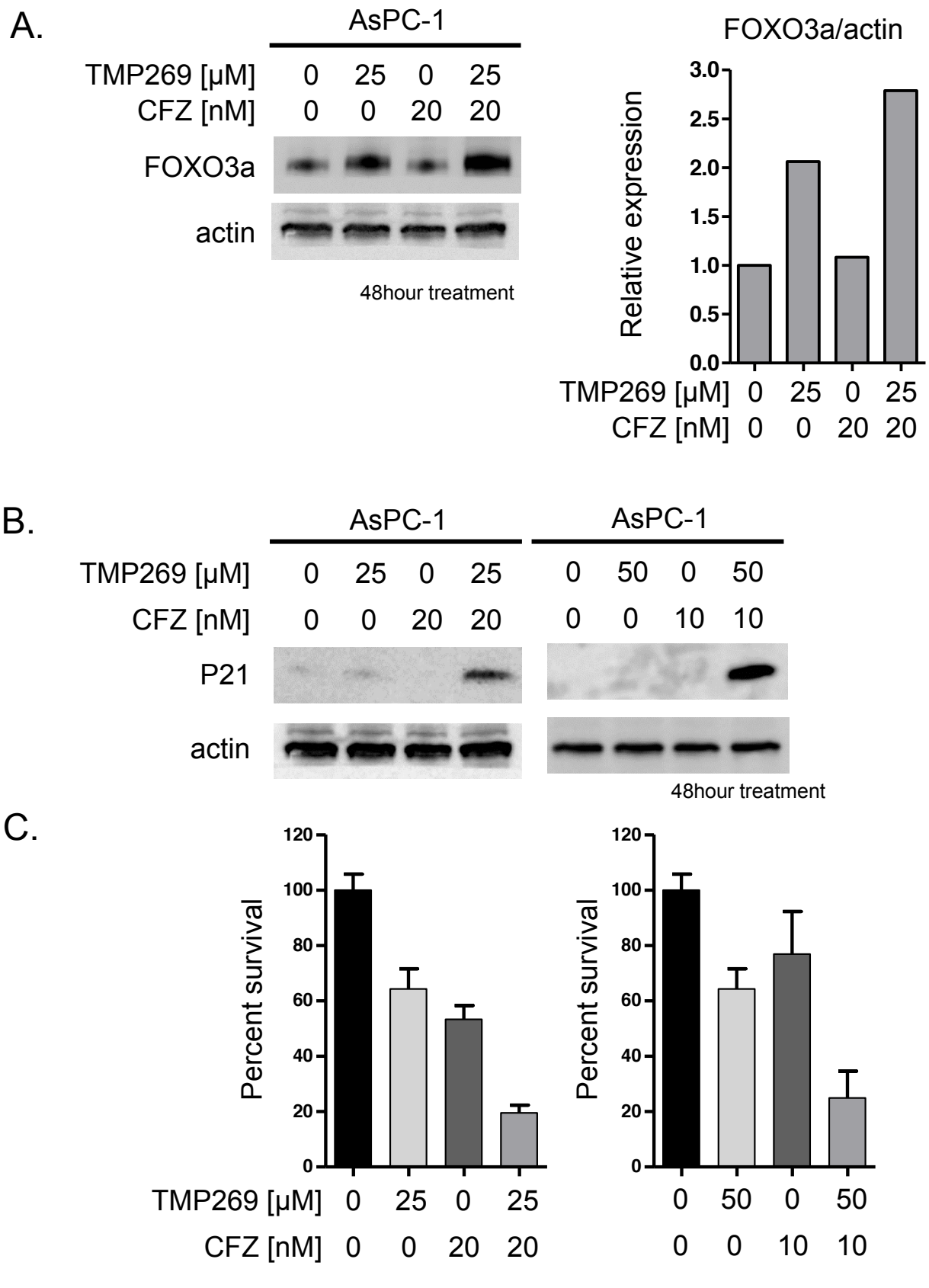

A.
AsPC-1
TMP269 [μM] 0 25 0 25
CFZ [nM] 0 0 20 20
FOXO3a
actin
48hour treatment
FOXO3a/actin
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TMP269 [μM] 0 25 0 25
CFZ [nM] 0 0 20 20
B.
AsPC-1
AsPC-1
TMP269 [μM] 0 25 0 25 0 50 0 50
CFZ [nM] 0 0 20 20 0 0 10 10
P21
actin
48hour treatment
C.
Percent survival
120
100
80
60
40
20
0
TMP269 [μM] 0 25 0 25
CFZ [nM] 0 0 20 20
Percent survival
120
100
80
60
40
20
0
0 50 0 50
0 0 10 10

Table 1.

Position	RefSeq Number	Gene Symbol	Description	Fold Regulation
B10	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	3.31
C04	NM_005311	GRB10	Growth factor receptor-bound protein 10	2.65
E03	NM_002576	PAK1	P21 protein (Cdc42/Rac)-activated kinase 1	2.43
G10	NM_000548	TSC2	Tuberous sclerosis 2	2.01
C02	NM_001455	FOXO3	Forkhead box O3	1.99
D07	NM_002746	MAPK3	Mitogen-activated protein kinase 3	1.97

Figure S1.

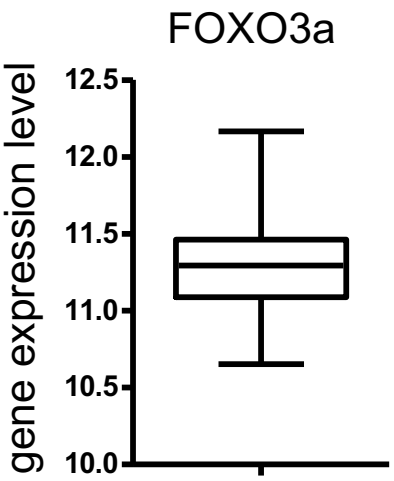


Table S1.

Position	RefSeq Number	Gene Symbol	Description	Fold Regulation
B12	NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog	-8.11
E04	NM_006206	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	-7.66
G05	NM_021966	TCL1A	T-cell leukemia/lymphoma 1A	-7.35
A08	NM_001229	CASP9	Caspase 9, apoptosis-related cysteine peptidase	-6.43
E06	NM_002611	PDK2	Pyruvate dehydrogenase kinase, isozyme 2	-5.82
A01	NM_001111	ADAR	Adenosine deaminase, RNA-specific	-4.77
A05	NM_000038	APC	Adenomatous polyposis coli	-4.53
F01	NM_002738	PRKCB	Protein kinase C, beta	-4.12
F10	NM_005614	RHEB	Ras homolog enriched in brain	-3.60
A06	NM_004322	BAD	BCL2-associated agonist of cell death	-3.55
D03	NM_002228	JUN	Jun proto-oncogene	-3.44
E12	NM_002737	PRKCA	Protein kinase C, alpha	-3.41
B03	NM_001904	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	-3.19
A03	NM_001626	AKT2	V-akt murine thymoma viral oncogene homolog 2	-3.17
B06	NM_001968	EIF4E	Eukaryotic translation initiation factor 4E	-3.16
A10	NM_000591	CD14	CD14 molecule	-3.12