Supplementary Information for

XP-524 is a Dual BET/EP300 Inhibitor that Represses Oncogenic KRAS and Potentiates Immune Checkpoint Inhibition in Pancreatic Cancer

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Materials and Methods

Cell Culture and Viability Assays

Human pancreatic cancer cells (Panc1 and MiaPaCa2) and murine pancreatic cancer cells (KPC105) were cultured in Dulbecco's Modified Eagle Medium (DMEM) DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), penicillin (100U/mL), and streptomycin (100mg/mL). ASPC1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% heat-inactivated FBS, penicillin (100U/mL), and streptomycin (100 mg/mL). Capan1 cells were grown in IMDM1 supplemented with 10% heat-inactivated FBS, penicillin (100U/mL), and streptomycin (100mg/mL). Human cancer cell lines were purchased from the American Type Culture Collection (ATCC), used within six months, and kept under passage 10. KPC105 cells were used as described in our previous study (1). All cell lines in the laboratory were tested for mycoplasma every 6 months via LookOut Mycoplasma PCR Detection Kit (Sigma Aldrich) and used only if negative.

For cell growth assays, a fixed number of serum starved tumor cells were seeded into each well of a 24 well plate to reach 10% confluence, the subsequent percent confluence evaluated every 4 hours until the control group reached 100% confluence. For cell viability assays, 2,000-4,000 cells were seeded into each well of a 96-well plate in serum free DMEM. After 16 hours, media/drug was added and cells cultured for 72 hours. At this time, we added 20ul of a 5mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution to each well (1:100). After two hours, media was aspirated and crystals dissolved in DMSO and 570 nm absorbance determined by plate reader.
BROMOscan Bromodomain Profiling

BROMOscan bromodomain profiling was provided by DiscoverX Corp. (Fremont, CA) dissociation constant (Kd) of test compounds with DNA-tagged bromodomains and determined through binding against a proprietary reference immobilized ligand.

Protein crystallization

Crystals of the BRD4 and CBP bromodomains complexed with XP-524 were grown by hanging drop vapor diffusion at 4°C. Prior to crystallization, 16 mg/mL BRD4 or CBP bromodomain was incubated with 2mM XP-524 for 60 min on ice and then centrifuged to remove precipitate. Crystals of the complex were grown by mixing 2μL of BRD4:XP-524 or CBP:XP-524 with 1-2μL of reservoir solution containing 17-19% polyethylene glycol (PEG) 3350 and 0.2 M lithium citrate tribasic tetrahydrate, pH 8.4.

Data collection and structure refinement

BRD4:XP-524 crystals were generated and analyzed as described previously (2). For CBP, crystals were cryo-preserved by soaking in mother liquor containing 10-15% glycerol and 200 μM of XP-524 before flash-freezing. Data were collected at the Life Sciences Collaborative Access Team 21-ID-F beamline at the Advanced Photon Source, Argonne National Laboratory. Data indexing, integration, and scaling were performed using XDS (3), and phases were determined by molecular replacement using first Phaser (4) and a CBP bromodomain structure (PDB entry: 5KTU) as search model. The assembly of four CBP:XP-524 monomers found by Phaser were then used as a search model by Molrep (5) to generate an asymmetric unit of two CBP:XP-524 “tetramers”. Rigid body refinement followed by iterative rounds of restrained
refinement and model building were performed with CCP4i (6) modules Refmac5 (7) and Coot (8). The coordinates and structure factors have been deposited with PDB accession code 7JUO.

RNA Sequencing and Gene Set Enrichment Analysis (GSEA)

Panc1 cells were treated with either a DMSO vehicle, 1μM of JQ-1, JQ-1 and SGC-CBP30, or XP-524. After 24 hours RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) per the manufacturer’s instructions. Quality control, sequencing, and data analysis were performed by Novogene. Data has been deposited under accession number GSE192903.

qPCR

Quantitative gene expression was performed with gene-specific TaqMan probes, TaqMan Universal PCR Master Mix, and the 7500 Fast Real-time PCR System from Applied Biosystems (Foster City, CA). These data were quantified with the comparative cycle threshold (C_T) method for relative gene expression as described in our previous study (9).

Western Blot and Immunoprecipitation

Cell or tissue lysates were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer (Cell Signaling) followed by sonication. Equal amounts of protein (15–50 μg) were mixed with loading dye, boiled for 8 min, separated on a 4-20% denaturing SDS–polyacrylamide gel electrophoresis (PAGE) gel and transferred to a Polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% milk/TBS/0.1% Tween for one hour and incubated with antibodies against pRB, pMEK1, MEK1, pERK1/2, ERK1/2, (Cell Signaling, Danvers, MA), H3K27ac (Active Motif, Carlsbad, CA), KRAS (Novus Bio, Saint Charles, MO), BRD4, EP300 (abcam, Cambridge, MA), or GAPDH (Santa Cruz Biotech, Santa Cruz, CA). The membrane was washed with tris-buffered saline (TBS)-0.1% Tween and then incubated with horseradish
peroxidase (HRP) conjugated secondary antibody (Cell Signaling) at room temperature for one hour and rewashed. Protein bands were visualized by an enhanced chemiluminescence method (Thermo Fischer, Waltham, MA) and resolved digitally per the manufacturer’s specifications.

For immunoprecipitation, cell lysates were collected using IP buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40 and 5% glycerol) with a protease and phosphatase inhibitory cocktail (Cell Signaling), and cell extracts were incubated overnight with the respective antibodies followed by incubation with protein A or G agarose beads for 4 h at 4°C. After washing 5–7 times with lysis buffer, immunocomplexes were resolved using SDS–PAGE and visualized by western blot. All antibodies were compared with isotype specific IgG controls to affirm specificity. All experiments were performed in triplicate unless otherwise specified.

**Transgenic Mice**

Nongenic B6 (Wild Type), P48-Cre x LSL-KrasG12D (KC), Pdx1-Cre x LSL-KrasG12D x LSL-TP53R172H+/− (KPC), and Pdx1 -Cre x LSL-Kras G12D x LSL -TP53R172H+/+ (KPPC) mice were generated as described previously (10). At 8 weeks of age, KC mice were administered an intraperitoneal injection (IP) of either a PBS vehicle or daily XP-524 (5mg/kg). Mice were euthanized at 6 months of age and tissues collected for analysis. For studies involving KPC mice, animals were enrolled at 15 weeks of age, at which point they received IP injections of a PBS vehicle, XP-524 (5mg/kg), anti-PD-1, or XP-524 and anti-PD-1 as described in the text. KPC mice were sacrificed when moribund or showing clear signs of health decline e.g fur loss, weight loss, or lethargy, or for non-survival studies at the fixed endpoint shown in the number of days after enrollment. For studies involving KPPC mice, animals were enrolled either when developing a 0.5 cm, palpable tumor or at 4 weeks of age. At this time, mice were administered an intraperitoneal injection (IP) of either a PBS vehicle or daily XP-524 (5mg/kg) and sacrificed.
when moribund or showing clear signs of health decline including ascites, hunched posture, lethargy, and/or weight loss. For euthanasia, animals were deeply anesthetized with isoflurane until unresponsive to toe tap and/or agonal breathing was observed. Thoracotomy served as the primary method of euthanasia and exsanguination the secondary method. For all mouse studies, mice were age matched within 2 weeks and males and females randomized at a 50:50 ratio.

**Primary Cell Line-Derived Xenografts**

G-68 primary cell line-derived xenografts were generated as described previously (1). Also as described (1), tumor size was measured twice weekly with digital caliper. Mice were euthanized when moribund, when the maximum tumor size allowed per institutional policy (2 cm), or when tumors became ulcerated. For euthanasia, animals were scarified by CO₂ suffocation followed by cervical dislocation, and tumors subsequently harvested and processed as described in the “transgenic mice” section.

**PDAC Slice Cultures**

Following the previously published protocol (11, 12), punch biopsies of ~6mm in diameter from human PDAC tumors were sectioned into 250 µm thick slices using the Leica VT1000S Vibrating blade microtome. The slices were then placed atop collagen-coated, 0.4 µm pore membrane inserts placed in 6-well plates. The next day, the slice cultures were treated with different drugs at the indicated doses. Fresh treatment media was replaced every 2-3 days. At the end of the experiment, slices were fixed with 4% PFA and processed by the Pathology Core at Northwestern University. The embedded tissues were subsequently embedded and sectioned for staining following the protocols described in the “histology, IHC, and immunofluorescence” section.
Histology, Immunohistochemistry, and Immunofluorescence

Mice were euthanized and the pancreas, colon, lungs, small bowel, liver, and spleen were subjected to pathologic examination. Tissues were fixed in 10% formalin, paraffin-embedded, and sections at 4 mm interval were cut from each tissue, and stained with hematoxylin and eosin (H&E), trichrome (Sigma Aldrich), or via immunohistochemistry (IHC) or immunofluorescence (IF). For immunohistochemistry, slides were deparaffinized by xylenes and rehydrated by ethanol gradient, then heated in a pressure cooker using DAKO retrieval buffer (DAKO, Santa Clara, CA). Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol for 30 minutes. Tissues were blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 minutes and incubated with primary antibodies against: BRD4, EP300/CBP, CD45, CD8 (abcam), RAS\textsuperscript{G12D} (Genetex, Irvine, CA), pERK, or Cleaved Caspase 3 (Cell Signaling, Danvers, MA) at 1:50–1:200 overnight at 4°C. Slides were developed using HRP conjugated secondary antibodies followed by 3,3'-Diaminobenzidine (DAB) substrate/buffer (DAKO).

For immunofluorescence, slides were heated via pressure cooker in DAKO retrieval buffer and tissues blocked with 0.5% BSA in PBS for 1 hour at room temperature. Sections were exposed to primary antibodies against CK19 (University of Iowa Hybridoma Bank), E-Cadherin (Cell Signaling), PCNA, CD3, (Santa Cruz Biotechnology, Dallas, TX), or Granzyme B (abcam, Cambridge, MA) at 1:50–1:200 overnight at 4°C. Slides were developed using AlexaFluor 488-or 594-conjugated secondary antibodies (1:200–1:1,000, Abcam), mounted in DAPI-containing media (Santa Cruz Biotechnology), exposed to DAPI, Fluorescein isothiocyanate (FITC), and Texas Red filters.
Microscopy

All images were acquired using a Nikon 40x-400x Epi-Fluorescent Inverted Microscope with Phase Contrast Kit and Nikon bright field camera attachment. Negative slides were used for white balance, and for all images no analog or digital gain was used. For fluorescent imaging, we used positive control slides for each experiment and auto-exposed slides using Nikon NIS elements software using a gain setting of zero. Gain was similarly set to zero and Lookup Tables (LUTs) were used to reduce background based on negative control slides. These LUT values and exposure times were standardized and used for all other similarly stained slides. Images were superimposed also using Nikon NIS elements software.

Tissue Slide Counts and Measurements

All counts were performed by a minimum of three blinded, investigators and each value displays includes the average of at least three high power fields per specimen as described in our previous publications (1, 10, 13-16). Score distributions were visualized via Minitab express software, showing the median value as a solid line and all individual values excluding any statistical outliers.

Flow Cytometry

Spleens were isolated, ruptured, washed in cold PBS, and contents filtered. For tumor specimens, samples were digested with Collagenase IV/DNase at 37°C for one hour, mechanically dissociated, and filtered at 100μm. Two million cells were seeded into a round-bottom 96-well plate, washed in PBS, incubated with a Golgi plug/protein transport inhibitor (BD biosciences, San Jose, CA) and stained with Anti-CD45-FITC, Anti-CD11b-APC, Anti-CD206-PerCP/Cy5.5, Anti-GR-1-APC/Cy7 (BioLegend, San Diego, CA), Anti-CD4-APC/Cy7, Anti-CD25-PE, Ant-CD8A-PerCP/Cy5.5 (BD biosciences), Anti-CD69-APC, Anti-FoxP3-FITC
(eBiosciences, San Diego, CA), anti-CD4-APC-Cy7, CD25-PE (BD biosciences), as well as an Alive/Dead kit (Invitrogen, Grand Island, NY) at 1:200-1:1500 in PBS over ice for 30 minutes. Cells were then fixed with 1% PFA in PBS for 10 minutes at room temperature, and for panels evaluating cytotoxic T-cells, stained with anti-Perforin-APC (Thermo Fischer) and anti-Interferon-γ-PE (BioLegend) at 1:500-150 in perm/stain buffer (BD biosciences) for 30 minutes over ice and washed three times with perm/wash buffer (BD biosciences). Analysis was conducted similarly using the same gating parameters and instrumentation, with a minimum of three mice included in each arm of analyses. Cells were analyzed with a BD Fortessa Cytometer, gating exclusively to cells within acceptable forward scatter (FSC)/side scatter (SSC) parameters. All subsequent flow plots correspond to live, single cells based on Live/Dead assay and SSC-W gating, and are representative of 100,000 events unless otherwise stated. High and low populations were identified based on the geometric mean of the control group, based on unstained and isotype controls for each antibody. All other experiments were compared to both unstained, single cell, and isotype controls.

**Genomic Database Analysis**

The Badea et al. and Pei et al. cohorts of PDAC patients were was downloaded and visualized using the using the Oncomine platform (https://www.oncomine.org/). Detailed information regarding these datasets and DNA/RNA sequencing analyses and protocols can be found on the data portal webpage listed above. All mRNA expression values are plotted in log scale unless otherwise noted.

**Authentication of Key Resources**

All mouse genotypes were verified by tail biopsy followed by PCR for all relevant transgenes (Pdx1-Cre, P48-Cre, LSL-\(Kras^{G12D}\), LSL-\(TP53^{R172H}\)). The anti-PD-1 antibody RMP1-
14 is verified by the manufacturer (BioXcell) for the ability to recognize purified mouse PD-1 by western blot and has been successfully used to neutralize PD-1 signaling in murine pancreatic ductal adenocarcinoma (10, 17). Validation of the compound XP-524 is described in the manuscript text, as well as in the original work describing its synthesis (2).
| Antibody                  | Company     | Clone  | Product Number |
|--------------------------|-------------|--------|----------------|
| Anti-BRD4                | abcam       | EPR5150(2) | ab128874       |
| Anti-EP300/CBP           | -           | Not Provided | ab10485       |
| Anti-MHC Class 1         | -           | ERMP42 | ab15680       |
| Anti-HLA,A,B,C           | -           | EMR8-5 | ab70328       |
| Anti-GranzymeB           | -           | Not Provided | ab4059       |
| Anti-Pancreatic Amylase  | -           | Not Provided | ab21156       |
| Anti-CTLA4               | -           | CAL49 | ab237712       |
| Anti-FoxP3               | -           | Not Provided | ab54501       |
| Anti-Rat 488             | -           | Not Provided | ab150153       |
| Anti-Mouse 488           | -           | Not Provided | ab150117       |
| Anti-Rat 488             | -           | Not Provided | ab96971       |
| Anti-Rabbit 594          | Invitrogen  | Not Provided | A11037       |
| Anti-pRB                 | CST         | D20B12 | 8516S         |
| Anti-pERK1/2             | -           | Not Provided | 9101S       |
| Anti-ERK1/2              | -           | 137F5 | 4695S         |
| Anti-pMEK1               | -           | Not Provided | 9127S       |
| Anti-MEK1                | -           | 61B12 | 2352S         |
| Anti-E-Cadherin          | -           | 4A2   | 14472S        |
| Anti-Cleaved Caspase 3   | -           | Asp175 | 9661S         |
| Anti-PD-1                | -           | D7D5W | 84651S        |
| Anti-VISTA               | -           | D5L5T | 54979S        |
| Anti-H3K27ac             | Active Motif | Not Provided | 39133       |
| Anti-CD3                 | SCBT        | PC3/188A | sc-20047       |
| Anti-CD8                 | -           | H-160 | sc-7188        |
| Anti-PCNA                | -           | PC10  | sc-56         |
| Anti-GAPDH               | -           | 0411  | sc-47724       |
| Anti-CK19                | University of Iowa | TROMA-III | TROMA-III-c |
| Anti-Mouse HRP           | DAKO        | Not Provided | K4001       |
| Anti-Rabbit HRP          | -           | Not Provided | K4003       |
| Anti-CD45-FITC           | BioLegend   | 30-F11 | 103108       |
| Anti-CD11b-APC           | -           | M1/70 | 101212       |
| Anti-CD206-Cy5.5         | -           | C068C2 | 141716       |
| Anti-GR-1- Cy7           | -           | RB6-8C5 | 108424        |
| Anti-CD4-Cy7             | BD          | GK1.5  | 552051       |
| Anti-CD25-PE             | -           | PC61  | 553866       |
| Anti-CD8A-Cy5.5          | -           | 53-6.7 | 100734       |
| Anti-IFNy-PE             | -           | XMG1.2 | 505808       |
| Anti-PD-1-FITC           | -           | 29F.1A12 | 135213     |
| Anti-CD69-APC            | eBiosciences | H1.2F3 | 17-0691-82   |
| Anti-FoxP3-FITC          | -           | FJK-16s | 11-5773-82   |
| Anti-Perforin-APC        | -           | eBioOMAK-D | 17-9392-80 |

Table S1. Antibodies arranged by vendor
Figure S1. BRD4 and EP300/CBP traffic to the nucleus in murine models of PDAC

(A,B) Pancreas tissue from either non-genic wild type (WT), the Pdx1-Cre x LSL-Kras\textsuperscript{G12D} (KC) model of PanIN disease, the Pdx1-Cre x LSL-Kras\textsuperscript{G12D} x LSL-TP53\textsuperscript{R172H/+} (KPC) model of advanced PDAC, the Pdx1-Cre x LSL-Kras\textsuperscript{G12D} x LSL-TP53\textsuperscript{R172H/+} (KPPC) model of extremely aggressive PDAC, or subcutaneous tumor tissue from the G-68 cell derived xenograft (CDX) model were collected and stained for either BRD4 or EP300/CBP. The number of positive nuclei per 40X field was quantified by three blinded investigators and divided by the total number of nuclei in each field. These values were averaged and displayed as an individual value plot. (*p < 0.05)
Figure S2. XP-524 restrains tumor cell growth in a similar manner to JQ-1 combined with EP300 inhibition

(A) An equal number of Panc1 cells were seeded in 24 well plates and treated with either a DMSO vehicle or 1μM of SGC-CBP30. Cell growth was evaluated every four hours until the control group reached 100% confluence (N=4/group). (B,C) An equal number of MiaPaCa2 or ASPC1 cells were seeded in 24 well plates and treated with either a DMSO vehicle, 1μM JQ-1, 1μM of SGC-CBP30, JQ-1 and SGC-CBP30, 1μM XP-524, or XP-524 and SGC-CBP30. Cell growth was evaluated every four hours until the control group reached 100% confluence (N=4/group). (D) Panc1 cells were incubated with increasing concentrations of SGC-CBP30 and cell viability measured after 48 hours by MTT assay.
Figure S3. XP-524 fails to promote a functional anti-tumor immune response in KPC mice

(A) Pdx1-Cre x LSL-Kras$^{G12D}$ x LSL-TP53$^{R172H+/−}$ (KPC) mice were generated as a model of advanced PDAC. Starting at 15 weeks of age, mice were administered daily intraperitoneal injections of either PBS vehicle or 5mg/kg XP-524. Pancreas tissues were collected when the animals were moribund, and stained for CD3 and CTLA-4, CD3 and VISTA, or CK19 and Granzyme B (GzmB).

(B) KPC mice were enrolled as described and treated with either a PBS vehicle or 5mg/kg XP-524. After two months on treatment, spleens were collected and analyzed by flow cytometry for tumor infiltrating CD4+ and CD8+ T-cells, respectively (N=4/group).

(C) Also following two months on treatment, tumor tissues were collected and analyzed by flow cytometry for CD45+CD11b+GR-1+ macrophages, as well as those positive for the M2 marker CD206 (N=4/group). Abbreviations: Forward scatter (FSC); Side scatter (SSC).
Figure S4. The combination of XP-524 and PD-1 inhibition leads to a modest increase in splenic T-cells and a relative decrease in tumor-infiltrating M2 macrophages

(A) Pdx1-Cre x LSL-Kras\textsuperscript{G12D} x LSL-TP53\textsuperscript{R172H+/−} (KPC) mice were generated as a model of advanced PDAC. Starting at 15 weeks of age, mice were administered either twice weekly intraperitoneal injections of 200μg of anti-PD-1, or daily injections of 5mg/kg XP-524 with twice weekly injections of anti-PD-1. Mice were sacrificed after two months on their respective treatments, at which time spleens were collected and analyzed by flow cytometry for tumor CD4+ and CD8+ T-cells, respectively. CD4+ T-cells were also evaluated for and analyzed for expression of Treg markers CD25 and FoxP3 (N=4/group). (B) Also following two months on treatment, tumor tissues were collected and analyzed by flow cytometry for CD45+CD11b+GR-1+ macrophages, as well as those positive for the M2 marker CD206 (N=4/group). Abbreviations: Forward scatter (FSC); Side scatter (SSC).
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