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Title

Drosophila screening identifies dual inhibition of MEK and AURKB as an effective therapy for pancreatic ductal adenocarcinoma

Authors

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Running title

Combination therapy for PDAC identified in *Drosophila*

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Significant progress has been made in understanding the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) by generating and utilizing murine models. In order to accelerate drug discovery by identifying novel therapeutic targets on a systemic level, here we generated a *Drosophila* model mimicking the genetic signature in PDAC (*KRAS*, *TP53*, *CDKN2A*, and *SMAD4* alterations), which is associated with the worst prognosis in patients. The ‘4-hit’ flies displayed epithelial transformation and decreased survival. Comprehensive genetic screening of their entire kinome revealed kinases including MEK and AURKB as therapeutic targets. Consistently, a combination of the MEK inhibitor trametinib and the AURKB inhibitor BI-831266 suppressed the growth of human PDAC xenografts in mice. In PDAC patients, the activity of AURKB was associated with poor prognosis. This fly-based platform provides an efficient whole-body approach that complements current methods for identifying therapeutic targets in PDAC.

Statement of Significance

Development of a *Drosophila* model mimicking genetic alterations in human pancreatic ductal adenocarcinoma provides a tool for genetic screening that identifies MEK and AURKB inhibition as a potential treatment strategy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant forms of cancer, with a five-year relative survival rate of merely 9% (1). At present, PDAC accounts for the third leading cause of cancer-related mortality. However, projections suggest that the incidence of PDAC keeps increasing, potentially propelling it to the second cause of cancer-associated death by 2025 in the US (2). The current standard of care for PDAC includes surgical resection and cytotoxic chemotherapy utilizing gemcitabine combined with nab-paclitaxel and FOLFIRINOX (a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin). However, they provide patients with only limited survival benefits due to their marginal efficacy and significant toxicity. In an effort to supplement these remedies, targeted therapy has been extensively studied for PDAC. Nonetheless, therapeutic targets remain largely unexplored, and even the approved EGFR inhibitor drug erlotinib combined with gemcitabine has had a minimal impact on patient survival (3). Furthermore, immune checkpoint therapy has failed to elicit responses in the majority of PDAC patients (4). Collectively, PDAC represents an urgent and clinical unmet need.

One of the significant problems hindering drug development for the PDAC treatment is the lack of an efficient and comprehensive screening platform utilizing whole-body animal models. Namely, cultured human PDAC cells frequently used in chemical screening are inadequate in modeling PDAC as a systemic disorder involving heterotypic inter-cellular/-organ interactions as well as drug metabolism. On the other hand, mouse models are labor-intensive and costly to generate, maintain, and analyze. To overcome these limitations, we have been employing the fruit fly *Drosophila* in conjunction with these models in cancer research. Indeed, flies possess highly-conserved genes and signaling pathways as well as tissue/organ functions with mammals; over 70% of human genes associated with diseases including cancer are also present in the fly genome. In addition, flies that are naturally mutated or genetically modified for such genes can be easily generated or are already available from resource banks, offering a useful genetic toolkit for whole-body analyses (5). Furthermore, flies mature rapidly with high reproductive capacity. With their low husbandry cost (< 0.1% of mice per animal), flies allow for fast and cheap screening.

Based on these and other advantages, *Drosophila* models mirroring human cancer genotypes have been developed for specific cancer types to delineate their mechanisms and novel therapeutic targets. For example, *Ret*^{M955T} flies modeling the genotype of medullary thyroid cancer (MTC) to demonstrate epithelial transformation proved efficient in evaluating chemical efficacy, supporting the development of vandetanib as the first targeted therapy for MTC as well as other kinase inhibitor leads (6,7). Furthermore, we have developed a new method ‘Rational Polypharmacology’ to broaden the therapeutic window of the approved kinase inhibitor drug sorafenib which exhibits significant toxicity in the clinic. The key to this method is to perform chemical genetic screening for the whole kinome in *Ret*^{M955T} flies to determine ‘anti-target’ kinases of sorafenib, whose inhibition is responsible for sorafenib toxicity. Subsequent *in silico* modeling and *Drosophila* screening provided sorafenib analogs with significantly decreased binding

capacity to the anti-targets thus higher efficacy than sorafenib in a mouse model of RET-dependent MTC (8–11). Furthermore, flies have proven invaluable in identifying the disease mechanisms and therapeutic seeds for cancers including lung and colorectal cancers (12–14). These studies demonstrate that flies offer an effective whole-body toolkit for delineating the cancer mechanisms and enhancing therapy development.

Here, we present the first PDAC study leveraging the platform combining flies and mice: we generated and analyzed flies mimicking genetic alterations in PDAC to unveil novel therapeutic targets MEK and Aurora kinase B (AURKB) for PDAC treatment.

Materials and Methods

Construction of 4-hit flies modeling PDAC genotypes

Messenger RNA was extracted using RNeasy Mini Kit (Qiagen; Hilden, Germany) from non-transgenic flies [w¹¹¹⁸, Bloomington *Drosophila* Stock Center (BDSC; Bloomington, IN)] and reverse-transcribed using ReverTra Ace® qPCR RT Master Mix (Toyobo; Osaka, Japan). This complementary DNA (cDNA) pool was used to amplify *Drosophila Ras85D* (*dRas85D*; a KRAS ortholog) by PCR. Taking this amplicon as template, GGA at codon 12 was substituted to GAT using QuikChange site-directed mutagenesis kit (Agilent; Santa Clara, CA). Resulting *Ras*^{G12D} was cloned into an expression vector (14) containing two UAS (Upstream activation sequence) cloning cassettes along with a short hairpin RNA (shRNA) knockdown sequence for *p53* (TGCTGAAGCAATAACCACCGA). This plasmid was injected into *y1w67c23;P{CaryP}attP2* embryos to generate *UAS-Ras*^{G12D},*UAS-p53*^{shRNA} flies which harbor the transgenes on the left arm of the 3rd chromosome. Similarly, *Drosophila Cyclin E* (*dCycE*) was obtained and cloned with HA-tag into the empty expression vector along with a knockdown sequence for *Medea* (*Med*; *SMAD4* ortholog in *Drosophila*) (TTCAGTGCATGAACATTGCT). Upon injecting into *PBac{yellow[+]-attP-9A}VK00027* embryos, we obtained *UAS-dCycE*,*UAS-Med*^{shRNA} flies with transgenes on the right arm of the 3rd chromosome. Then resulting flies were crossed to generate *UAS-Ras*^{G12D},*UAS-p53*^{shRNA},*UAS-dCycE*,*UAS-Med*^{shRNA} (*UAS-4-hit*) flies through meiotic recombination.

Drosophila genetic screening

All fly studies were conducted under protocols approved by Hokkaido University Safety Committee on Genetic Recombination Experiments (approval numbers: 2019-007 and 2022-029). Fly stocks carrying kinase gene mutation or small interfering RNA (siRNA) were obtained from BDSC, KYOTO Stock Center (Kyoto, Japan), and National Institute of Genetics (Mishima, Japan). Stocks balanced with *FM6*, *FM7a*, *FM7c* or *FM7i* balancer were outcrossed with the balancer stock *FM7c-Tb-RFP*. Similarly, *CyO*-, *SM5*- or *SM6a*-balanced stocks were rebalanced with *CyO-Tb-RFP*, while *TM3*-, *TM6C*- or *MKRS*-balanced flies were rebalanced with *TM6B* balancer carrying *Tubby* (*Tb*) as a visible marker. In genetic screening for kinase genes located on X chromosome, *4-hit* males were crossed with *w*⁻ (control) or kinase-mutant females, whereas *4-hit* females were crossed with *w*⁻ (control) or kinase-mutant males for testing heterozygosity of kinase genes on 2nd, 3rd, or 4th chromosome (schemes in Fig. S1A-I). In screening for siRNA, *4-hit* females were crossed with males harboring *UAS*-siRNA for kinase knockdown (scheme in Fig. S1J). Resulting *4-hit* progenies with or without kinase heterozygosity or knockdown were cultured until adulthood on fly food for 11 days at 27-degrees C. The number of adults was divided by that of total pupae to determine percent viability. For wing disc analyses, developing discs were collected from L3 offspring obtained and observed as described in the supplementary method section. Human orthologs of fly genes were predicted by *Drosophila* RNAi Screening Center (DRSC) integrative ortholog predictive tool (DIOPT: https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl).

Drosophila chemical testing

All chemicals were dissolved in DMSO (Sigma Aldrich; St. Louis, MO) to prepare stock solutions (Table S1). Fly food with vehicle and/or chemicals (0.1% final DMSO concentration) was aliquoted into plastic vials (Thermo Fisher Scientific; Waltham, MA). Before screening, maximum tolerated dose (MTD) of each chemical was determined by evaluating viability of non-transgenic control flies fed with the chemical. Then virgin females of *UAS-4-hit* were crossed with *Serrate (Ser)-gal4* males to obtain *Ser-gal4;UAS-4-hit (Ser>4-hit)* offspring, and their viability was determined as in genetic screening at 22-degrees C.

Tissue microarray

All patient studies were conducted under protocols approved by Hokkaido University Ethical Review Board for Life Science and Medical Research (approval number: 019-0154). Patients with PDAC who had undergone surgical resection in Department of Gastroenterological Surgery II at Hokkaido University Hospital from 2000 to 2010 were retrospectively evaluated via medical records and pathology reports. Written informed consents to provide surgical samples were obtained from all the patients before surgery. Among 87 specimens obtained from patients without preoperative nor postoperative adjuvant treatments, one specimen was excluded due to the lack of sufficient clinical information. A total of 86 specimens were examined in tissue microarray (TMA) (Tables S2-4). TMA blocks were constructed using a manual tissue microarrayer JF-4 (Sakura Finetek Japan; Tokyo, Japan) with a 2.0-mm-diameter needle from representative tumor areas. Tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Heat-induced antigen retrieval was carried out in high-pH antigen retrieval buffer (Agilent). Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 5 min, and sections were incubated with the primary antibody against phosphorylated histone H3 (pHH3, 1:100, Sigma Aldrich; RRID, AB_2335642) or Ki-67 (ready to use, Agilent; RRID, AB_2890068) for 30 min. Staining was visualized by EnVision FLEX system (Agilent). Immunostained sections were counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. Specimens were scored for a ratio of the number of pHH3- or Ki-67-positive cancer cells to that of cancer cells in the whole core by two researchers (SS and KH) who were blinded to the clinical information of patients. The patients were classified into pHH3-positive or -negative groups (cutoff: > 0%), and overall survival and other characteristics were compared between two groups using appropriate statistical methods.

Mouse xenograft assay

All mouse studies were conducted under protocols approved by Hokkaido University Safety Committee on Genetic Recombination Experiments (approval number: 2022-029) and Hokkaido University Animal Research Committee (approval numbers: 19-0121, 2022-0117, and 2022-0118). Female BALB/c-*nu/nu*

mice (6-week-old, CLEA; Tokyo, Japan) were housed under specific pathogen-free conditions on a 12:12 hours light:dark cycle. All surgical processes were done in mice anesthetized by subcutaneous dosing of 15 mg/kg of medetomidine (Kyoritsuseiyaku; Tokyo, Japan), 200 mg/kg of midazolam (Astellas pharma; Tokyo, Japan) and 250 mg/kg of butorphanol tartrate (Meiji Seika Pharma; Tokyo, Japan). The anesthesia was reversed with 150 mg/kg of atipamezole (Kyoritsuseiyaku) after the procedure. To generate murine subcutaneous tumors, 5×10^6 cells in 100 μ L of chilled Matrigel (Corning; Tewksbury, MA):PBS (1:1) were injected subcutaneously to the right flank of mice. Body weight was calculated by subtracting tumor weight (mg, equal to mm^3 tumor volume) from total body weight. Tumor volume was calculated through the formula $\text{length} \times \text{width} \times \text{width} / 2$. When tumor volume achieved $\sim 100 \text{ mm}^3$, mice were randomly grouped into four arms, and each arm was dosed orally 5 days per week with vehicle (5% DMSO in saline) or tram (1 mg/kg/day; MedChemExpress, NJ; Table S1), B8 (10 mg/kg/day; Boehringer Ingelheim, Ingelheim, Germany) or tram (1 mg/kg/day) combined with B8 (10 mg/kg/day). To generate murine orthotopic tumors, a small incision was made in the left abdominal flank. AsPC-1 cells (ATCC; Manassas, VA) transduced with *luciferase* cDNA were adjusted to a concentration of 1×10^6 cells in 30 μ L of Hanks' Balanced Salt Solution (HBSS, Life technologies; Carlsbad, CA) supplemented with 50% Matrigel and were then injected into the pancreatic tail using a 30 G needle (Nipro; Osaka, Japan) affixed to a 100 μ L Hamilton microsyringe. A cotton swab was held for 30 seconds over the injection site to prevent any possible leakage. The abdominal incision was subsequently sutured, and animals were allowed to recover. The mice were randomly assigned to one of the four arms ($n = 6/\text{group}$) based on similar average tumor size, as determined by the bioluminescence measured using IVIS Spectrum Imaging System (Caliper Life Science; Mountain View, CA) 15 min after administration of D-luciferin (150 mg/kg intraperitoneal injection; BioVision, Palo Alto, CA). The mice were then dosed orally five times a week for a period of four weeks with vehicle (5% DMSO in saline), tram (1.0 mg/kg/day), B8 (10 mg/kg/day), or a combination of tram (1.0 mg/kg/day) and B8 (10 mg/kg/day). Bioluminescent signals were analyzed on weekly basis by the IVIS system and quantified using Living Image Ver.4.2 (Caliper; Hopkinton, MA). On or before day 28 after starting treatment, mice were sacrificed for analyses following the endpoints approved by Institutional Animal Care and Use Committee (IACUC): (i) Animals showing signs of significant discomfort, (ii) ascites or overt signs of tumor metastasis or gastrointestinal bleeding (blood in stool), (iii) animals losing $> 15\%$ of their body weight, or (iv) animals with tumors > 2 cm in diameter.

Cell culture

Human PDAC cell lines MIA PaCa-2 and PANC-1 were authenticated and provided by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. Authenticated Capan-1 and AsPC-1 were purchased from ATCC. Patient-derived PCI-55 cells were established from primary PDAC resected surgically at Hokkaido University Hospital and was kindly provided by Department of

Pathology, Hokkaido University (Sapporo, Japan). All cell lines were maintained in appropriate media (Table S5) supplemented with fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Nacalai Tesque; Kyoto, Japan) at 37-degrees C under 5% CO₂. No Mycoplasma tests were performed for these cell lines. All cell lines were used within 2 months after thawing.

Statistical analysis

All statistical analyses were performed using R version 4.1.1. All probability values were two-tailed, and the significance level was set at $P < 0.05$. Bliss synergy scores in combination assay were calculated using SynergyFinder (version 3.0.14; RRID, SCR_019318).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. All other raw data are available upon request from the corresponding author.

Results

Establishing a Drosophila model for PDAC genotype

PDAC frequently harbors somatic mutation combinations of four specific genes, including activation of the oncogene *KRAS* and inactivation of the tumor suppressor genes *TP53*, *CDKN2A*, and/or *SMAD4*. These mutations are proposed to underlie the progression of pancreatic ductal epithelium into benign tumors and PDAC (15). Consistently, patient prognosis deteriorates as the number of mutated genes increases. In fact, patients with PDAC harboring mutations in all of these genes exhibit the poorest prognosis among patients with other mutation patterns (16).

Animal models generating endogenous tumors can advance our understanding of the PDAC mechanisms and therapeutic targets. Currently, however, there are no available genetically-engineered mouse models (GEMMs) for this ‘4-hit’ genotype, largely owing to technical difficulties. Therefore, we employed *Drosophila* to generate the first 4-hit animal model efficiently (Fig. 1A). To this end, we utilized *Drosophila Ras*^{G12D} (*dRas*^{G12D}) cDNA as a transgene, which is equivalent functionally to the active *KRAS*^{G12D} mutant found in most PDAC patients (16). We also exogenously expressed *Drosophila Cyclin E* (*dCycE*) in this model, which phenocopies loss of *CDKN2A* in mammals (17). In addition, we knocked down a *TP53* ortholog *p53* and a *SMAD4* ortholog *Med* using transgenic shRNA in these flies. To induce these transgenes, we employed the binary GAL4-UAS system, which utilizes a combination of ‘driver’ flies and ‘UAS’ flies. Namely, driver flies carry GAL4, the temperature-sensitive yeast transcription factor, driven by a specific enhancer. On the other hand, UAS flies contain the GAL4-inducible UAS sequence upstream of transgenes. Crossing these two strains allows for spatiotemporal control of the aforementioned transgenes.

In previous reports, we have demonstrated that the developing wing disc in flies is a useful tissue to model epithelial transformation caused by RTK-Ras signaling. Wing discs are composed of epithelial monolayers and are easy for evaluating inhibitors against RTK-Ras and other kinase signaling pathways (7–11). We employed the *patched* (*ptc*)-*gal4* driver to target transgenes to an organized central region in wing discs to evaluate morphological changes including proliferation and migration of the transformed cells as in the previous reports (9–11). First, we found that the *dRas*^{G12D} transgene alone in ‘1-hit (*ptc>dRas*^{G12D})’ flies caused epithelial transformation inducing stripe expansion (Fig. 1B-C and Fig. S2A). Intriguingly, ‘4-hit’ flies expressing all four transgenes (*dRas*^{G12D}, *p53*^{shRNA}, *dCycE*, and *Med*^{shRNA}) displayed more severe disorganization of the *ptc* domain and an aggressive migration of transformed cells than 1-hit flies (Fig. 1B-C). Consistent with these phenotypes, fly viability decreased as the transgene number accumulated and 4-hit flies were lethal at the pupal stage (Fig. 1D), which is in line with the clinical setting where patient prognosis worsens as their PDAC acquires more mutations (16). To functionally validate transgene cDNAs, we employed the *Serrate* (*Ser*)-*gal4* driver, which resulted in higher fly viability than *ptc-gal4* allowing us to obtain sufficient animals for large-scale experiments such as immunostaining and genetic screening. As expected, we detected increased levels of phosphorylated

ERK (pERK) as a downstream effector of *Ras*^{G12D} and HA-tagged dCycE by immunofluorescence (Fig. 1E-H and Fig. S2B, Table S6) (18). Subsequently, we utilized the *765-gal4* driver, which is active in the majority of the disc (14). This protocol allowed us to confirm knockdown of the endogenous expression levels of *p53* and *Med* genes by RT-qPCR in *4-hit* discs as compared with control (Fig. 1I-J and Fig. S2C, Table S7). The knockdown efficiencies for *p53* and *Med* were modest, with an approximate reduction of 75% and 60%, respectively. This observation could be attributed to the inactivity of the *765-gal4* driver in a small fraction of the disc cells, resulting in a reduction of the overall knockdown efficiency within the discs. These results indicate that we have successfully generated novel fly models recapitulating PDAC genotypes, which manifests epithelial transformation and animal lethality.

Identifying MEK and Aurora kinases as therapeutic targets through genetic screening in 4-hit flies

Kinases play pivotal roles as central nodes in various signaling pathways that govern cancer progression, serving attractive therapeutic targets for treating cancers (19). In order to identify novel therapeutic targets for PDAC, we conducted ‘dominant modifier screening’, a gold standard method in the field of fly genetics (Fig. 2A). By introducing a heterozygous mutation of a gene into a fly model, the contribution of the gene to a particular phenotype can be determined in a whole-animal manner. Also, this genetic approach to reduce the activity of a specific kinase throughout the body is useful to imitate the inhibition of the kinase upon drug administration. Indeed, this type of screening has provided key genes thus mechanistic insights in biologic processes such as organismal development and tumorigenesis (11). Particularly, we have successfully determined therapeutic target kinases and signaling networks in MTC pathogenesis through this screening in a fly model of MTC (9,10).

To perform this assay, we employed the *Ser-gal4* driver to induce the *4-hit* transgenes in tissues including wing discs to reduce pupal viability by 80~90% at 27-degrees C. In fact, fly viability of 10~20% turned out to be useful as a practical baseline to identify both suppressors and enhancers of transformation phenotypes in our previous work (9), which was also true in this study analyzing the *4-hit*-dependent transformation. Here, we introduced a heterozygous mutation of each of 220 kinase genes covering ~90% of the entire kinome into *Ser>4-hit* flies in a similar manner to our prior MTC study (9) (Fig. 2A and Fig. S1A-I).

Through this assay, we found that heterozygosity of *Dsor1* (a fly ortholog of human *MEK*) rescued lethality of *4-hit* flies almost completely (Fig. 2B and Table S8). This result is consistent with the established role of MEK in RAS-dependent transformation (15). Furthermore, our findings have led us to identify other kinases whose inhibition suppressed *4-hit* fly lethality. Of particular interest were *Src42A*, *wee*, *rok*, and *aurA* due to the existence of inhibitors targeting their human orthologs *FRK* (*fyn related Src family tyrosine kinase*), *WEE1*, *ROCK1* (*Rho associated coiled-coil containing protein kinase 1*), and *AURK* (*Aurora kinase A/B/C*), respectively (Fig. 2B and Tables S8-9). To delineate the role of these kinases in transformed cells, we employed also *UAS-kinase*^{siRNA} stocks, which enabled knockdown of the

kinases through GAL4-dependent induction of siRNA (Fig. S1J). In this assay, knockdown of another aurora kinase member *aurB* (a fly ortholog of human *AURKB*) significantly rescued lethality in *4-hit* flies (Fig. 2C). Consistent with this rescue, reducing the activity of *Dsor1* or *aurB* suppressed expansion of transformed cells in wing discs (Fig. 2D). Collectively, our findings demonstrate the utility of genetic screening for the entire kinome to pinpoint key drivers of transformation in a whole-body context.

Synergistic effects between kinase inhibitors on 4-hit fly viability

The aforementioned findings suggest that inhibiting MEK and AURK kinases presents a potential therapeutic avenue for PDAC patients. Therefore, we proceeded to investigate whether inhibitors targeting these kinases suppress the transformation phenotypes in *4-hit* flies (Fig. S3A). Administered via fly food, the orally available MEK inhibitor drug trametinib weakly increased the viability of lethal *4-hit* flies, to a maximum of 25% even at the maximum tolerated dose (MTD) of 1 μ M determined in wild-type control flies (Fig. 3A). Thus, we hypothesized that additional inhibition of the aforementioned candidates was useful to enhance the trametinib efficacy.

To test this hypothesis, we dosed trametinib concomitantly with an AURKB inhibitor BI-831266 (hereafter referred to as B8; Table S1) (20) to *4-hit* flies. As expected, this combination treatment rescued *4-hit* fly lethality more efficiently than trametinib monotherapy (Fig. 3A). The Bliss synergy score confirmed that the two chemicals worked synergistically, while B8 alone was not effective (Fig. 3B). When cultured at a permissive temperature of 16-degrees C, *4-hit* flies were able to survive due to decreased GAL4 activity. This survival, however, was accompanied by wing malformation resulting from significantly enhanced wing venation, a well-established marker of Ras signaling activation (21). Consistent with the viability rescue, the combination treatment suppressed this abnormality (Fig. 3C).

Our findings suggest that the combined inhibition of MEK and AURKB offers a novel therapeutic option to PDAC patients. Additionally, we confirmed the efficacy of trametinib in combination with inhibitors targeting other candidates of therapeutic targets identified in our genetic screening (Fig. S3B). In this study, however, our focus was on targeting AURKB in PDAC, because the potential significance of this strategy had not been fully evaluated, despite the established role of AURKB as an oncogenic regulator of cell division in other types of cancers (22).

Association between AURKB activation and poor prognosis of PDAC patients

Aurora kinases comprise a highly conserved family of serine/threonine kinases that were identified first in *Drosophila* (23). Among the three members in mammals (AURKA, AURKB, and AURKC), AURKB is a key regulator of mitosis through phosphorylating histone H3 (HH3) as its substrate (24). Although *AURKB* is upregulated in PDAC as compared with normal pancreatic tissues (Fig. S4), it remained unclear whether AURKB activity was associated with the prognosis of PDAC patients.

To investigate this issue, we conducted immunohistochemistry in PDAC tissues for phosphorylated HH3 (pHH3), a surrogate marker of AURKB activity (25). Out of the 86 specimens examined, 70 (81.4%) contained cancer cells exhibiting pHH3 expression in the nucleus (Fig. 4A and Tables S2-3). Significantly, patients with detectable pHH3 expression showed a significantly lower overall survival rate as compared with those with negligible pHH3 expression (HR = 2.12, 95% CI = 1.10 - 4.08, $P = 0.026$). Notably, this finding retained its significance even after adjusting for covariates such as patient age, sex, and Ki-67 scores (Fig. 4B and Table S4). These results indicate that AURKB activation correlates with an unfavorable prognosis in PDAC patients.

Dual inhibition of MEK and AURK suppresses growth of human PDAC xenograft in mice

To validate the efficacy of the trametinib/B8 combination (hereafter referred to as TB) in mammals, we employed an established subcutaneous PDAC xenograft model in nude mice using MIA PaCa-2 human PDAC cells (26). Following the determination of the oral MTD of trametinib and B8, respectively (Fig. S5A), as well as pharmacokinetics of B8 in mice (Fig. S5B-C), we administered trametinib and/or B8 to mice with MIA PaCa-2 xenograft for a period of four weeks. We observed that treatment with trametinib or B8 alone suppressed xenograft growth. Moreover, their concomitant dosing retarded tumors significantly (Fig. 5A). Out of eight mice treated with TB, one displayed complete response, while four achieved partial response (Fig. 5B). Despite mice experienced body weight loss during TB treatment, their body weight did not exhibit any significant difference as compared with other groups at the end of the assay, indicating that TB is well-tolerated (Fig. S5D). On the other hand, we conducted an orthotopic xenograft assay using an alternative PDAC cell line AsPC-1 carrying the *4-hit* genotype. While trametinib monotherapy suppressed growth of AsPC-1 xenograft and pERK expression, B8 did not enhance the efficacy of trametinib or decrease pHH3 expression (Fig. S5E-F). A potential mechanism behind the lack of the B8 efficacy can involve additional genetic abnormalities beside the four-gene alterations that AsPC-1 cells harbor (see Discussion). Overall, our results reveal the anti-PDAC effects of trametinib alone or in combination with B8.

TB combination suppresses PDAC cell proliferation and induces cell death

To further elucidate the mechanisms behind the anti-PDAC efficacy of TB, we conducted cultured cell experiments. Consistent with the xenograft data, trametinib and B8 effectively suppressed proliferation of MIA PaCa-2 and other PDAC cells (commercially available Capan-1 and PANC-1, as well as patient-derived PCI-55) (Fig. 6A-B and Fig. S6A-F, Tables S5 and S10). In line with the fly results, the combination of trametinib and kinase inhibitors interfering with other therapeutic targets also suppressed the MIA PaCa-2 proliferation (Fig. S6G-J). As expected, trametinib treatment suppressed pERK in MIA PaCa-2, Capan-1, PANC-1, and PCI-55 (Fig. 6C and Fig. S6K-M, respectively). Additionally, we

confirmed that B8 suppressed pHH3 in these cells (Fig. 6D and Fig. S6N-P). Moreover, the TB combination convincingly decreased levels of both pERK and pHH3 (Fig. 6C-D and Fig S6K-P).

Upon TB treatment, MIA PaCa-2 cells exhibited cellular swelling and vacuole accumulation (Fig. 6E). These observations led us to hypothesize that TB caused cell death. Previous reports have demonstrated that MEK inhibitors including trametinib induce apoptosis (27) or autophagy (28), while an AURKA inhibitor CCT137690 induces necroptosis (29) in cultured and xenografted PDAC cells. Therefore, we conducted marker screening to identify the type of cell death in MIA PaCa-2 cells following chemical treatment. Consistently with previous reports, trametinib induced apoptosis as determined by increased cleaved PARP in MIA PaCa-2 cells, and we observed similar induction also in TB-treated cells (Fig. 6F). In addition, TB caused autophagy as indicated by a decrease in p62 levels and the ratio of LC3-I/LC3-II (Fig. 6G-H). Collectively, these results suggest that TB suppresses PDAC cell proliferation by activating apoptosis and autophagy through inhibiting MAPK and AURKB signaling pathways.

Discussion

To date, significant progress has been made in the search for drug candidates for PDAC. Especially, efforts to model endogenous PDAC have generated GEMMs such as the 1-hit strain *Kras* (30), the 2-hit strains *Kras-Trp53* (31), *Kras-Cdkn2a* (32), and *Kras-Smad4* (33), as well as the 3-hit strain *Kras-Trp53-Cdkn2a* (34). These models have provided significant insights into the mechanisms of PDAC development, such as the critical role of *Kras*^{G12D} in inducing precursor lesions in the mouse pancreas (30), and the progression mechanisms of PDAC through *Kras* and *Trp53* alterations (31). Additionally, patient-derived xenografts (PDXs) in mice have been elaborately established and utilized in drug testing, and these models have contributed to the identification of saracatinib as a drug candidate for PDAC upon chemical testing (35). However, GEMMs are cost- and labor-intensive for efficient genetic and drug screenings, and there is currently no mouse model for endogenous transformation caused by the *4-hit* alteration. To address this issue, we established series of transgenic *Drosophila* as a novel screening platform in this study. Consistent with clinical findings, transgenic flies exhibited worsened phenotypes as the number of mutations increased. Furthermore, *4-hit* flies enabled us to determine MEK and AURKB as therapeutic targets in PDAC in a whole-body manner. Importantly, their inhibitors effectively suppressed the growth of xenografted human PDAC MIA PaCa-2 cells. These results collectively offer a proof-of-concept that the *4-hit* model holds great promise in identifying novel drug candidates for the PDAC treatment.

While we have not conducted comprehensive assays to elucidate the mechanisms underlying the lethality observed in *ptc>4-hit* flies, we speculate that the proliferation and migration phenotypes within the wing disc disrupt the disc development, thereby arresting animal development. Additionally, a previous study has demonstrated that transformed cells induced by *Ras* secrete IL-6 to suppress cell competition, resulting in tumor formation in wing disc (12). As IL-6 is a cachexia-inducing factor (36), it is also possible that the four-gene alterations lead to a deficiency in homeostasis in flies. Conversely, the lower lethality in *Ser>4-hit* flies than *ptc>4-hit* flies can be attributed to lower levels of transgene expression induced by the activity of the endogenous *Ser* gene enhancer than the *ptc* gene enhancer. We would like to propose this possibility, supported by both the fact that the activity of the yeast gal4 transactivator increases proportionally with temperature (11) and by our observation of comparable signals of gal4-induced GFP between *ptc>4-hit* (raised at 16-degrees C; Fig. 1B) and *Ser>4-hit* discs (raised at 27-degrees C; Fig. 1E).

The key experiment in this study to pinpoint therapeutic targets of PDAC involved the comprehensive genetic screening of the entire kinome in *4-hit* flies. Previous studies including our own have collectively demonstrated that genetic screening is valuable in cancer research aimed at elucidating the disease mechanisms and identifying novel seeds (9,10). In this study, we identified five candidates of therapeutic targets through the kinome screening. In line with this, trametinib combinations with inhibitors targeting other candidates suppressed tumor traits in both *4-hit* flies and human MIA PaCa-2

cells, underscoring the predictive power of genetic screening for identifying chemical combinations as promising therapeutic candidates. In a previous clinical trial for PDAC, the combination of trametinib with the standard therapy gemcitabine demonstrated only marginal benefits (37). We expect that our genetic and drug screening platforms are invaluable in determining the most effective chemicals to combine to improve efficacy compared with monotherapy. Nonetheless, we acknowledge that there may be species-specific differences in the mechanisms of cellular transformation and drug response, which may present potential limitations in our study. However, we and others have demonstrated that the *Drosophila* wing disc offers a useful tissue for studying RTK-Ras signaling. Namely, activating RTK in this tissue causes cell proliferation and/or migration (7,9,10). In addition, we and others have successfully developed a novel kinase inhibitor lead that suppressed growth of human MTC xenograft in mice using *RET*-driven transformation as a reliable readout of chemical efficacy in flies (7,9,10). Based upon these achievements, in conjunction with those in this study, we speculate that *Drosophila* offers a valuable animal platform for the drug development to treat diseases caused by evolutionarily conserved genes such as kinases. Moreover, our results offer a novel strategy using *4-hit* flies to explore uncharted territories in treating kinase-dependent cancers exhibiting the same mutation profile, including lung and colorectal cancers, which currently lack mouse models for endogenous tumorigenesis depending on the four-gene alterations.

Our findings indicated that dual inhibition of MEK and AURKB offers a novel therapeutic strategy for PDAC. Trametinib has been recognized for its toxicity such as skin rash and diarrhea in patients. Oral administration of 3 mg of trametinib in patients led to $C_{\max}$ of 54.3 nM (38). On the other hand, dosing mice with 1 mg/kg of trametinib led to $C_{\max}$ of 503.7 nM (39). In the present study, we treated mice carrying xenografts with 1 mg/kg/day of trametinib, which is a substantially lower dose than MTD we determined (50 mg/kg/day; Fig. S5A) and a consistent dose with previous studies to demonstrate its anti-tumor effects (40). Notably, at the end of the dosing experiment, we did not observe any hematuria, diarrhea, or body weight loss, which are useful indicators to monitor chemical toxicity in mice. Likewise, we did not observe these symptoms in mice treated with the combination. Therefore, we speculate that both trametinib monotherapy and the combination in this study were tolerable for at least four weeks in mice although the plasma concentration of trametinib is speculated to be 9 times higher than in patients. Based on the results from a PDAC trial that demonstrated the ineffectiveness of trametinib combined with gemcitabine (37), it is possible that the concentration of trametinib in patients may need to be elevated to a sufficient level to exert therapeutic effects against PDAC. Conversely, a recent study has demonstrated the efficacy of a MEK/AURKB dual inhibitor BI-847325 in xenograft mouse models of melanoma and lung cancer (25). Nevertheless, the clinical development of BI-847325 was discontinued due to its insufficient inhibition of MEK and AURKB in a phase I trial (41). Our results hold promise of combining different chemicals to suppress PDAC growth by achieving effective inhibition of these two targets with tolerable toxicity.

Intriguingly, B8 alone failed to rescue *4-hit* flies, whereas reducing *AURKB* demonstrated significant efficacy. The polypharmacological nature of B8, which inhibits other kinases besides *AURKB*, may account for this discrepancy and hinder its therapeutic benefits. In a previous study, we successfully generated an effective lead by determining an ‘anti-target’ of the kinase inhibitor drug sorafenib through chemical genetic screening in flies and by changing its chemical structure to evade such anti-target inhibition (9). It will be intriguing to apply this strategy to B8 to determine its anti-target(s) and derive a novel lead for PDAC treatment with a widened therapeutic window. We speculate that such an optimization of a therapeutic strategy targeting AURK holds promise in generating novel therapeutics for other malignancies such as gastroenteropancreatic neuroendocrine tumors. This idea is supported by a previous report, which demonstrated that an AURK inhibitor danusertib suppressed the growth and liver metastasis of these tumors in an orthotopic xenograft model (42).

In this study, we found that the efficacy of TB combination was synergistic in both flies and cultured cells. Our PDAC cell experiments demonstrated that B8 alone activated MAPK signaling (Fig. 6C and Fig. S6K-M), which can reduce the anti-tumor effect by *AURKB* inhibition. Furthermore, it has been demonstrated that a multi-kinase inhibitor BMS-777607 reduced chemosensitivity in PDAC cells through inhibiting *AURKB* (43). Therefore, it is interesting to hypothesize that additional treatment with trametinib increases the B8 efficacy in reducing cell proliferation more effectively than B8 alone by interfering with this bypass survival mechanism in PDAC. On the other hand, MAPK signaling is known to upregulate expression of *AURKB* mRNA in melanoma cells (44), consistent with our results that trametinib inhibition of MAPK signaling suppressed phosphorylation of HH3 in PDAC cells (Fig. 6D and Fig. S6N-P). It is also reported that long-term treatment with MEK inhibitors reactivates MAPK signaling in *RAS*-mutant cancers (45). This reactivation could potentiate *AURKB*, but we expect that adding B8 to the trametinib regimen can help sustain the treatment’s effectiveness. We also found that TB suppressed the proliferation of PDAC cells with various genetic alterations (MIA PaCa-2 and PANC-1 as *3-hit*; Capan-1 and PCI-55 as *4-hit*; Fig. S6A-F and Table S10). These results suggest that the therapeutic candidates identified in our study are efficacious to treat PDAC with diverse genotypes. We observed either synergistic or additive effects by the two chemicals in these cells. The factors governing the chemical sensitivity of these cells remains unclear currently, but it is possible that genetic alterations in these cells determine their drug response, as implicated by previous reports (46). On the other hand, a previous report conducting a comprehensive CRISPR screening found that targeting the function of genes related to mitotic cell cycle and kinetochore function enhanced the responsiveness of PDAC cells to trametinib (47). The authors also demonstrated the anti-PDAC effects by a combination of a pan-aurora kinase inhibitor and trametinib. In conjunction with our own results, we conclude that the concurrent inhibition of *AURKB* as the key aurora kinase subtype with MEK is crucial for the suppression of PDAC growth.

Curiously, we did not detect suppression of AsPC-1 xenografts or the pHH3 level by B8 treatment alone or in combination with trametinib (Fig. S5E-F). A potential explanation for these results is an additional mutation in the *FBXW7* gene. FBXW7 is a member of the F-box protein family involved in protein ubiquitination and has been established as a tumor suppressor (48). In fact, the R465C missense mutation in *FBXW7*, which AsPC-1 carries, is one of the hot spot mutations in human cancers including PDAC (49). Intriguingly, a report demonstrated that FBXW7 targets AURKB for degradation (50). Based on these findings, we speculate that the mutated *FBXW7* contributes to the reduction of chemical sensitivity in AsPC-1 cells by upregulating thus activating AURKB, which B8 could not fully suppress. Elucidating the precise molecular mechanisms behind this drug resistance can pave the way for the development of personalized medicine based on patients' genotypes in the future.

In summary, we have successfully established a novel platform for PDAC research. Specifically, modeling PDAC genotype in *Drosophila* allows for both comprehensive screening of therapeutic targets and rapid testing of chemicals as therapeutic candidates in a whole-body context (Fig. 6I). Our efficient and inexpensive *Drosophila* platform, combined with established mammalian models such as GEMMs and PDXs, will accelerate the understanding of PDAC pathogenesis and the development of novel drugs for this devastating disease.

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Figure Legend

Figure 1. Modeling PDAC genotype in *Drosophila*.

(A) Generating a *Drosophila* model for '4-hit' PDAC genotype associated with the worst prognosis among PDAC patients. G12D, G to D missense activation mutation. LOF, loss of function. shRNA, short hairpin RNA. HA, Hemagglutinin tag. (B) Accumulating mutations promote transformation in *Drosophila*. The *patched* (*ptc*) enhancer drove expression of GFP to mark the center area of a wing disc in a third instar larva (control). Magenta, propidium iodide staining outlining the disc margin. Wing discs in 4-hit flies displayed a more disorganized stripe than those of 1-hit (*dRas^{G12D}*) flies, with increased cell migration (representative cells indicated by arrowheads). Scale bars, 50 μ m. (C) Expanded relative *ptc* area in 4-hit wing discs. Cont, control flies. (D) Pupal lethality of 4-hit flies. $*P < 0.001$ in one-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test (C, D). Error bars, S.D. in technical triplicate. (E-J) Validation of transgenes. Immunofluorescence revealed induction of phosphorylated ERK (pERK) as downstream of dRas^{G12D} (E; magenta) as well as CycE (F; magenta) in transgene-expressing areas (green). Fluorescent signals were quantified with ImageJ software (G, H). While, RT-qPCR verified knockdown of *p53* (I) and *Med* (J) expression in wing discs. $*P < 0.01$, $**P < 0.001$ in Student's *t*-test compared with control flies. Error bars, S.D. in 3-5 disc samples.

Figure 2. Identifying MEK and aurora kinases as therapeutic targets for PDAC in genetic screening using 4-hit flies.

(A) Screening scheme for the entire *Drosophila* kinome to determine genetic modifiers of 4-hit transformation. A heterozygous mutation of each kinase gene was introduced to 4-hit flies through crossing. The viability of their progenies was compared with that of progenies obtained from a cross between 4-hit flies and kinase-proficient control flies at 27-degrees C. Detailed scheme in Fig. S2. *white*⁻, non-transgenic strain as control. Balancer, an artificial *SM5^{tubP}-GAL80-TM6B* chromosome carrying *tubulin* promoter-driven GAL80 that suppresses GAL4 transcriptional activator. (B) Kinase genes whose reduction rescued pupal lethality of 4-hit flies. $*P < 0.01$ and $**P < 0.001$ in Dunnett's test compared with control *Ser>4-hit* flies. Error bars, S.D. in technical triplicate. Parentheses, human ortholog of fly gene predicted by DIOPT. (C) Knockdown of *aurB* by siRNA rescued lethality of 4-hit flies. Legend the same as in (B). (D) *Dsor1* heterozygosity or *aurB* knockdown suppressed transformation in wing disc of *ptc>4-hit* larvae. Transformed cells were marked by GFP. Scale bars, 20 μ m.

Figure 3. Combination of trametinib and BI-831266 as a novel therapeutic candidate as determined by drug screening using 4-hit flies.

(A) Concurrent treatment with the MEK inhibitor drug trametinib and an AURK inhibitor BI-831266 (B8) increased viability of 4-hit flies. MicroM, chemical concentration in fly food. Error bars, standard deviation (S.D.). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ in one-way ANOVA with Tukey's HSD post hoc test compared

with the results for the same concentration of B8. # $P < 0.05$ in Dunnett's test compared with trametinib alone. Error bars, S.D. in technical triplicate. **(B)** Trametinib and B8 worked synergistically in *4-hit* flies. Bliss synergy score was employed to generate a heatmap to indicate the effect as synergistic (red), additive (white), or antagonistic (green). Bliss synergy score, its average, and P -value were calculated using SynergyFinder. **(C)** Combination treatment rescued wing defects in *4-hit* flies. Compared with the wings of wild-type adults, those of *4-hit* adults showed disorganization with a sac-like structure (arrowhead). Trametinib alone rescued this phenotype partially, which was potentiated by B8. Scale bars, 500 μ m.

Figure 4. AURKB activity associating with unfavorable prognosis of PDAC patients.

(A) Phosphorylated histone H3 (pHH3) in PDAC specimen as detected by immunohistochemistry. Arrowheads, cancer cells with pHH3 immunoreactivity (magenta staining). Scale bars, 20 μ m. **(B)** Expression of pHH3 is associated with unfavorable overall survival of PDAC patients. Kaplan-Meier analyses were conducted by classifying the patients into two groups based on the presence or absence of pHH3-positive cells. P values were calculated by log-rank test. Patient characteristics provided in Tables S2-3.

Figure 5. Anti-tumor effect of a combination between trametinib and BI-831266 in a mouse xenograft model for human PDAC.

(A) Trametinib (tram) and BI-831266 (B8) inhibited growth of MIA PaCa-2 xenograft in nude mice. Trametinib (1 mg/kg/day) suppressed growth of subcutaneous MIA PaCa-2 xenografts, and co-treatment with B8 (10 mg/kg/day) potentiated the anti-tumor activity of trametinib. * $P < 0.05$ and ** $P < 0.01$ in Mann-Whitney U test on day 21. Error bars, S.D. in 8 mice. **(B)** Trametinib and B8 combination suppressed xenograft growth more efficiently than monotherapies. Waterfall plot showing percent changes in tumor volume on day 21 relative to pretreatment baselines; each bar represents a single mouse. Yellow bars indicate partial response (at least a 30% tumor size reduction from baseline), whereas orange bars indicate complete response (tumor eradication), according to the RECIST criteria.

Figure 6. Suppression of PDAC cell proliferation by trametinib/B8 combination through induction of cell death.

(A) Trametinib (tram) and BI-831266 (B8) suppressed MIA PaCa-2 proliferation. Cells were treated with tram or B8 (concentration as indicated) alone or in combination for 72 h. **(B)** Synergistic suppression of MIA PaCa-2 proliferation by the tram and B8 combination (TB). Legend the same as in Fig. 3B. **(C, D)** TB inhibited MAPK and AURKB signaling. The expression levels of phosphorylated ERK (pERK) were normalized by that of total ERK (tERK) (C). Both tram and B8 suppressed upregulation of pHH3 (D). TB inhibited both pathways. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in one-way ANOVA with Tukey's HSD post hoc test. **(E)** TB induced cell death of MIA PaCa-2. Treated cells displayed morphological changes such as

swelling (arrows) and cytoplasmic vacuoles (arrowheads). Scale bars, 10 μ m. **(F-H)** Enhanced apoptosis and autophagy upon TB treatment. Trametinib alone or TB increased expression of the apoptotic marker cleaved poly ADP-ribose polymerase (cPARP) (F), while these treatments reduced p62 expression (G) and the light chain 3 (LC3)-I/LC3-II ratio (H), which are hallmarks of autophagy. The identical loading control, α -Tub, is displayed in F and G, as the identical set of samples was analyzed on a single membrane. α -Tub, α -tubulin. Error bars show S.D. of three experiments. A.U., arbitrary unit. **(I)** Scheme illustrating the platforms and findings in this study. Modeling PDAC genotype in *Drosophila* allowed for comprehensive genetic screening to identify novel therapeutic targets MEK and AURK in a whole-body manner. Following chemical testing in flies and in a PDAC mouse model validated the efficacy of the MEK inhibitor drug trametinib in combination with the AURKB inhibitor BI-831266.

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Analyzing the mouse model and the cultured human PDAC cells: SS, JF, RY, TO, YS, SK, RS, MS

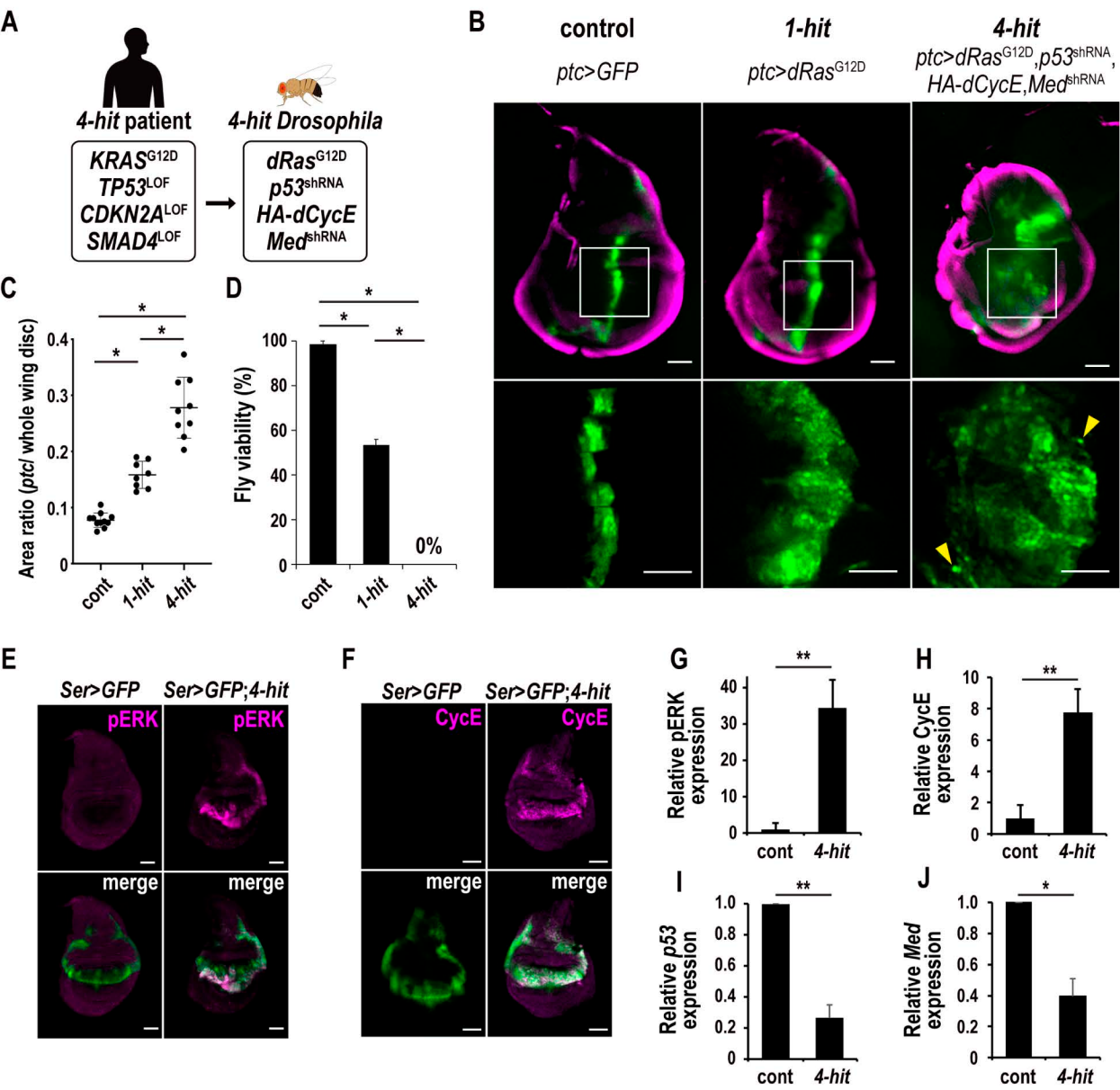
Analyzing patient tissue: SS, RY, KCH, YH, TM, TN, YM, SH, MS

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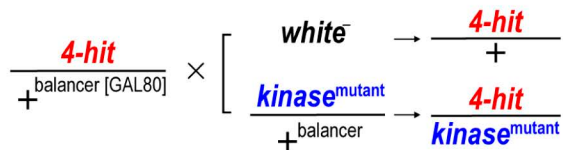
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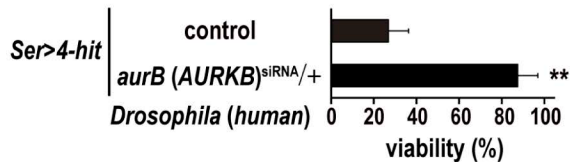
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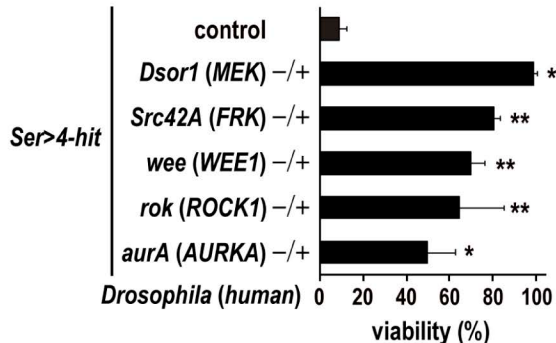
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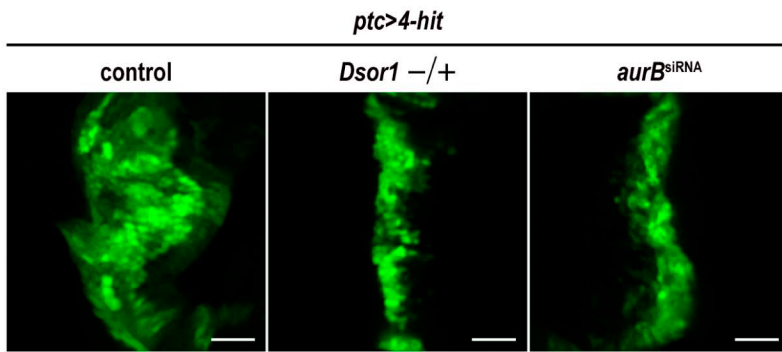
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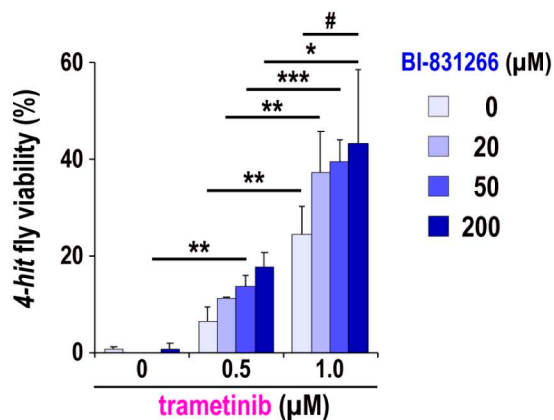
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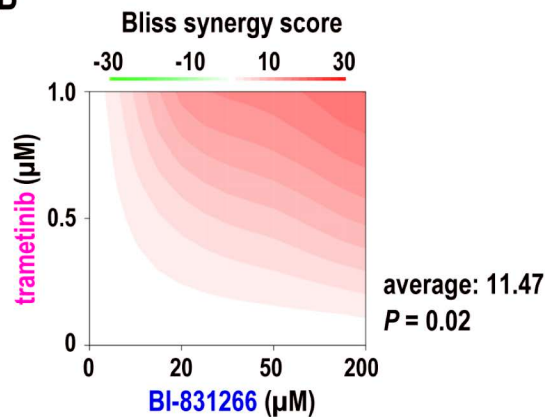
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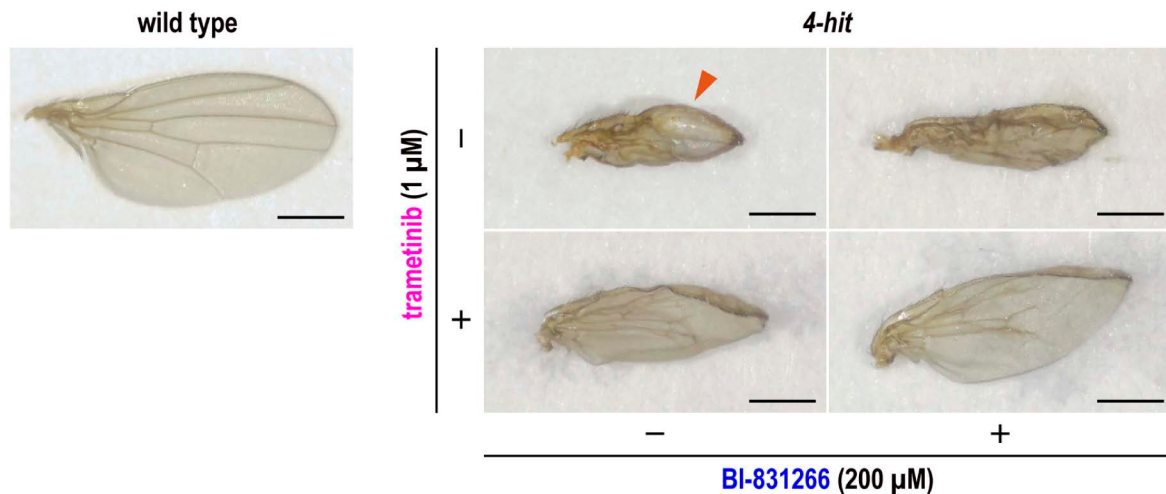


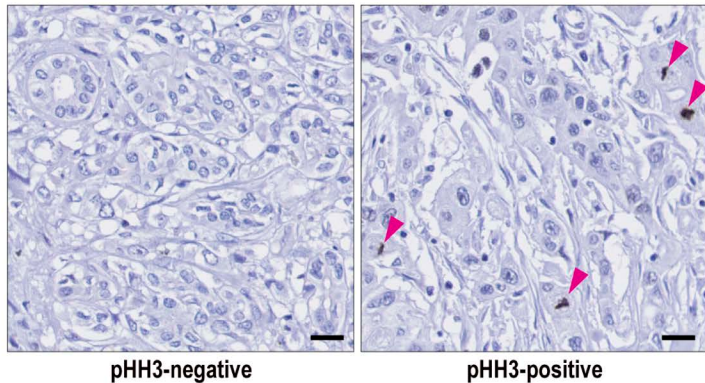
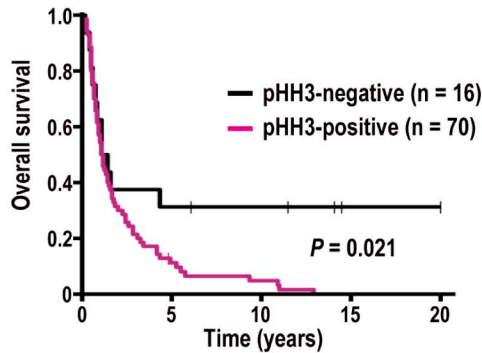
Fig. 4**A****PDAC patient specimens****B**

Fig. 5

