G3, 2023, 13(5), jkad053
https://doi.org/10.1093/g3journal/jkad053
Advance Access Publication Date: 6 March 2023
Mutant Screen Report

# A targeted genetic modifier screen in *Drosophila* uncovers vulnerabilities in a genetically complex model of colon cancer

Ishwaree Datta, Tajah Vassel, Benjamin Linkous,† Tyler Odum,† Christian Drew,† Andrew Taylor,† Erdem Bangi*

Department of Biological Science, Florida State University, Tallahassee, FL 32304, USA

*Corresponding author: Department of Biological Science, Florida State University, Tallahassee, FL 32304 USA. Email: ebangi@bio.fsu.edu
†These authors contributed equally to this work.

## Abstract

Received on 16 January 2023; accepted on 21 February 2023Kinases are key regulators of cellular signal transduction pathways. Many diseases, including cancer, are associated with global alterations in protein phosphorylation networks. As a result, kinases are frequent targets of drug discovery efforts. However, target identification and assessment, a critical step in targeted drug discovery that involves identifying essential genetic mediators of disease phenotypes, can be challenging in complex, heterogeneous diseases like cancer, where multiple concurrent genomic alterations are common. *Drosophila* is a particularly useful genetic model system to identify novel regulators of biological processes through unbiased genetic screens. Here, we report 2 classic genetic modifier screens focusing on the *Drosophila* kinome to identify kinase regulators in 2 different backgrounds: KRAS TP53 PTEN APC, a multigenic cancer model that targets 4 genes recurrently mutated in human colon tumors and KRAS alone, a simpler model that targets one of the most frequently altered pathways in cancer. These screens identified hits unique to each model and one shared by both, emphasizing the importance of capturing the genetic complexity of human tumor genome landscapes in experimental models. Our follow-up analysis of 2 hits from the KRAS-only screen suggests that classical genetic modifier screens in heterozygous mutant backgrounds that result in a modest, nonlethal reduction in candidate gene activity in the context of a whole animal—a key goal of systemic drug treatment—may be a particularly useful approach to identify the most rate-limiting genetic vulnerabilities in disease models as ideal candidate drug targets.

Keywords: *Drosophila*, colon cancer, AKT, MEK, KRAS, genetic modifier screen, kinome

## Introduction

Cancer is a genetically complex and heterogeneous disease. For most solid tumors, tumorigenesis and progression into metastatic disease typically require the accumulation of multiple genomic alterations (Bailey *et al.* 2018; Sanchez-Vega *et al.* 2018). For instance, broad alterations in multiple signaling networks and global changes in transcriptomic, proteomic, phospho-proteomic, and epigenomic profiles have consistently emerged from recent tumor profiling studies as hallmarks of most advanced tumors (Das *et al.* 2020; Weber *et al.* 2020; Zhang *et al.* 2022). Individual genes and pathways recurrently altered in human tumors have been well characterized in multiple experimental systems, and multigenic cancer models have started to shed light on emergent interactions between concurrent cancer-driving genetic alterations (Kersten *et al.* 2017; Guerin *et al.* 2020; Sajjad *et al.* 2021). However, the complex, multigenic nature of tumor genome landscapes makes it particularly challenging to identify genes required to establish and maintain tumor phenotypes and prioritize candidate targets for drug discovery approaches (Haley and Roudnicky 2020).

With its sophisticated genetic tools and practical advantages, *Drosophila* has a strong track record as a disease model and has also emerged as a useful drug discovery platform (Cheng *et al.* 2018; Mohr and Perrimon 2019; Munnik *et al.* 2022). Disease models generated either by genetically manipulating *Drosophila* orthologs of human disease genes or directly introducing disease-associated variants of human genes into *Drosophila* have provided important insights into molecular mechanisms underlying human diseases, including cancer (Verheyen 2022). Furthermore, multiple studies over the past decade have demonstrated a high degree of conservation of compound activity in *Drosophila* (Su 2019; Munnik *et al.* 2022); several compounds identified through these studies have been effective in vertebrate experimental models and human patients (Dar *et al.* 2012; Bangi *et al.* 2016, 2019, 2021; Das *et al.* 2018; Sonoshita *et al.* 2018).

We have previously leveraged the genetic simplicity and power of *Drosophila* as a model system to functionally explore genome landscapes of human colon tumors (Bangi *et al.* 2016, 2019). These multigenic, tumor genome-based models capture many hallmarks of human tumors and demonstrate that intrinsic drug resistance is an emergent feature of genetic complexity. In this study, we take advantage of a classic genetic modifier screen

Received: January 16, 2023. Accepted: February 21, 2023

approach to identify bona fide regulators of a 4-hit model targeting *Drosophila* orthologs of KRAS, TP53, PTEN, and APC, 4 genes recurrently mutated in colon tumors, as well as KRAS alone, which represents one of the most commonly observed pathway alterations in human tumors (Cancer Genome Atlas Network 2012). As kinases are critical regulators of signaling networks and frequent targets of cancer drug discovery efforts (Attwood *et al.* 2021), we focused our genetic screen on the *Drosophila* kinome, which consists of 377 kinases representing all branches of the human kinome tree (Manning *et al.* 2002; Gramates *et al.* 2022). Our parallel screens of these 2 models identified partially overlapping hits, indicating that as the genetic complexity of the model increases, its dependence on the activity of some kinases decreases and new genetic vulnerabilities that were absent in simpler models emerge. By comparing the sensitivity of our models to a genetic copy loss vs. strong, tissue-specific knockdown of kinase activity, we found that classic modifier screens in heterozygous mutant backgrounds, which result in a modest, nonlethal reduction of kinase activity in the whole animal may be a valuable approach for identifying most rate-limiting genetic vulnerabilities that could be exploited in therapy, while tissue-specific gene knockdown or knockout approaches can be used to uncover genetic dependencies.

# Materials and methods

## *Drosophila* strains

All *Drosophila* strains were maintained at room temperature on a standard *Drosophila* medium. $w^{1118}$ and all kinome stocks used for the screen were obtained from Bloomington Drosophila Stock Center (BDSC, see Supplementary Table 1 for details). Independent alleles used to test confirmed hits were $aPKC^{MI10848}$, $par\text{-}1^{k05603}$, $cdk2^{3}$, $hppy^{MI03637}$, $dsor^{G42}$, and $akt^{MI14526}$ (BDSC #s 56083, 10574, 6636, 41475, 7131, and 59527, respectively). TRIP RNAi lines (Zirin *et al.* 2020), UAS-$akt^{RNAi}$ (BDSC #31701, #33615) and UAS-$dsor^{RNAi}$ (BDSC #33639, #34830), used for *akt* and *dsor* knockdown, were also obtained from BDSC. UAS-$luciferase^{RNAi}$ (BDSC #31603) was used as a negative control. Transgenic *Drosophila* lines used to generate the KRAS TP53 PTEN APC combination are UAS-$ras^{G12V}$ (II, G. Halder), and 3 RNAi lines UAS-$p53^{RNAi}$ (II), UAS-$pten^{RNAi}$ (III), and UAS-$apc^{RNAi}$ (II) that were obtained from the Vienna Drosophila Resource Center (VDRC) (Dietzl *et al.* 2007). To build the UAS-$ras^{G12V}$UAS-$p53^{RNAi}$ UAS-$pten^{RNAi}$ UAS-$apc^{RNAi}$ quadruple multigenic combination, we first generated a second chromosome insertion of UAS-$pten^{RNAi}$ (III, VDRC), which also provided strong activation of the PI3K pathway, by P-element transposase-mediated mobilization. We then recombined it into the previously described UAS-$ras^{G12V}$ UAS-$p53^{RNAi}$ UAS-$apc^{RNAi}$ triple combination on the second chromosome (Bangi *et al.* 2016). The *byn-gal4 UAS-GFP tub-gal80*$^{ts}$ triple recombinant chromosome used for targeted expression in the hindgut has also been described previously (Bangi *et al.* 2012).

Fly lines that combine all transgenic elements required for targeted expression of KRAS TP53 PTEN APC or KRAS to generate the lethal screening phenotypes into a single genetic background (i.e. screening stocks) were generated using standard *Drosophila* genetic crosses: $w^{1118}$ *UAS-dcr2/Y, hs-hid*; UAS-$ras^{G12V}$ UAS-$p53^{RNAi}$ UAS-$pten^{RNAi}$ UAS-$apc^{RNAi}$; *byn-gal4 UAS-GFP tub-gal80*$^{ts}$*/S-T, Cy, tub-gal80, Hu, Tb* and $w^{1118}$ *UAS-dcr2/Y, hs-hid*; UAS-$ras^{G12V}$; *byn-gal4 UAS-GFP tub-gal80*$^{ts}$*/S-T, Cy, tub-gal80, Hu, Tb*. *UAS-GFP* and *UAS-dcr2* transgenes are included to fluorescently label targeted hindgut epithelial cells and to facilitate RNAi-mediated knockdown, respectively. A *tub-gal80*$^{ts}$ transgene on the third chromosome (*byn-gal4 UAS-GFP tub-gal80*$^{ts}$), which drives the ubiquitous expression of a temperature-sensitive (ts) allele of the Gal4 inhibitor Gal80 (McGuire *et al.* 2004), is used to temporally regulate transgene induction. A *tub-gal80* transgene (Lee and Luo 1999) introduced into the balancer chromosome (*S-T, Cy, tub-gal80, Hu, Tb*) is used to inhibit gal4 activity in screening stocks, preventing transgene expression and lethality. The Y chromosome *hs-hid* transgene, which results in ubiquitous activation of apoptosis when induced (Starz-Gaiano *et al.* 2001), is used to kill all male progeny and facilitate mass virgin female collection required for a large number of crosses of the genetic modifier screens. Kinase alleles were balanced using balancer chromosomes carrying a Tb marker, using *CyO, Cy, Tb, RFP* (CTR) and *FM7c, Tb, RFP* (FTR) balancers for the second and X chromosomes, respectively (Pina and Pignoni 2012), and the *TM6b, Hu, Tb* balancer for the third chromosome.

## Genetic screen

Mutant kinase alleles were introduced into the KRAS TP53 PTEN APC and KRAS backgrounds with a simple F1 cross (see Supplementary Fig. 1 for examples). For the 2nd, 3rd, and 4th chromosome screens, males from kinase stocks were crossed to virgin females from the KRAS TP53 PTEN APC and KRAS-only screening stocks. For kinases on the X chromosome, *kinase*$^{-}$*/FM7c, Tb, RFP* virgin females were crossed to males from the KRAS TP53 PTEN APC and KRAS-only screening stocks. KRAS TP53 PTEN APC and KRAS virgins crossed to $w^{1118}$/Y males (no kinase mutations) were used as baseline controls for our screening read-outs. Virgins required for these crosses were generated en masse by 2 independent 1-hour heat shocks of screening lines using a 37°C water bath during development using the following schedule: 3-day egg lay at room temperature in bottles with standard *Drosophila* medium followed by removal of parents, first heat-shock of the progeny on day 4, and second heat shock on either day 5 or day 7.

Crosses for the screen were set up on Bloomington semi-defined medium (Bangi *et al.* 2019) at 29°C using 18–20 virgin females and 8–12 males for each cross. Parents were removed after 2 days of egg laying. Progeny was scored 12–14 days after crosses were set up. Experimental pupae were identified by the absence of the Tb marker. Survival to the pupal stage was calculated by counting the experimental and control pupae in each vial (Supplementary Fig. 1). The fraction of empty experimental pupal cases (indicating an adult fly has emerged) was used to calculate survival to the adult stage. Each kinase allele was tested in triplicate. Kinase alleles that resulted in a statistically significant increase in survival compared to KRAS TP53 PTEN APC or KRAS alone (Fig. 1, c and d) were considered candidate hits (multiple *t*-tests with Holm-Sidak correction for multiple hypotheses, PRISM software). Candidate hits were then retested in an independent set of experiments, and kinases that showed statistically significant rescue in the retest experiments were considered confirmed hits. Screening crosses that were inconclusive due to low *n* were retested similarly. Each hit was also crossed to $w^{1118}$ *UAS-dcr2/Y, hs-hid; +; byn-gal4 UAS-GFP tub-gal80*$^{ts}$*/S-T, Cy, tub-gal80, Hu, Tb* to confirm that mutant alleles did not affect organismal lethality or the size of the hindgut imaginal ring area in an otherwise wildtype background. The hits obtained from the screen were also tested using different alleles to control for background mutations that may contribute to the phenotypic rescue.

## Dissections, imaging, and scoring

Crosses to analyze the overall size of the hindgut imaginal ring area were set up similarly on Bloomington semi-defined medium using 18–20 virgin females and 8–10 males for each cross, but at

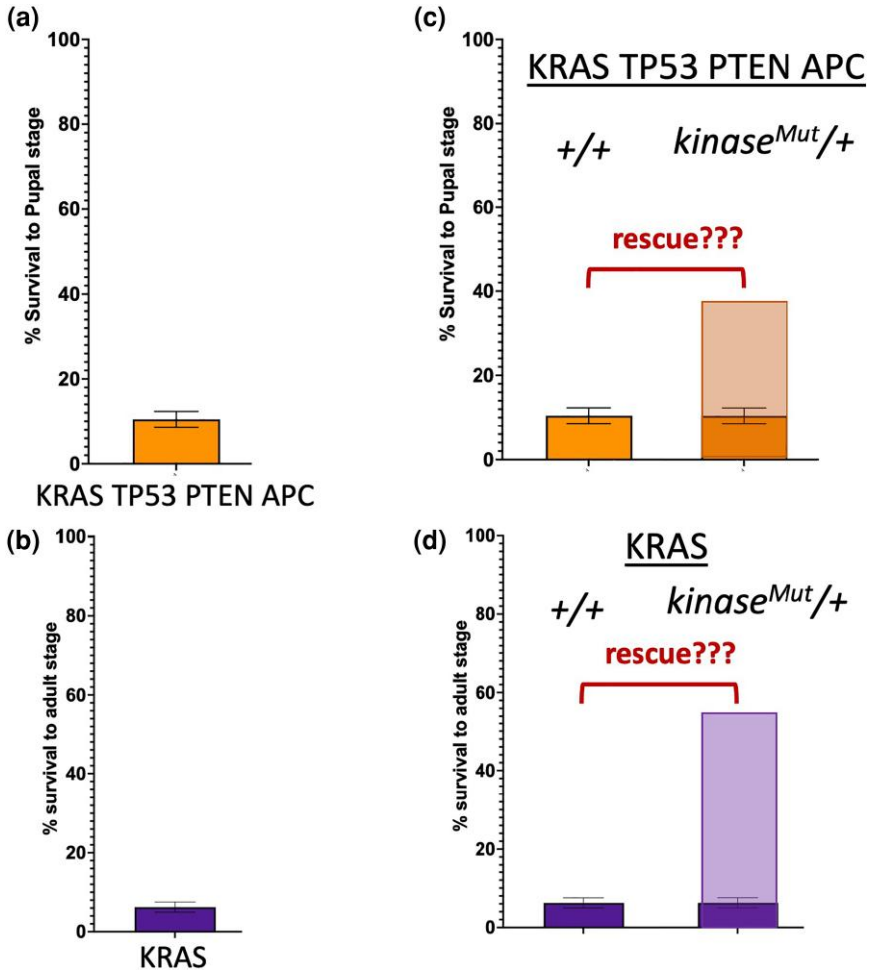


**Fig. 1.** Screening strategy using rescue of KRAS TP53 PTEN APC and KRAS induced lethality as a primary read-out. a and b) Lethal phenotypes induced by targeting *UAS-ras*$^{G12V}$ *UAS-p53*$^{RNAi}$ *UAS-pten*$^{RNAi}$ *UAS-apc*$^{RNAi}$ (KRAS TP53 PTEN APC, a) and *UAS-ras*$^{G12V}$ (KRAS alone, b) to the developing hindgut using the hindgut specific *byn-gal4* along with a *UAS-GFP* transgene. c and d) Screening strategy using rescue of lethality as a read-out. A mutant allele for each kinase is introduced into KRAS TP53 PTEN APC (c) and KRAS (d) backgrounds to evaluate their effect on lethality. Kinases with important, rate-limiting roles in these backgrounds may improve survival.

18°C to prevent early larval lethality. Parents were removed after 3 days of egg laying, and progeny were kept at 18°C for an additional 3 days before transgenes were induced by a temperature shift to 29°C. Experimental larvae were identified based on the absence of the Tb phenotype, and hindguts from larvae at the late third instar stage were dissected 3 days after induction. Dissections were performed in phosphate-buffered saline (PBS), and hindguts were fixed in ice-cold 4% paraformaldehyde in PBS for 15 min at room temperature, followed by 3 rinses and a 15-min wash in PBS. Hindguts were mounted the next day on Vectashield with DAPI and imaged at 10× magnification at 1.5 Zoom using Leica SPE DM6 confocal microscope using 488 and 405 nm lasers for visualizing the GFP labeled hindgut epithelium and nuclei (DAPI), respectively. About 10–12 hindguts of each genotype were imaged, and the area of the imaginal ring region of each hindgut was quantified using Fiji Image J Software in pixels (Schindelin *et al.* 2012). KRAS TP53 PTEN APC and KRAS screening stocks crossed to $w^{1118}$/Y was used as positive controls and GFP-only expressing hindguts generated by crossing $w^{1118}$ *UAS-dcr2/Y*, *hs-hid*; +; *byn-gal4 UAS-GFP tub-gal80*$^{ts}$/S-T, *Cy*, *tub-gal80*, *Hu*, and *Tb* virgins to $w^{1118}$/Y males served as negative controls. Experimental and control groups were compared using a one-way ANOVA (PRISM software).

## Western blot analysis

Crosses to generate experimental larvae for dissections were set up as described in the previous section. Hindguts for protein extraction were dissected from late third instar larvae lacking the Tb marker after 3 days of induction. Ten hindguts for KRAS TP53 PTEN APC and 15 hindguts for KRAS alone, along with matching GFP-only control larvae, were used for making protein lysates (3 biological replicates/genotype). Larval hindguts were homogenized with a motorized pestle (at 10-second pulses) in ice-cold lysis RIPA buffer (37.5 µl, Sigma-Aldrich #R0278) fortified with protease and phosphatase inhibitors (Sigma-Aldrich #4693132001 and Millipore Sigma #524627). Lysates were then centrifuged at 4°C for 10 min at 13,000 rpm. Thirty-five microliters of supernatants were transferred to a fresh tube, followed by the addition of 12.5 µl of Sample buffer (Bio-Rad #1610792) and 2.5 µl of 20× Reducing Agent (Bio-Rad #1610792). The lysates were boiled for 10 min in a 100°C heat block, centrifuged at 4°C for 5 min at 13,000 rpm, and 50 µl of supernatant were transferred to new tubes. Lysates were stored at −80°C until use. For western blot analysis, proteins were separated using 4–12% Criterion XT Bis-Tris Protein Gels (Bio-Rad #3450125). Primary antibodies used were Rabbit anti-phospho-AKT (1:1,000, Cell Signaling

Technology #4060), Mouse anti-diphospho-Erk (1:1,000 Sigma-Aldrich #M8159), and Mouse anti-Syntaxin as a loading control (1:1,000 Developmental Studies Hybridoma Bank #8C3). The secondary antibodies were Anti-rabbit IgG, HRP-linked Antibody (1:1,000, Cell Signaling Technology #7074), and Anti-mouse IgG, HRP-linked Antibody (1:5,000, Cell Signaling Technology #7076). Protein bands were developed with Immobilon Western Chemiluminescent HRP Substrate solutions (Millipore Sigma #WBKLS0050) and visualized under UV light using BioRad's ChemiDoc MP Imaging Platform. Bands were quantified using Fiji Image J (Schindelin *et al.* 2012). Statistical analysis was performed with a one-way ANOVA using PRISM software.

# Results and discussion

## Screen strategy

Rescue from lethality has been shown to serve as a useful read-out in high throughput chemical and genetic screens to identify novel regulators of disease-relevant phenotypes (Rudrapatna *et al.* 2014; Levinson and Cagan 2016; Bangi *et al.* 2019). To generate a robust, lethal phenotype suitable for large-scale screening, we used the *byn-Gal4* driver expressed in the hindgut epithelium (Takashima *et al.* 2008) to target our 4-hit model KRAS TP53 PTEN APC to the developing hindgut. This model comprises an oncogenic *UAS-ras*$^{G12V}$ transgene and 3 UAS-RNAi transgenes to knockdown *Drosophila* orthologs of tumor suppressors APC, TP53, and PTEN (Bangi *et al.* 2016). We found that targeting KRAS TP53 PTEN APC multigenic combination to the developing hindgut epithelium resulted in a highly penetrant and consistent early larval lethal phenotype (Fig. 1a), while targeting KRAS alone (*UAS-ras*$^{G12V}$) to the same tissue resulted in a similarly robust lethal phenotype, albeit at a later stage during late pupal development (Fig. 1b). We then used these lethal phenotypes as surrogate read-outs in parallel genetic screens to evaluate vulnerabilities of genetically complex and simple models and identify genes with essential roles in intestinal transformation.

Kinases are essential in governing cancer-relevant pathways (Gross *et al.* 2015; Cicenas *et al.* 2018); therefore, we focused our screens on the *Drosophila* kinome. Like in humans, most *Drosophila* loci are recessive; they show no phenotype or lethality when a single genomic copy is removed. However, losing a copy of a gene that promotes oncogenic transformation and organismal lethality in the context of a multigenic model may prove rate-limiting and improve survival. Therefore, we decided to adopt a classic genetic modifier screening approach by reducing the gene dosage of each kinase by introducing a mutant allele into our models using publicly available fly lines. Building on prior studies taking a similar approach (Levinson and Cagan 2016; Sonoshita *et al.* 2018), we reasoned that reducing the level of a kinase that may be critical for tumor progression would rescue the lethality we observe in our models (Fig. 1, c and d).

## Screen design

The ability to perform complex genetic manipulations and carry out large-scale in vivo screens are 2 key strengths of *Drosophila* as a model system. However, as our multigenic models are already genetically complex—for instance, experimental animals in our KRAS TP53 PTEN APC model carry 8 different transgenes—performing additional genetic manipulations in these backgrounds, especially in the context of a large-scale genetic screen, can be challenging. To address this problem, we established screening lines for KRAS TP53 PTEN APC and KRAS alone by consolidating all transgenic elements required to generate each lethal screening read-out into a single genetic background (Supplementary Fig. 1). These screening lines also include: (1) a ubiquitously expressed *gal80* transgene (Lee and Luo 1999) to prevent gal4 activity and lethality within screening stocks and (2) a *hs-hid* transgene on the Y chromosome (Starz-Gaiano *et al.* 2001) that leads to ubiquitous activation of apoptosis and lethality in male progeny when induced, a strategy that allows *en masse* virgin generation necessary for large-scale screens. This design allowed us to introduce mutant kinase alleles into KRAS TP53 PTEN APC and KRAS alone backgrounds with simple F1 genetic crosses (Supplementary Fig. 1).

## Screen results

The *Drosophila* kinome comprises 377 kinases (Gramates *et al.* 2022). We were able to obtain mutant lines for 206 kinases, covering 54.6% of the entire fly kinome (Supplementary Table 1). Our parallel screens of the same set of kinases identified 3 confirmed hits from the KRAS TP53 PTEN APC screen (*aPKC*, *par-1*, *cdk2*), while the KRAS screen resulted in 5 hits (*par-1*, *cdk2*, *hppy*, *dsor1*, *akt*) (Fig. 2, a and b, Supplementary Table 2). Confirmed hits did not affect organismal lethality in GFP-only control animals (Supplementary Fig. 2a). Retests of confirmed hits using a different mutant allele for each kinase provided results consistent with the original screen, except *par-1*, where the new allele resulted in the statistically significant rescue of KRAS TP53 PTEN APC-induced lethality but not that of KRAS alone (Supplementary Fig. S2, b and c). Given this discrepancy, we did not pursue *par-1* further as a hit for the KRAS-only screen in this study (Fig. 2c). However, these results do not rule out a role for *par-1* in KRAS-mediated intestinal transformation; additional experiments using other alleles and transgenic constructs will be necessary to investigate its function further.

The only shared hit between the 2 screens was *cdk2*, a highly conserved gene with cancer-relevant functions (Fig. 2c). *cdk2* regulates the G1/S transition of the cell cycle in association with *CycE* and plays an essential role in modulating cell division and differentiation (Sauer *et al.* 1995; Roesley *et al.* 2018). The human cdk2 ortholog, CDK2, is altered in various cancers, including colorectal, and its downregulation can have antitumor effects depending on the genetic context (Tadesse *et al.* 2020).

*aPKC*, 1 of the 2 hits unique to KRAS TP53 PTEN APC (Fig. 2, a and c, Supplementary Fig. 2b), is an atypical protein kinase with many downstream targets and a key regulator of cell polarity (Vorhagen and Niessen 2014; Hong 2018). *aPKC* overexpression is also linked to the progression and development of different cancers (Eder *et al.* 2005; Regala *et al.* 2005; Nayak *et al.* 2019). *par1*, the other hit unique to KRAS TP53 PTEN APC, is the single *Drosophila* ortholog of the PAR-1/MARK family of serine/threonine kinases in mammals (Wu and Griffin 2017) and a critical regulator of cytoskeleton structuring, cell polarity, and Hippo signaling (Huang et al. 2013; Doerflinger et al. 2022). PAR-1 has been implicated in cancer progression and is upregulated in various tumors like pancreatic, breast, and lung cancer (Boire *et al.* 2005; Cisowski *et al.* 2011; Tekin *et al.* 2018). Hits like *aPKC* and *par-1*, which are effective against genetically complex cancer models, are promising candidate drug targets as they may represent broad genetic sensitivities shared among cancer genome landscapes.

The 3 genes identified as unique hits from the KRAS-only screen were *hppy*, *akt*, and *dsor* (Fig. 2, b and c, Supplementary Fig. 2c), which are critical components of Hippo, PI3K, and MAPK signaling cascades, respectively (Zheng *et al.* 2015; Yang *et al.* 2019; Guo *et al.* 2020). Hppy, a serine–threonine kinase, activates Warts alongside Hippo and regulates JNK signaling, inflammation, cellular migration, and invasion (Meng *et al.* 2015; Zheng *et al.* 2015;

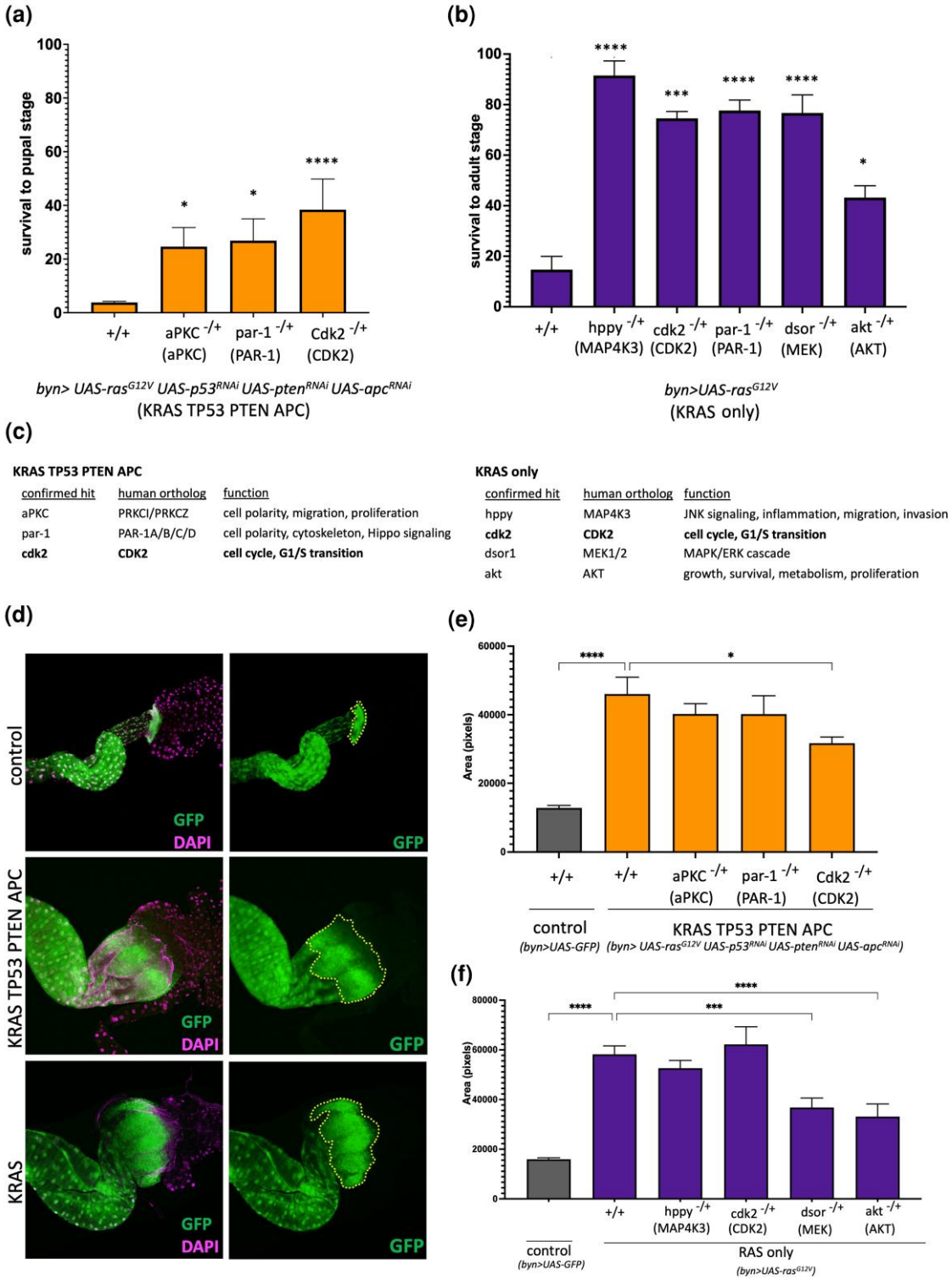


**Fig. 2.** Hit confirmation and subsequent analysis. a and b) Rescue of KRAS TP53 PTEN APC-induced larval lethality (a) and KRAS-induced pupal lethality (b) by confirmed hits from the kinome screens. Human orthologs are in parentheses. Error bars represent standard error of the mean (SEM). $^{*}P \leq 0.05$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ (multiple unpaired *t*-tests with Holm-Sidak Correction, PRISM Software). c) Confirmed hits from KRAS TP53 PTEN APC (left) and KRAS (right) screens, with their human orthologs and biological functions. d) Anterior regions of dissected control (GFP only), KRAS TP53 PTEN APC and KRAS alone hindguts. GFP labels the larval hindgut epithelium and DAPI marks the nuclei. e and f) Quantification of the imaginal ring area (outlined in yellow dashed lines in panels on the right in (d) in hindguts with indicated genotypes, measured in pixels. Error bars representSEM. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ (one-way ANOVA, PRISM Software). Mutant alleles: *aPKC*$^{k06403}$, *par-1*$^{k06323}$, *cdk2*$^{2}$, *hppy*$^{SH1261}$, *dsor*$^{LH110}$, *akt*$^{04226}$.

Liu *et al.* 2017; Chuang *et al.* 2018, 2019). Its human ortholog, MAP4K3, promotes cell migration and invasion in various cancers (Ho *et al.* 2016; Hsu *et al.* 2016; Varghese *et al.* 2016; Liu *et al.* 2017). *dsor*, the *Drosophila* ortholog of MEK1/2, is a central component of the MAPK signaling pathway, which phosphorylates ERK and regulates several cancer-relevant processes, including cell growth, proliferation, differentiation, migration, and apoptosis. Aberrant activation of MAPK signaling in tumors results in unregulated

cell proliferation and downregulation of antiproliferative genes (Chambard *et al.* 2007; Guo *et al.* 2020). The third hit, *akt*, lies downstream of PI3K/AKT signaling cascade, another pathway that regulates cell growth, survival, proliferation, and metabolism in *Drosophila* and mammals (Saxton and Sabatini 2017) and is dysregulated in various cancers (Yang *et al.* 2019). Our observation is that KRAS TP53 PTEN APC is not sensitive to a reduction in the gene dosage of these kinases is interesting, particularly in the case of *dsor* and *akt*, given the strong activation of both MAPK and AKT signaling pathways in RAS-PI3K co-activated tumors (Wee *et al.* 2009). Overall, our findings suggest that as the genetic complexity of the model increases, it may become less dependent on kinases that have essential roles in simpler genetic backgrounds.

## Characterizing hits using cancer-relevant secondary assays

While rescue from lethality is a useful surrogate screening read-out, it is critical to establish more disease-relevant secondary assays to test whether hits from such screens can also modify disease phenotypes and explore their mechanisms of action (Rudrapatna *et al.* 2014; Levinson and Cagan 2016; Bangi *et al.* 2019). We have previously demonstrated that multigenic cancer models in *Drosophila* capture multiple key hallmarks of human tumors and using multiple phenotypic read-outs is particularly important for exploring mechanisms of action of hits identified from genetic and drug screens (Bangi *et al.* 2012, 2016, 2019). To illustrate this principle, we use one such assay to test our hits from the genetic screens as a proof of concept.

Targeting KRAS TP53 PTEN APC and KRAS to the larval hindgut epithelium results in the expansion of the hindgut imaginal ring zone (Fig. 2d), the anterior region of the hindgut where proliferating progenitor cells reside (Murakami and Shiotsuki 2001). We next tested whether hits from our screens could reduce KRAS TP53 PTEN APC or KRAS-induced expansion of this region as a secondary read-out. We found that a reduction in *cdk2* resulted in a significant decrease in the size of the imaginal ring area in the KRAS TP53 PTEN APC background but not in KRAS alone (Fig. 2, e and f). We also found that reduction in *akt* or *dsor* levels resulted in a significant reduction in the imaginal ring zone size in KRAS alone (Fig. 2f), further confirming the essential roles these 2 kinases play as downstream mediators of the RAS pathway. None of the hits affected the size of the anterior hindgut of GFP-only control animals (Supplementary Fig. 2d).

Combined, these results show that not all hits that rescued KRAS TP53 PTEN APC or KRAS-induced lethality were able to reduce the size of the transformed hindgut area, demonstrating that the expansion of the imaginal ring area cannot fully account for the lethal phenotype we observe in our models. As the size of this area is likely to be determined by a combination of proliferation, apoptosis, senescence, and cell growth, subsequent assays that quantitatively analyze these markers will be necessary to investigate the mechanisms of action of these hits. Furthermore, given the complex nature of intestinal transformation, hits that had no effect in this particular assay are likely to contribute to intestinal transformation by affecting other hallmarks of cancer. Future studies using additional phenotypic read-outs of intestinal transformation, such as epithelial–mesenchymal transition and cell polarity, will be necessary to explore the mechanisms of action of these hits further. Overall, these results demonstrate that a single secondary assay is unlikely to be sufficient to confirm hits from lethality-based screens and emphasize the importance of using multiple assays that capture different hallmarks of tumors to fully explore the mechanisms of action of hits generated from genetic screens.

## Tissue-specific knockdowns uncover genetic dependencies on *dsor* and *akt*

Ras/MAPK and PI3K/AKT signaling cascades are 2 of the most common pathways dysregulated in human cancers and multiple experimental models, including our previous work (Ericson *et al.* 2010; Bangi *et al.* 2016, 2019; Reggiani Bonetti *et al.* 2018; Jiang *et al.* 2020; Chen *et al.* 2021), making them relevant drug targets in cancer therapy. Still, we found that reducing *dsor* or *akt* gene dosage using the loss of function alleles is insufficient to rescue KRAS TP53 PTEN APC-induced lethality in our screen, suggesting that this model was not very sensitive to changes in *akt* or *dsor* levels. To investigate the genetic dependency of our multigenic model on these 2 genes, we tested the effect of strongly knocking down each gene in the hindgut on KRAS TP53 PTEN APC-induced lethality. RNAi-mediated knockdown of *dsor* or *akt* in the hindgut epithelium using 2 independent RNAi lines for each significantly improved survival to the pupal stage in KRAS TP53 PTEN APC background (Fig. 3, a and c, Supplementary Fig. 3b) and reduced the expansion of the imaginal ring area (Fig. 3, e and g, Supplementary Fig. 3c). Furthermore, an RNAi line targeting *luciferase* had no effect on KRAS TP53 PTEN APC-induced lethality or the expansion of the anterior hindgut area (Supplementary Fig. 3, b and c), indicating that the rescue observed upon *dsor* and *akt* knockdown is not an indirect effect of introducing another UAS-transgene into this already complex genetic background.

Consistent with our KRAS-only screen results, RNAi knockdown of *akt* and *dsor* also rescued KRAS-induced lethality and expansion of the imaginal ring area (Fig. 3, b, d, f, and h). These results demonstrate that even though both *akt* and *dsor* are required for intestinal transformation in KRAS TP53 PTEN APC background, this model is more resistant to moderate changes in *dsor* or *akt* levels; a more substantial reduction in their activity is necessary to lead to the rescue. This contrasts with the KRAS-only background, where reducing the *akt* or *dsor* gene dosage is sufficient to mount a rescue.

Identifying genetic dependencies of tumors is a crucial step in target identification and prioritization in drug discovery approaches. Our findings demonstrate that genetically complex models may not be sensitive to moderate reductions in the activity of all genes, on which they are dependent. This has important implications for target prioritization for drug discovery, as it may not always be feasible to achieve a strong enough reduction in target activity comparable to what we observe in our tissue-specific knockdowns by systemic drug treatment without significant side effects. As genetic modifier screens performed in heterozygous genetic backgrounds select for hits that suppress disease phenotypes without causing any organismal lethality by design, they may represent a valuable approach to identifying the most rate-limiting genetic vulnerabilities that may be more suitable candidate drug targets.

## Molecular correlates of genetic dependency and sensitivity to *akt* and *dsor*

To further investigate how reducing the gene dosage of *akt* or *dsor* and their RNAi knockdowns alter Ras/MAPK and AKT pathway activity in our models, we directly analyzed the levels of phoshpo-Akt (pAKT) and diphospho-Erk (dpERK), the main read-outs of the Ras/MAPK and AKT pathways, respectively, in KRAS TP53 PTEN APC and KRAS-only hindguts (Fig. 4). Heterozygous loss of *akt* or *dsor* resulted in a modest but statistically significant

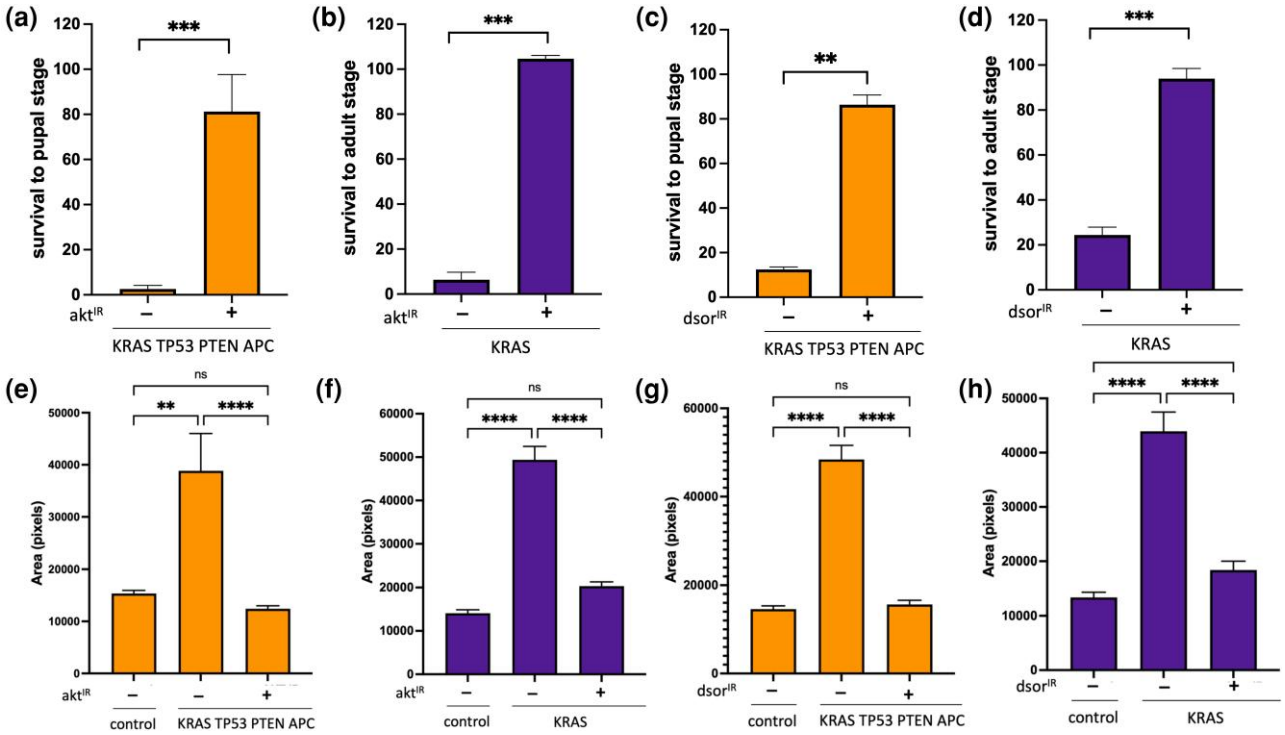


**Fig. 3.** Strong-tissue specific knockdowns reveal requirements for both *dsor* and *akt* in KRAS TP53 PTEN APC. a–d) Rescue of KRAS TP53 PTEN APC induced larval lethality (a and c) and KRAS induced pupal lethality (b and d) by knocking down *akt* (a and b) or *dsor* (c and d) in the hindgut epithelium. Error bars represent SEM. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ (*t*-tests, PRISM software). e–h) Quantification of hindgut imaginal ring area in hindguts with indicated genotypes. Error bars represent SEM. $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$ (one-way ANOVA, PRISM Software).

downregulation of dpERK in KRAS TP53 PTEN APC hindguts but had no significant effect on pAKT levels (Fig. 4, a, c, and e), suggesting that increased AKT activity may be more critical than ERK activity in the context of KRAS TP53 PTEN APC. *dsor* knockdown in KRAS TP53 PTEN APC hindguts was not sufficient to alter pAKT levels but resulted in a much stronger reduction in pERK levels, indicating that KRAS TP53 PTEN APC is still dependent on ERK activity and can be rescued if a strong enough reduction in its activity is achieved (Fig. 3, c and g). *akt* knockdown, on the other hand, resulted in a significant decrease in both pAKT and pERK levels, albeit a much stronger reduction in pAKT compared to dpERK (Fig. 4, c and e). These results demonstrate that in KRAS TP53 PTEN APC, a strong decrease in either AKT or MAPK pathway activity is sufficient to result in rescue; however, this level of reduction in activity could not be achieved in heterozygous mutant backgrounds for either *akt* or *dsor*.

In comparison, in KRAS-only hindguts, we observed a more significant decrease in MAPK and AKT pathway activities in *dsor* and *akt* heterozygous mutant backgrounds, respectively (Fig. 4, b, d, and f). We also noted a significant increase in pAKT levels in *dsor* heterozygous mutant background, most likely due to the disruption of a previously established negative feedback regulation of AKT activity by MEK (Turke *et al.* 2012). Our finding that heterozygous *dsor* loss is sufficient to rescue both KRAS-induced lethality and imaginal ring area expansion despite an increase in pAKT levels suggests that in a KRAS-only background, transformed tissue is more dependent on MAPK activity than AKT signaling. In contrast, KRAS TP53 PTEN APC transformed tissue is more dependent on the AKT pathway. Combined, these results show that strong downregulation of either MAPK or AKT signaling pathways results in the rescue of lethality and a significant decrease in the size of the transformed tissue (Fig. 3), demonstrating that both pathways are essential for KRAS TP53 PTEN APC and KRAS alone. However, it is easier to achieve a level of reduction in MAPK or AKT pathway activity sufficient to rescue lethality or overgrowth in KRAS alone compared to KRAS TP53 PTEN APC.

## Concluding remarks

AKT and MEK have been attractive targets for cancer drug discovery given their central roles in Ras/MAPK and PI3K/AKT pathways and functional studies demonstrating a requirement for these 2 genes in tumor progression and development (Yap *et al.* 2011; Caunt *et al.* 2015; Saura *et al.* 2017; Bose and Kalinsky 2021). Despite extensive efforts, single-agent MEK and AKT inhibitors have not been very successful in clinical trials for most solid tumor types in part due to severe toxicities (Chandarlapaty *et al.* 2011; Serra *et al.* 2011; Yap *et al.* 2011; Faião-Flores *et al.* 2019; Zhan *et al.* 2019). In addition, moderate inhibition of the MAPK pathway by MEK inhibitors can also lead to the development of resistance mainly via upregulation of the PI3K/AKT pathway (Balmanno *et al.* 2009; Turke *et al.* 2012; Irvine *et al.* 2018; Tsubaki *et al.* 2019). Importantly, AKT and MEK inhibitor combinations designed to inhibit both pathways simultaneously and prevent the activation of feedback loops that promote resistance have also been unsuccessful due to adverse effects that were more severe than single-agent trials and observed at lower doses (Tolcher *et al.* 2015, 2020). Overall, our findings are consistent with clinical studies suggesting that, despite the importance of these genes in cancer, pharmacological inhibition of their activity to a level that could be detrimental to genetically complex tumors may not be possible in most cases due to significant side effects and toxicities (Altomare *et al.* 2010; Greenwell *et al.* 2017; Ma *et al.* 2017; Mondaca *et al.* 2018; Glen *et al.* 2022).

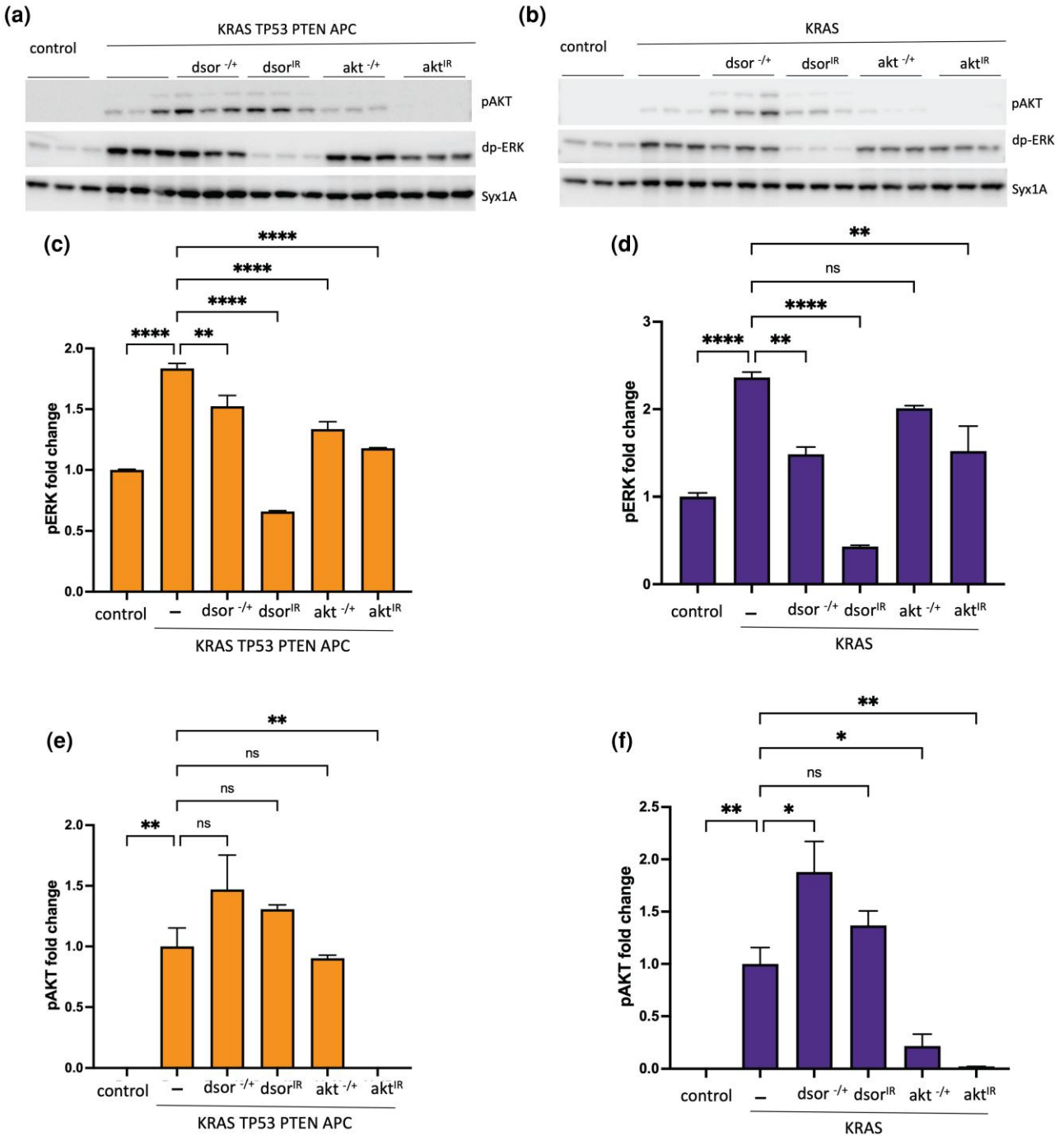


**Fig. 4.** Analysis of MAPK and AKT pathway activity in response to tissue specific knockdown vs. heterozygous loss. a and b) Western blot analysis of pAKT and pERK levels in protein extracts from hindguts with indicated genotypes (3 biological replicates/genotype). Syntaxin: loading control. c–f) Quantification of western blot data presented in (a) and (b). For quantification of pERK levels, all genotypes are normalized to GFP-only negative controls (c and d). As there were no detectable pAKT bands in GFP-only negative controls (top blots in a and b), all genotypes were normalized to KRAS TP53 PTEN APC (e) or KRAS (f). Error bars represent SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$ (one-way ANOVA, PRISM Software).

As most drugs are administered systemically, identifying doses that provide a clinical benefit without any adverse effects and toxicities can be challenging. (Muller and Milton 2012). In fact, a significant fraction of drugs that enter clinical trials fail due to unmanageable toxicities or lack of clinical efficacy at maximum tolerable doses, even though they target critical cancer genetic dependencies (Sun *et al.* 2022). While tissue-specific gene knockdown and knockout approaches are helpful in identifying genes essential for mediating disease phenotypes, the dependence of a disease on a gene does not necessarily reflect its potential as a drug target. Our results suggest that classic genetic modifier screens performed in heterozygous mutant backgrounds that result in a modest, nonlethal reduction in gene activity in the context of a whole animal may be a particularly useful complementary strategy to identify promising genetic vulnerabilities that can be successfully exploited by therapy.

## Data availability statement

Fly stocks generated through this work are available upon request. The BDSC IDs and full genotypes of the kinome stocks used in this study are available in Supplementary Table 1 and online at https://bdsc.indiana.edu/index.html. More information on the kinase alleles used in this study can be found online at https://flybase.org/.

Supplemental material is available at G3 online.

## Acknowledgments

Stocks and transgenic TRIP RNAi lines (Office of the Director R24 OD030002: "TRiP resources for modeling human disease") obtained from the BDSC (NIH P40OD018537) and transgenic fly stocks obtained from the VDRC (www.vdrc.at) were used in this study. We also thank Jason Cassara, Juan Martin Portilla, Justin Brown, and Dr Fateme Karimi Dermani for their technical assistance.

## Conflicts of interest

The authors declare no conflict of interest.

## Literature cited

Altomare DA, Zhang L, Deng J, Di Cristofano A, Klein-Szanto AJ, Kumar R, Testa JR. GSK690693 delays tumor onset and progression in genetically defined mouse models expressing activated Akt. Clin Cancer Res. 2010;16(2):486–496. doi:10.1158/1078-0432.CCR-09-1026.

Attwood MM, Fabbro D, Sokolov AV, Knapp S, Schiöth HB. Trends in kinase drug discovery: targets, indications and inhibitor design. Nat Rev Drug Discov. 2021;20(11):839–861. doi:10.1038/s41573-021-00252-y.

Bailey MH, Tokheim C, Porta-Pardo E, Sengupta S, Bertrand D, Weerasinghe A, Colaprico A, Wendl MC, Kim J, Reardon B, *et al.* Comprehensive characterization of cancer driver genes and mutations. Cell. 2018;173(2):371–385.e18. doi:10.1016/j.cell.2018.02.060.

Balmanno K, Chell SD, Gillings AS, Hayat S, Cook SJ. Intrinsic resistance to the MEK1/2 inhibitor AZD6244 (ARRY-142886) is associated with weak ERK1/2 signalling and/or strong PI3K signalling in colorectal cancer cell lines. Int J Cancer. 2009;125-(10):2332–2341. doi:10.1002/ijc.24604.

Bangi E, Ang C, Smibert P, Uzilov AV, Teague AG, Antipin Y, Chen R, Hecht C, Gruszczynski N, Yon WJ, *et al.* A personalized platform identifies trametinib plus zoledronate for a patient with KRAS-mutant metastatic colorectal cancer. Sci Adv. 2019;5(5):eaav6528. doi:10.1126/sciadv.aav6528.

Bangi E, Murgia C, Teague AGS, Sansom OJ, Cagan RL. Functional exploration of colorectal cancer genomes using Drosophila. Nat Commun. 2016;7(1):13615. doi:10.1038/ncomms13615.

Bangi E, Pitsouli C, Rahme LG, Cagan R, Apidianakis Y. Immune response to bacteria induces dissemination of Ras-activated Drosophila hindgut cells. EMBO Rep. 2012;13(6):569–576. doi:10.1038/embor.2012.44.

Bangi E, Smibert P, Uzilov AV, Teague AG, Gopinath S, Antipin Y, Chen R, Hecht C, Gruszczynski N, Yon WJ, *et al.* A Drosophila platform identifies a novel, personalized therapy for a patient with adenoid cystic carcinoma. iScience. 2021;24(3):102212. doi:10.1016/j.isci.2021.102212.

Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell. 2005;120(3):303–313. doi:10.1016/j.cell.2004.12.018.

Bose S, Kalinsky K. Durable clinical activity to the AKT inhibitor ipatasertib in a heavily pretreated patient with an AKT1 E17K mutant metastatic breast cancer. Clin Breast Cancer. 2021;21(3):e150–e153. doi:10.1016/j.clbc.2020.10.002.

Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487-(7407):330–337. doi:10.1038/nature11252.

Caunt CJ, Sale MJ, Smith PD, Cook SJ. MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road. Nat Rev Cancer. 2015;15(10):577–592. doi:10.1038/nrc4000.

Chambard J-C, Lefloch R, Pouysségur J, Lenormand P. ERK implication in cell cycle regulation. Biochim Biophys Acta. 2007;1773(8):1299–1310. doi:10.1016/j.bbamcr.2006.11.010.

Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, *et al.* AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011;19(1):58–71. doi:10.1016/j.ccr.2010.10.031.

Chen K, Zhang Y, Qian L, Wang P. Emerging strategies to target RAS signaling in human cancer therapy. J Hematol Oncol. 2021;14(1):116. doi:10.1186/s13045-021-01127-w.

Cheng L, Baonza A, Grifoni D. Models of human disease. Biomed Res Int. 2018;2018:7214974. doi:10.1155/2018/7214974.

Chuang H-C, Chang C-C, Teng C-F, Hsueh C-H, Chiu L-L, Hsu P-M, Lee M-C, Hsu C-P, Chen Y-R, Liu Y-C, *et al.* MAP4K3/GLK promotes lung cancer metastasis by phosphorylating and activating IQGAP1. Cancer Res. 2019;79(19):4978–4993. doi:10.1158/0008-5472.CAN-19-1402.

Chuang H-C, Tsai C-Y, Hsueh C-H, Tan T-H. GLK-IKKβ signaling induces dimerization and translocation of the AhR-RORγt complex in IL-17A induction and autoimmune disease. Sci Adv. 2018;4(9):eaat5401. doi:10.1126/sciadv.aat5401.

Cicenas J, Zalyte E, Bairoch A, Gaudet P. Kinases and cancer. Cancers (Basel). 2018;10(3):63. doi:10.3390/cancers10030063.

Cisowski J, O'Callaghan K, Kuliopulos A, Yang J, Nguyen N, Deng Q, Yang E, Fogel M, Tressel S, Foley C, *et al.* Targeting protease-activated receptor-1 with cell-penetrating pepducins in lung cancer. Am J Pathol. 2011;179(1):513–523. doi:10.1016/j.ajpath.2011.03.025.

Dar AC, Das TK, Shokat KM, Cagan RL. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. Nature. 2012;486(7401):80–84. doi:10.1038/nature11127.

Das T, Andrieux G, Ahmed M, Chakraborty S. Integration of online omics-data resources for cancer research. Front Genet. 2020;11:578345. doi:10.3389/fgene.2020.578345.

Das TK, Esernio J, Cagan RL. Restraining network response to targeted cancer therapies improves efficacy and reduces cellular resistance. Cancer Res. 2018;78(15):4344–4359. doi:10.1158/0008-5472.CAN-17-2001.

Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, *et al.* A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature. 2007;448(7150):151–156. doi:10.1038/nature05954.

Doerflinger H, Zimyanin V, St Johnston D. The Drosophila anterior-posterior axis is polarized by asymmetric myosin activation. Curr Biol. 2022;32(2):374–385.e4. doi:10.1016/j.cub.2021.11.024.

Eder AM, Sui X, Rosen DG, Nolden LK, Cheng KW, Lahad JP, Kango-Singh M, Lu KH, Warneke CL, Atkinson EN, *et al.* Atypical PKCι contributes to poor prognosis through loss of apical-basal polarity and cyclin E overexpression in ovarian cancer. Proc

Natl Acad Sci U S A. 2005;102(35):12519–12524. doi:10.1073/pnas.0505641102.

Ericson K, Gan C, Cheong I, Rago C, Samuels Y, Velculescu VE, Kinzler KW, Huso DL, Vogelstein B, Papadopoulos N. Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation. Proc Natl Acad Sci U S A. 2010;107(6):2598–2603. doi:10.1073/pnas.0914018107.

Faião-Flores F, Emmons MF, Durante MA, Kinose F, Saha B, Fang B, Koomen JM, Chellappan SP, Maria-Engler SS, Rix U, *et al.* HDAC inhibition enhances the in vivo efficacy of MEK inhibitor therapy in uveal melanoma. Clin Cancer Res. 2019;25(18):5686–5701. doi:10.1158/1078-0432.CCR-18-3382.

Glen C, Tan YY, Waterston A, Evans TRJ, Jones RJ, Petrie MC, Lang NN. Mechanistic and clinical overview cardiovascular toxicity of BRAF and MEK inhibitors: JACC: CardioOncology state-of-the-art review. JACC CardioOncol. 2022;4(1):1–18. doi:10.1016/j.jaccao.2022.01.096.

Gramates LS, Agapite J, Attrill H, Calvi BR, Crosby MA, dos Santos G, Goodman JL, Goutte-Gattat D, Jenkins VK, Kaufman T, *et al.* Flybase: a guided tour of highlighted features. Genetics. 2022;220(4):iyac035. doi:10.1093/genetics/iyac035.

Greenwell IB, Ip A, Cohen JB. PI3K Inhibitors: understanding toxicity mechanisms and management. Oncology. 2017;31(11):821–828. PMID: 29179250.

Gross S, Rahal R, Stransky N, Lengauer C, Hoeflich KP. Targeting cancer with kinase inhibitors. J Clin Invest. 2015;125(5):1780–1789. doi:10.1172/JCI76094.

Guerin MV, Finisguerra V, Van den Eynde BJ, Bercovici N, Trautmann A. Preclinical murine tumor models: a structural and functional perspective. Elife. 2020;9:e50740. doi:10.7554/eLife.50740.

Guo Y-J, Pan W-W, Liu S-B, Shen Z-F, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. Exp Ther Med. 2020;19(3):1997–2007. doi:10.3892/etm.2020.8454.

Haley B, Roudnicky F. Functional genomics for cancer drug target discovery. Cancer Cell. 2020;38(1):31–43. doi:10.1016/j.ccell.2020.04.006.

Ho C-H, Chuang H-C, Wu I-C, Tsai H-W, Lin Y-J, *et al.* Prediction of early hepatocellular carcinoma recurrence using germinal center kinase-like kinase. Oncotarget. 2016;7(31):49765–49776. doi:10.18632/oncotarget.10176.

Hong Y. aPKC: the kinase that phosphorylates cell polarity. F1000Res. 2018;7:F1000 Faculty Rev-903. doi:10.12688/f1000research.14427.1.

Hsu C-P, Chuang H-C, Lee M-C, Tsou H-H, Lee L-W, Li J-P, Tan T-H. GLK/MAP4K3 overexpression associates with recurrence risk for non-small cell lung cancer. Oncotarget. 2016;7(27):41748–41757. doi:10.18632/oncotarget.9410.

Huang H-L, Wang S, Yin M-X, Dong L, Wang C, Wu Wei, Lu Y, Feng M, Dai C, Guo X, *et al.* Par-1 regulates tissue growth by influencing hippo phosphorylation status and hippo-salvador association. PLoS Biol. 2013;11(8):e1001620. doi:10.1371/journal.pbio.1001620.

Irvine M, Stewart A, Pedersen B, Boyd S, Kefford R, Rizos H. Oncogenic PI3K/AKT promotes the step-wise evolution of combination BRAF/MEK inhibitor resistance in melanoma. Oncogenesis. 2018;7(9):72. doi:10.1038/s41389-018-0081-3.

Jiang N, Dai Q, Su X, Fu J, Feng X, Peng J. Role of PI3K/AKT pathway in cancer: the framework of malignant behavior. Mol Biol Rep. 2020;47(6):4587–4629. doi:10.1007/s11033-020-05435-1.

Kersten K, de Visser KE, van Miltenburg MH, Jonkers J. Genetically engineered mouse models in oncology research and cancer medicine. EMBO Mol Med. 2017;9(2):137–153. doi:10.15252/emmm.201606857.

Lee T, Luo L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron. 1999;22(3):451–461. doi:10.1016/S0896-6273(00)80701-1.

Levinson S, Cagan RL. Drosophila cancer models identify functional differences between Ret fusions. Cell Rep. 2016;16(11):3052–3061. doi:10.1016/j.celrep.2016.08.019.

Liu L, Lu L, Zheng A, Xie J, Xue Q, Wang F, Wang X, Zhou H, Tong X, Li Y, *et al.* MiR-199a-5p and let-7c cooperatively inhibit migration and invasion by targeting MAP4K3 in hepatocellular carcinoma. Oncotarget. 2017;8(8):13666–13677. doi:10.18632/oncotarget.14623.

Ma CX, Suman V, Goetz MP, Northfelt D, Burkard ME, Ademuyiwa F, Naughton M, Margenthaler J, Aft R, Gray R, *et al.* A phase II trial of neoadjuvant MK-2206, an AKT inhibitor, with anastrozole in clinical stage II or III *PIK3CA*-mutant ER-positive and HER2-negative breast cancer. Clin Cancer Res. 2017;23(22):6823–6832. doi:10.1158/1078-0432.CCR-17-1260.

Manning G, Plowman GD, Hunter T, Sudarsanam S. Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci. 2002;27(10):514–520. doi:10.1016/S0968-0004(02)02179-5.

McGuire SE, Mao Z, Davis RL. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci STKE. 2004;2004(220):l6. doi:10.1126/stke.2202004pl6.

Meng Z, Moroishi T, Mottier-Pavie V, Plouffe SW, Hansen CG, Hong AW, Park HW, Mo J-S, Lu W, Lu S, *et al.* MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the hippo pathway. Nat Commun. 2015;6(1):8357. doi:10.1038/ncomms9357.

Mohr SE, Perrimon N. A simple system for understanding complexity. Dis Model Mech. 2019;12(10):dmm041871. doi:10.1242/dmm.041871.

Mondaca S, Lacouture M, Hersch J, Yaeger R. Balancing RAF, MEK, and EGFR inhibitor doses to achieve clinical responses and modulate toxicity in BRAF V600E colorectal cancer. JCO Precis Oncol. 2018;2018:PO.18.00088. doi:10.1200/PO.18.00088.

Muller PY, Milton MN. The determination and interpretation of the therapeutic index in drug development. Nat Rev Drug Dis. 2012;11(10):751–761. doi:10.1038/nrd3801.

Munnik C, Xaba MP, Malindisa ST, Russell BL, Sooklal SA. Drosophila melanogaster: a platform for anticancer drug discovery and personalized therapies. Front Genet. 2022;13:949241. doi:10.3389/fgene.2022.949241.

Murakami R, Shiotsuki Y. Ultrastructure of the hindgut of Drosophila larvae, with special reference to the domains identified by specific gene expression patterns. J Morphol. 2001;248(2):144–150. doi:10.1002/jmor.1025.

Nayak RC, Hegde S, Althoff MJ, Wellendorf AM, Mohmoud F, *et al.* The signaling axis atypical protein kinase C λ/ι-satb2 mediates leukemic transformation of B-cell progenitors. Nat Commun. 2019;10(1):46. doi:10.1038/s41467-018-07846-y.

Pina C, Pignoni F. Tubby-RFP balancers for developmental analysis: FM7c 2xTb-RFP, CyO 2xTb-RFP, and TM3 2xTb-RFP. Genesis. 2012;50(2):119–123. doi:10.1002/dvg.20801.

Regala RP, Weems C, Jamieson L, Khoor A, Edell ES, *et al.* Atypical protein kinase Cι is an oncogene in human non-small cell lung cancer. Cancer Res. 2005;65(19):8905–8911. doi:10.1158/0008-5472.CAN-05-2372.

Reggiani Bonetti L, Barresi V, Maiorana A, Manfredini S, Caprera C, Bettelli S. Clinical impact and prognostic role of KRAS/BRAF/PIK3CA mutations in stage I colorectal cancer. Dis Markers. 2018;2018:2959801. doi:10.1155/2018/2959801.

Roesley SNA, La Marca JE, Deans AJ, Mckenzie L, Suryadinata R, Burke P, Portela M, Wang H, Bernard O, Sarcevic B, *et al.* Phosphorylation of Drosophila Brahma on CDK-phosphorylation sites is important for cell cycle regulation and differentiation. Cell Cycle. 2018;17(13):1559–1578. doi:10.1080/15384101.2018.1493414.

Rudrapatna VA, Bangi E, Cagan RL. A Jnk-Rho-Actin remodeling positive feedback network directs Src-driven invasion. Oncogene. 2014;33(21):2801–2806. doi:10.1038/onc.2013.232.

Sajjad H, Imtiaz S, Noor T, Siddiqui YH, Sajjad A, Zia M. Cancer models in preclinical research: a chronicle review of advancement in effective cancer research. Animal Model Exp Med. 2021;4(2): 87–103. doi:10.1002/ame2.12165.

Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, Dimitriadoy S, Liu DL, Kantheti HS, Saghafinia S, *et al.* Oncogenic signaling pathways in the cancer genome atlas. Cell. 2018;173(2):321–337.e10. doi:10.1016/j.cell.2018.03.035.

Sauer K, Knoblich JA, Richardson H, Lehner CF. Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of Drosophila embryogenesis. Genes Dev. 1995;9(11):1327–1339. doi:10.1101/gad.9.11.1327.

Saura C, Roda D, Roselló S, Oliveira M, Macarulla T, Pérez-Fidalgo JA, Morales-Barrera R, Sanchis-García JM, Musib L, Budha N, *et al.* A first-in-human phase I study of the ATP-competitive AKT inhibitor ipatasertib demonstrates robust and safe targeting of AKT in patients with solid tumors. Cancer Discov. 2017;7(1):102–113. doi: 10.1158/2159-8290.CD-16-0512.

Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell. 2017;169(2):361–371. doi:10.1016/j.cell.2017.03.035.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676–682. doi:10.1038/nmeth.2019.

Serra V, Scaltriti M, Prudkin L, Eichhorn PJA, Ibrahim YH, Chandarlapaty S, Markman B, Rodriguez O, Guzman M, Rodriguez S, *et al.* PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene. 2011;30(22):2547–2557. doi:10.1038/onc.2010.626.

Sonoshita M, Scopton AP, Ung PMU, Murray MA, Silber L, Maldonado AY, Real A, Schlessinger A, Cagan RL, Dar AC. A whole-animal platform to advance a clinical kinase inhibitor into new disease space. Nat Chem Biol. 2018;14(3):291–298. doi:10.1038/nchembio.2556.

Starz-Gaiano M, Cho NK, Forbes A, Lehmann R. Spatially restricted activity of a Drosophila lipid phosphatase guides migrating germ cells. Development. 2001;128(6):983–991. doi:10.1242/dev.128.6.983.

Su TT. Drug screening in Drosophila; why, when, and when not? Wiley Interdiscip Rev Dev Biol. 2019;8(6):e346. doi:10.1002/wdev.346.

Sun D, Gao W, Hu H, Zhou S. Why 90% of clinical drug development fails and how to improve it? Acta Pharm Sin B. 2022;12(7): 3049–3062. doi:10.1016/j.apsb.2022.02.002.

Tadesse S, Anshabo AT, Portman N, Lim E, Tilley W, Caldon C, Wang S. Targeting CDK2 in cancer: challenges and opportunities for therapy. Drug Discov Today. 2020;25(2):406–413. doi:10.1016/j.drudis.2019.12.001.

Takashima S, Mkrtchyan M, Younossi-Hartenstein A, Merriam JR, Hartenstein V. The behaviour of Drosophila adult hindgut stem cells is controlled by Wnt and Hh signalling. Nature. 2008; 454(7204):651–655. doi:10.1038/nature07156.

Tekin C, Shi K, Daalhuisen JB, Ten Brink MS, Bijlsma MF, Spek CA. PAR1 signaling on tumor cells limits tumor growth by maintaining a mesenchymal phenotype in pancreatic cancer. Oncotarget. 2018;9(62):32010–32023. doi:10.18632/oncotarget.25880.

Tolcher AW, Kurzrock R, Valero V, Gonzalez R, Heist RS, *et al.* Phase I dose-escalation trial of the oral AKT inhibitor uprosertib in combination with the oral MEK1/MEK2 inhibitor trametinib in patients with solid tumors. Cancer Chemother Pharmacol. 2020; 85(4):673–683. doi:10.1007/s00280-020-04038-8.

Tolcher AW, Patnaik A, Papadopoulos KP, Rasco DW, Becerra CR, *et al.* Phase I study of the MEK inhibitor trametinib in combination with the AKT inhibitor afuresertib in patients with solid tumors and multiple myeloma. Cancer Chemother Pharmacol. 2015; 75(1):183–189. doi:10.1007/s00280-014-2615-5.

Tsubaki M, Takeda T, Noguchi M, Jinushi M, Seki S, *et al.* Overactivation of Akt contributes to MEK inhibitor primary and acquired resistance in colorectal cancer cells. Cancers (Basel). 2019;11(12):1866. doi:10.3390/cancers11121866.

Turke AB, Song Y, Costa C, Cook R, Arteaga CL, Asara JM, Engelman JA. MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. Cancer Res. 2012;72(13): 3228–3237. doi:10.1158/0008-5472.CAN-11-3747.

Varghese RT, Liang Y, Guan T, Franck CT, Kelly DF, Sheng Z. Survival kinase genes present prognostic significance in glioblastoma. Oncotarget. 2016;7(15):20140–20151. doi:10.18632/oncotarget.7917.

Verheyen EM. The power of Drosophila in modeling human disease mechanisms. Dis Model Mech. 2022;15(3):dmm049549. doi:10.1242/dmm.049549.

Vorhagen S, Niessen CM. Mammalian aPKC/par polarity complex mediated regulation of epithelial division orientation and cell fate. Exp Cell Res. 2014;328(2):296–302. doi:10.1016/j.yexcr.2014.08.008.

Weber J, Braun CJ, Saur D, Rad R. In vivo functional screening for systems-level integrative cancer genomics. Nat Rev Cancer. 2020;20(10):573–593. doi:10.1038/s41568-020-0275-9.

Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, *et al.* PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res. 2009;69(10):4286–4293. doi:10.1158/0008-5472.CAN-08-4765.

Wu Y, Griffin EE. Regulation of cell polarity by PAR-1/MARK kinase. Curr Top Dev Biol. 2017;123:365–397. doi:10.1016/bs.ctdb.2016.11.001.

Yang J, Nie J, Ma X, Wei Y, Peng Y, *et al.* Targeting PI3K in cancer: mechanisms and advances in clinical trials. Mol Cancer. 2019;18(1): 26. doi:10.1186/s12943-019-0954-x.

Yap TA, Yan L, Patnaik A, Fearen I, Olmos D, Papadopoulos K, Baird RD, Delgado L, Taylor A, Lupinacci L, *et al.* First-in-man clinical trial of the oral pan-AKT inhibitor MK-2206 in patients with advanced solid tumors. J Clin Oncol. 2011;29(35):4688–4695. doi:10.1200/JCO.2011.35.5263.

Zhan T, Ambrosi G, Wandmacher AM, Rauscher B, Betge J, Rindtorff N, Häussler RS, Hinsenkamp I, Bamberg L, Hessling B, *et al.* MEK inhibitors activate Wnt signalling and induce stem cell plasticity in colorectal cancer. Nat Commun. 2019;10(1):2197. doi:10.1038/s41467-019-09898-0.

Zhang Y, Chen F, Chandrashekar DS, Varambally S, Creighton CJ. Proteogenomic characterization of 2002 human cancers reveals pan-cancer molecular subtypes and associated pathways. Nat Commun. 2022;13(1):2669. doi:10.1038/s41467-022-30342-3.

Zheng Y, Wang W, Liu B, Deng H, Uster E, Pan D. Identification of happyhour/MAP4K as alternative Hpo/Mst-like kinases in the hippo kinase cascade. Dev Cell. 2015;34(6):642–655. doi:10.1016/j.devcel.2015.08.014.

Zirin J, Hu Y, Liu L, Yang-Zhou D, Colbeth R, Yan D, Ewen-Campen B, Tao R, Vogt E, VanNest S, *et al.* Large-scale transgenic resource collections for loss- and gain-of-function studies. Genetics. 2020;214(4):755–767. doi:10.1534/genetics.119.302964.

*Editor: M. Boutros*