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Systematic screen of chemotherapeutics in *Drosophila* stem cell tumors

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Contributed by Norbert Perrimon, January 21, 2014 (sent for review December 19, 2013)

Here we report the development of an in vivo system to study the interaction of stem cells with drugs using a tumor model in the adult *Drosophila* intestine. Strikingly, we find that some Food and Drug Administration-approved chemotherapeutics that can inhibit the growth of *Drosophila* tumor stem cells can paradoxically promote the hyperproliferation of their wild-type counterparts. These results reveal an unanticipated side effect on stem cells that may contribute to tumor recurrence. We propose that the same side effect may occur in humans based on our finding that it is driven in *Drosophila* by the evolutionarily conserved Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway. An immediate implication of our findings is that supplementing traditional chemotherapeutics with anti-inflammatories may reduce tumor recurrence.

cancer stem cell | drug screening | *Drosophila* intestinal stem cell | whole-animal screening

A vexing problem in cancer therapeutics is tumor recurrence: tumors that initially respond to chemotherapy ultimately return resistant to chemotherapy. Drug-resistant tumors emerge because drugs select for the survival of cells with either preexisting or newly acquired drug resistance properties (1). For example, tumors that recur in the wake of treatment with the ABL-BCR inhibitor Gleevec typically have mutations in ABL-BCR that prevent Gleevec from inhibiting it (2). Sometimes these mutations exist in small populations of tumor cells before drug treatment, whereas other times they arise spontaneously in cells during treatment. Another form of drug resistance comes from "bypass" mutations that activate multiple oncogenes, thereby rendering drug inhibition of a specific oncogene inconsequential (3, 4). This form of resistance is believed to be prevalent due to the genomic instability of most tumors (5). Additionally, there is mounting evidence that selection may act on yet a third level, in which selection is not for specific mutations or oncogenes, but instead for a class of cells with stem cell properties, called cancer stem cells (CSCs) (6, 7). CSCs, like wild-type (WT) stem cells, are defined by their ability to give rise to all of the cell types in a tissue, which in the case of CSCs are all of the cell types of its cognate tumor. CSCs have been identified as rare populations of cells in several cancers including breast, brain, and colorectal cancers. Based on similarities between the biology of CSCs and WT stem cells, including drug resistance (8–12), a relatively new field is emerging to identify small molecules that can target the underlying biology of "stemness."

To date, screens for drugs that target stemness have been largely in vitro using either cultured cell lines induced to become stem cells or stem cells isolated from freshly dissected tissue and cultured in conditions that permit stem cell survival (13). These approaches have identified stem-cell–selective drugs such as salinomycin (14) and metformin (15) for breast CSCs and neurotransmitter inhibitors that suppress neuronal CSCs (16, 17). However, although in vitro stem cell screens have proven successful in identifying drugs that directly act on stem cells, they cannot in their present form identify drugs that act on the stem cell microenvironment (18). Because stem cells rely on their microenvironment for cues to divide, differentiate, and die, this omission from drug screens could miss the identification of drugs with potent effects on stem cells.

However, to include the stem cell microenvironment in chemical screens requires methods to culture stem cells in entirely new ways. Current methods that enable stem cells to be cultured either supply niche signals in lieu of the niche itself or use stem-like cells engineered to retain stem cell characteristics autonomously. Efforts are underway to more precisely culture and screen stem cells cocultured with their niche (19, 20). However, an alternative approach that is more immediately available is to use the ready-made stem cell microenvironments found in living animals, which can be probed by performing whole-animal screens (21).

We set out to develop a whole-animal approach to screen for drugs affecting stemness, using the fruit fly *Drosophila melanogaster*. A strength of *Drosophila* as an organism for whole-animal screening is its small size: adults can fit into the wells of 96-well plates, opening the possibility of using flies to identify drugs that affect adult stem cells in vivo. To take full advantage of this feature, we developed methods to handle flies in 96-well plates. First, we developed a method to house and feed flies drugs in 1-mL deep 96-well plates, containing as little as 100 μL of food. In addition, we developed a method to score the size of stem cell tumors within adults using a luciferase reporter assay adapted to 96-well plates (see below). Together, these methods make it possible to perform chemical screens in adult *Drosophila* for stem-cell–modifying drugs.

We chose to focus on drug interactions with the stem cells of the adult *Drosophila* intestine because they have molecular, physiological, and cellular properties in common with their mammalian counterparts (22–24). Moreover, because they line the digestive

Significance

In this article we report a large-scale chemical screen in adult *Drosophila* to find inhibitors of stem-cell–derived tumors. To our surprise, we found that some Food and Drug Administration-approved chemotherapy drugs have the dual property of reducing growth of stem-cell–derived tumors while also stimulating hyperproliferation of their wild-type counterparts. Since hyperproliferation is one of the hallmarks of cancer cells, this side effect could contribute to refueling the growth of the very tumors that these chemotherapeutics are intended to inhibit. We show that this side effect is driven by the evolutionarily conserved Janus kinase-signal transducers and activators of transcription (JAK-STAT) inflammatory pathway, raising the possibility that the JAK-STAT pathway may also be activated in humans who are treated with some chemotherapeutics.

Author contributions: M.M. designed research; M.M., S.D., J.C., R.A.N., and S.C.-M. performed research; M.M., J.C., R.A.N., and N.P. analyzed data; and M.M. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1401160111/-/DCSupplemental.

track, they are optimally placed to come into direct contact with ingested drugs.

Several features make *Drosophila* intestinal stem cells (ISCs) a compelling model for mammalian ISCs: they are multipotent, giving rise to cell types similar to those in mammals, large absorptive enterocytes (ECs) and a wide array of secretory cell types (25, 26) (Fig. 1*A*); they are similarly situated in a single-layered epithelium that abuts the muscle layer; they differentiate based on stochastic competition (29); and they use evolutionarily conserved pathways for similar processes—the Wnt pathway for stem cell proliferation (30, 31) and the Notch pathway for stem cell differentiation (25, 26, 28). Additional evolutionarily conserved pathways, including the EGFR, Hippo, AKT, and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways, are at play in *Drosophila* ISCs, each of which are linked to human cancers (24, 32). Thus, *Drosophila* ISCs provide both a model for their mammalian counterparts and a multicellular context in which to dissect the interplay of drugs with human oncogenic pathways.

Results

Building an in Vivo Stem-Cell–Derived Tumor Model. Based on the parallels between *Drosophila* and mammalian ISCs, we built a "screenable" tumor model using the ISC-expressed *esg*-Gal4 transcription factor to express transgenes engineered with upstream Gal4-binding sites called upstream activating sequence (UAS) sites (33). We constructed flies to simultaneously express three UAS transgenes under control of the esg-Gal4 transcription factor: UAS-human RAF^{gof} (gain-of-function allele of the serine-threonine kinase Raf) to hyperactivate the downstream oncogenic MAPK pathway (34), UAS-luciferase to estimate tumor size from whole-animal homogenates (35), and UAS-GFP to visualize stem-cell–initiated tumors in dissected intestinal tissue (36) (Fig. 1*B*).

We found that expression of human RAF^{gof} drives the formation of large heterogeneous tumors characterized by the persistent expression of the progenitor marker *esg* and continued activation of the MAPK pathway, which we detected with a phospho-specific antibody against the active di-phosphorlyated form of dpERK, a downstream kinase that mediates MAPK signaling (Fig. 1*C*). These tumors, which we refer to as RAF^{gof} ISC tumors, comprise both mitotic stem-like cells that express the Notch ligand Delta and nondividing daughter cells with large polyploid nuclei (Fig. 1*C*). We observed that only the Delta+ cells stain positively for the mitotic marker phospho-histone 3 (PH3), suggesting that they alone drive proliferation of the tumors (Fig. S1). These results are consistent with observations reported by Jiang et al. (27). In addition, we found that these cells have a great regenerative capacity; they not only maintain the tumor, but also, when we transplanted the tumors to wild-type hosts by injection into the host abdomen, about 10% of the time (n > 100 transplants) the tumors continued growing until they filled the abdomen and killed the host (Fig. 1*D* and Fig. S2).

Although the tumors are heterogeneous, luciferase expression from the tumors is largely consistent from animal to animal, comprising about two-thirds of the total luciferase signal in whole-animal homogenates (Fig. 1*E*). Consequently, changes in luciferase expression can be used to approximate changes in tumor growth. For example, feeding flies the mitotic inhibitor colchicine results in complete tumor loss (Fig. 1*F*) and a corresponding loss of tumor-expressed luciferase activity that can be readily measured in whole-animal homogenates (Fig. 1*G*). These results demonstrate that expressing RAF^{gof} in the ISCs creates heterogeneous tumors that are amenable to screening with the luciferase assay.

Screen of Food and Drug Administration-Approved Chemotherapeutics Identifies Drugs That Inhibit *Drosophila* Tumors. With the screenable RAF^{gof} ISC tumor model in hand we systematically screened the effects of 88 Food and Drug Administration (FDA)-approved chemotherapy drugs [National Cancer Institute (NCI) Drug Therapeutics Program set] to determine the sensitivity of *Drosophila* ISCs to human drugs (Fig. 2*A*). We diluted each drug in a food we developed for mixing chemicals, which we call low-melt fly food (*Materials and Methods*). We decided to test drugs at the highest concentration feasible, 100 μM, to maximize our ability to detect drugs with putative antitumorigenic activity. To determine if the flies would consume the drugs, we added human-grade red food coloring to the low-melt fly food. We found that within 1 h of feeding the dye could be visualized through the abdomen of each fly, indicating that the low-melt fly food and chemotherapeutics were palatable to the flies. However, because dyes in food coloring can have deleterious effects in humans (37), we omitted them from the food used in the screens.

Because mammalian stem cells and their CSC equivalents are largely resistant to chemotherapy (8–12), we expected that the *Drosophila* WT ISCs would likewise be resistant. However, because mammalian stem cells can be sensitized to chemotherapy when induced to actively proliferate (16, 38), we expected that

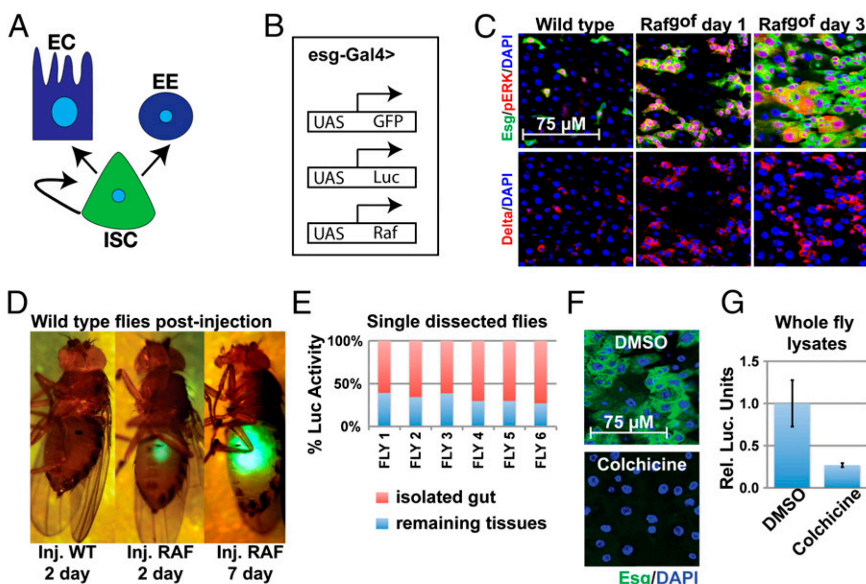

Fig. 1. Characterization of the screenable stem cell tumor model. (*A*) Diagram of intestinal stem cell (ISC) lineage showing polyploid enterocytes (EC) and diploid enteroendocrine cells (EE). (*B*) Genotype of the screenable tumor model showing the esg-Gal4 transcription factor driving the expression of GFP, luciferase, and RAF^{gof} UAS-linked transgenes. (*C*) WT ISCs and RAF^{gof} ISC tumors. ISCs are labeled by esg-Gal4 driving UAS-GFP (green). Nuclei are visualized with the DNA dye DAPI (blue). WT ISCs express dpERK (27) (*Upper*: red cytoplasmic staining) and Delta (28) (*Lower*: red membrane staining). Expression of UAS-RAF^{gof} with the esg-Gal4 driver increases dpERK and proliferation of ISC-like Delta-expressing cells. (*D*) Fate of WT and RAF^{gof} intestinal fragments injected into WT hosts (n > 100). The *esg*+ cells within the injected intestines are marked with GFP (green). (*E*) Measurements of luciferase activity from individually dissected flies shows that gut tumors contribute about 66% of the total luciferase activity in each animal, which correlates with the amount that is absent from colchicine-treated animals (*F* and *G*).

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rapidly dividing RAF^{gof} ISC tumors would be sensitive to at least some chemotherapy drugs.

Consistent with the expectation that RAF^{gof} ISC tumors would be sensitive to human chemotherapy drugs, we identified 14 drugs from the luciferase screen with putative tumor suppressor activity. These drugs, when fed to flies with RAF^{gof} ISC tumors, resulted in a 50% or greater loss of luciferase activity in whole-animal lysates compared with DMSO controls (rank sum $P < 0.001$) (Fig. 2*B*). To validate the luciferase results, we dissected the intestines from flies treated with these drugs to visualize GFP-expressing tumor cells. We also dissected and visualized the intestines of flies treated with drugs that did not reduce luciferase expression. In each case, the GFP observations validated the luciferase results: the drugs that scored as hits in the luciferase screen each reduced tumor burden, whereas drugs, like bleomycin, that failed to score as a hit had no apparent effect on the tumors (Fig. 2*C*). The tumor inhibitors constitute a wide-spectrum of cytotoxic cell cycle inhibitors, including S-phase inhibitors and the pathway-specific mTOR inhibitor rapamycin (39) (Fig. 2*C*, *Upper*), and transcriptional, proteasome, and mitotic inhibitors, as well as inducers of DNA damage (Fig. 2*C*, *Lower*). Together, these results establish that *Drosophila* RAF^{gof} ISC tumors are sensitive to a broad range of compounds of clinical significance.

Side Effect of Class II Drugs Drives Stem Cell Hyperproliferation. We next tested the effects of the 14 RAF^{gof} ISC tumor inhibitors on WT ISCs. Our expectation was that the WT ISCs, like mammalian WT stem cells and mammalian CSCs, would be resistant to traditional chemotherapy drugs. Indeed, under the same conditions as the screen, none of the drugs had obvious inhibitory effects on the WT ISCs (Fig. 2*D*). Because WT ISCs in *Drosophila*, as well as in mammals, divide on average once a day (22), this result is not due to stem cell quiescence. At least within the parameters of our experiment, WT ISCs are less susceptible than their tumor counterparts to the destructive effects of the chemotherapy drugs that we tested.

Although we did not observe inhibitory effects of the drugs on WT ISCs, to our surprise, we found that a diverse spectrum of the drugs induced overgrowth of WT ISCs, including the transcriptional inhibitor actinomycin; the proteasome inhibitor bortezomib; the mitotic inhibitors paclitaxel, vinblastine, and vincristine; and two inducers of DNA damage, mitomycin, and daunorubicin (Fig. 2*D*, *Lower*). The overgrowth of WT ISCs was also observed with bleomycin, as previously reported (40) (Fig. 2*D*).

To determine whether the overgrowth of WT ISCs was due to an increase in proliferation, we stained dissected intestines with antibodies against the mitotic marker PH3. We observed PH3+ staining in the ISCs in both DMSO-treated and drug-treated animals, as seen, for example, in a cluster of esg+ cells after treatment with bortezomib (Fig. 3*A*). PH3+ staining was specific to these cells, evident by focusing on the surface of the intestine where the ISC nuclei are in focus, and it was absent from the EC nuclei, evident by focusing about 1 μm below the ISC nuclei, where the EC nuclei are in focus.

Consistent with the possibility that the increase in WT ISCs could be due to an increase in cell proliferation, we observed a statistically significant increase in the number of PH3+ cells per gut between the cohort of animals treated with DMSO (46 flies) and animals treated with drugs that increased WT ISCs (ranging from 6 to 24 flies per cohort; rank sum <0.001) (Fig. 3*B*).

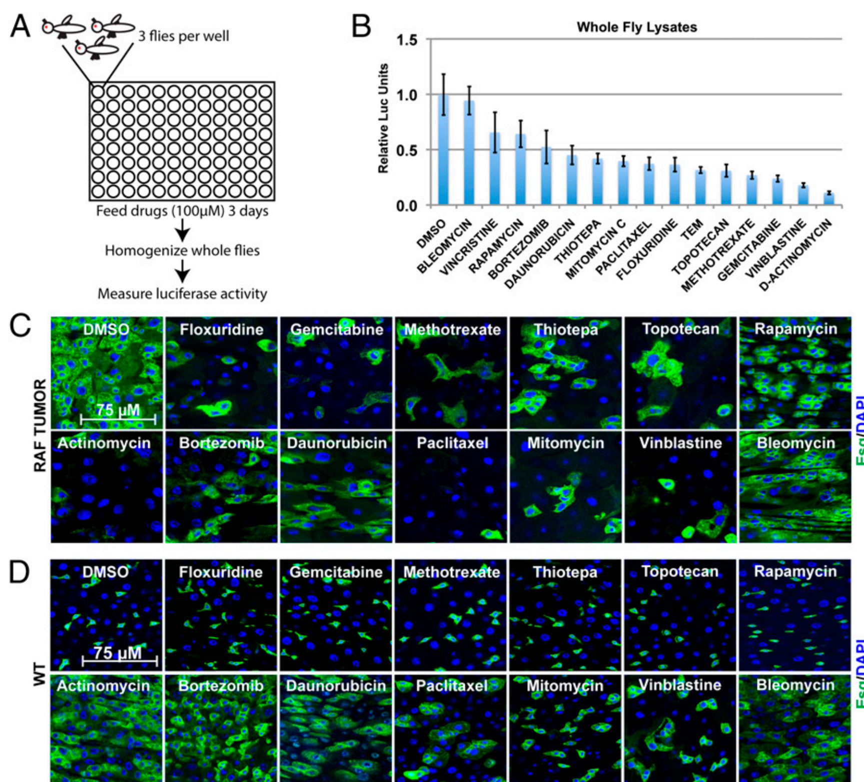

Fig. 2. Screen of 88 FDA-approved oncology drugs identifies two classes of drugs that inhibit *Drosophila* RAF^{gof} tumors. (*A*) Schematic of drug screen. (*B*) Replicate whole-animal luciferase assays from flies fed either DMSO controls or drugs identified as hits from the screen. Bleomycin did not score as a hit and is included as a negative control. Each bar represents the average of 12 biological replicates; error bar = 1 SD; $P < 0.001$ by rank-sum analysis. (*C*) Confocal images of posterior midguts dissected from RAF^{gof} flies treated with the compounds that scored as hits, with bleomycin included as a negative control. (*D*) Confocal images of posterior midguts dissected from WT flies fed with drugs that scored as hits from the drug screen. (*Upper*) Drugs that have no effect and are termed class I drugs. (*Lower*) Drugs that induce an increase in esg+ cells (green) and are termed class II drugs.

Conversely, we did not see a statistically significant difference in the number of PH3+ cells/gut between the cohorts of animals treated with DMSO and animals treated with drugs that did not increase ISCs (ranging from 6 to 10 flies/cohort) (Fig. 3*D*).

Our results thus distinguish two classes of chemotherapy drugs: class I drugs inhibit the growth of RAF^{gof} ISC tumors but do not affect WT ISCs, whereas class II drugs not only inhibit growth of RAF^{gof} ISC tumors but also paradoxically increase growth of their wild-type counterparts (Table 1). Because class II drugs inhibited growth of ISC-induced tumors, we did not expect them to also induce the hyperproliferation of ISCs. Indeed, the finding that drugs that can kill rapidly dividing stem cells can paradoxically also induce them to hyperproliferate was, to our knowledge, unprecedented. However, as noted in the discussion, similar results were recently reported in mammals, thus highlighting the generality of our findings (41).

Class II Side Effect Is Mediated by the Stem Cell Microenvironment. In previous studies, ISCs had been shown to proliferate in response to damage to their EC daughter cells, the major constituency of the ISC microenvironment. We thus explored the possibility that hyperproliferation was a "side effect" of chemotherapy effects on the microenvironment.

We envisioned that the class II side effect might be mediated by the JAK-STAT–signaling pathway because this pathway has been shown to mediate ISC proliferation in response to a variety of agents that can damage the EC daughter cells, including genetically induced apoptosis and stress, bacterial infection, and treatment with the DNA-damaging drug bleomycin (40, 42–46). Interestingly, bleomycin had failed to inhibit the growth of RAF^{gof} ISC tumors in our screen (Fig. 2 *B* and *C*). This result thus shows that induction of the JAK-STAT pathway, although sufficient to induce ISC proliferation, is not sufficient to kill RAF^{gof} ISC tumors. Thus, the possibility that the JAK-STAT pathway might underlie the ability of class II drugs to induce ISC proliferation was an appealing prospect because it would indicate that class II drugs elicit not only a side effect in the ECs, but also a side effect that is mechanistically separable from their ability to kill the tumor.

In response to bacterial infection, genetically induced stress, and cell death, ECs have been shown to express Unpaired (Upds), IL-6–like cytokines that activate the JAK-STAT pathway (42). To investigate whether the same mechanism is triggered by treatment with class II chemotherapy drugs, we used an Upd-3 Gal4 enhancer trap (47) to track expression of Upd-3. We found that Upd-3 expression correlated precisely with the effects of class I and class II chemotherapy drugs on WT proliferation: none of the class I drugs induced Upd-3 expression whereas each of the class II drugs did induce EC expression of Upd-3 (Fig. 4*A*).

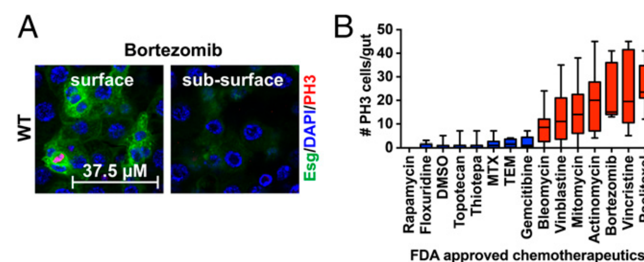

Fig. 3. Class II drugs increase stem cell proliferation. (*A*) PH3 staining (red) after treatment with the class II drug bortezomib. PH3 is evident in the nuclei of esg+ cells (green), visible in the surface view, and is absent from the nuclei of polyploid EC cells, visualized 1 μM below subsurface. (*B*) Box plot showing the number of PH3+ cells/gut: the class I drugs are labeled in blue, the class II in red. Note: vinblastine, vincristine, and paclitaxel arrest the cell cycle in M-phase and are therefore expected to increase PH3+ cells regardless of their effect on proliferation.

Table 1. Hits in screen of 6,100 compounds

Class I compounds	Class II compounds	New class I
Gemcitabine	D-actinomycin	Halcinonide
Methotrexate	Bortezomib	Harmalol hydrochloride
Thiotepa	Paclitaxel	Senecíphylline
Topotecan	Vincristine	Heliotrine
Rapamycin	Vinblastine	Chinese medicinal herbs (3)
	Mitomycin	Fungal extracts (3)
	Daunorubicin	

Similarly, we found that bleomycin, which was previously shown to induce Stat activation in the ISCs, stimulated Upd-3 expression in the ECs (Fig. 4*A*). In all cases, Upd-3 induction was specific to the EC cells, evident by focusing on either the surface of the intestine where the diploid ISC nuclei are in focus or by focusing 1 μM down, at the "subsurface" layer where the EC nuclei are in focus (Fig. S3).

In addition to observing the expression of Upd-3 in the ECs, we found that activation of the JAK-STAT–signaling cascade within ISCs was required for their proliferation. For example, when we reduced JAK-STAT signaling in ISCs, either by RNAi against the Upd-3 receptor, *domeless*, or by overexpression of Socs36E, a repressor that acts downstream of *domeless*, we found that the hyperproliferation response was reduced when treated with one of the strongest class II drugs, bortezomib (Fig. 4*B*). These results indicate that the JAK-STAT pathway is required specifically in the ISC hyperproliferating cells.

Collectively, these results demonstrate that class II drugs stimulate expression of Upd-3 in the EC daughter cells, culminating in JAK-STAT–mediated proliferation in WT ISC cells. Our finding that bleomycin induces ISC proliferation by the same mechanism and yet fails to kill RAF^{gof} ISC tumors indicates that the neither the induction of Upd-3 from the ECs nor the stimulation of JAK-STAT signaling in the ISCs is sufficient to kill the tumor. These results suggest that the side effect of class II drugs on the ISC microenvironment is mechanistically separable from their ability to kill RAF^{gof} ISC tumors.

Separation of Class II Tumor Inhibition from Tumor Initiation. Our finding that chemotherapy drugs that block tumor growth can also induce growth of WT stem cells was not only an unforeseen side effect, but also possibly a deleterious side effect with the potential to fuel tumor recurrence by multiple mechanisms. For example, in humans, the JAK-STAT pathway mediates the inflammation response, which correlates strongly with the onset of cancer (48). In addition, the induction of stem cell hyperproliferation could conceivably drive CSCs to regenerate tumors or even drive the formation of de novo stem-cell–initiated tumors.

Given that we identified the JAK-STAT pathway as driving WT stem cell hyperproliferation, one mechanism to suppress the potential for stem-cell–mediated tumor recurrence would be to couple traditional chemotherapy with anti-inflammatory drugs. However, a complementary approach and one that may be more broadly applicable would be to circumvent the side effect altogether. Along these lines, we tested class II drugs over a range of concentrations to identify a therapeutic window in which specific doses could inhibit tumors without also stimulating stem cell proliferation. In no instance were we able to separate the tumor inhibitory effects from the stem cell proliferation side effect (Fig. S4). These results indicate that the therapeutic windows of class II drugs may not be easily separable from their side effect on WT ISCs, suggesting that the best way to circumvent the side effect is to find drugs that avoid the side effect altogether.

In our screen of FDA-approved chemotherapy drugs, we identified seven drugs that can block RAF^{gof} ICS tumors without

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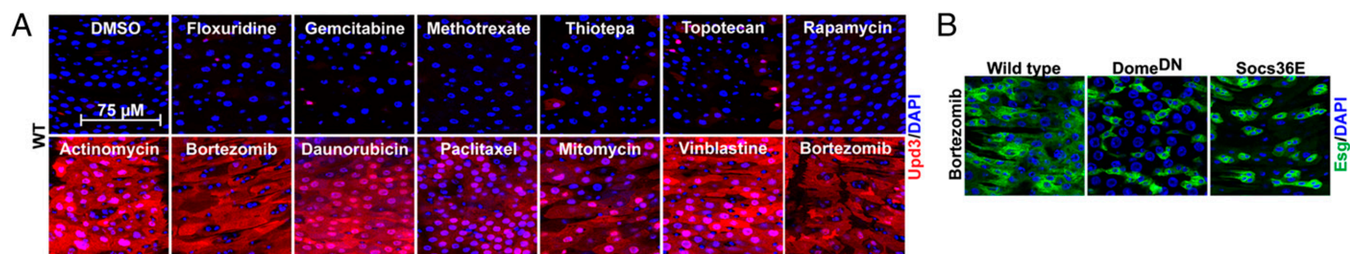

Fig. 4. JAK-STAT mediates the side effect of class II drugs. (*A*) Confocal images of posterior midguts dissected from WT flies fed with class I and class II drugs. Expression of Upd-3 is detected by Upd-3 Gal4 driving UAS-GFP (false-colored red to distinguish from GFP-labeled esg+ cells in other panels). Upd-3 is induced by class II drugs, not by class I drugs. (*B*) Reduction of JAK-STAT signaling in ISCs by expression of pathway repressors reduces the proliferation response to the strong class II drug bortezomib.

also stimulating WT ISC hyperproliferation. However, these drugs constitute only a narrow slice of the drug spectrum: six are inhibitors of DNA synthesis, and one, rapamycin, is an inhibitor of the TOR pathway. To expand the repertoire of anticancer drugs lacking the proliferation side effect, we screened a library of 6,100 small molecules for inhibitors of RAF^{gof} ISC tumors (Table S1). Based on the success of our original screen and the finding that some clinically relevant drugs, such as vincristine, inhibit tumors only when supplied at high doses (Fig. S4), we decided to screen the drugs at 100 μM. The screen identified 35 compounds that reduced luciferase activity by 50% or more in at least two of three biological replicates (Dataset S1). Of these, six were previously identified and confirmed as tumor suppressors in our FDA drug screen. We obtained material to retest 22 of the remaining hits and found that 10 validated as bona fide inhibitors of the RAF^{gof} ISC tumors, and, moreover, they did not induce the WT stem cell side effect (Table 1). These newly identified class I compounds include synthetic kinase inhibitors, known cytotoxics, and unclassified natural products extracted from fungi and Chinese medicinal herbs. These hits thus expand the repertoire of small molecules that in our model can block tumors without inducing WT stem cell hyperproliferation.

Discussion

Here we have established the use of *Drosophila* as an organism for large-scale drug screening of stem cell tumors. *Drosophila* has not yet achieved the status of a conventional organism for drug screening, but it is emerging as one based on a growing list of successful screens (49, 50). Our systematic study of FDA-approved chemotherapy drugs shows that *Drosophila* stem cell tumors are sensitive to a wide range of clinically relevant drugs, including 14 of the 88 tested chemotherapy drugs and an additional 10 uncharacterized compounds from our screen of 6,100 small molecules. Our finding that these drugs suppress growth of rapidly dividing RAF^{gof} ISC tumors but not of their wild-type counterparts is consistent with the antiproliferative activity that typifies classical chemotherapy drugs: they are potent against rapidly dividing tumor cells, which march through the cell cycle in an unregulated fashion, but not against normally dividing cells, which keep regulatory checkpoints intact.

A strength of using *Drosophila* for in vivo drug screening is that it enables the study of stem cells in the context of their natural microenvironment. The importance of this feature is highlighted by our discovery that a subset of classic chemotherapy drugs that inhibit growth of RAF^{gof} ISC tumors also elicits a side effect on the stem cell microenvironment, driving wild-type stem cells to hyperproliferate. Because hyperproliferation is a hallmark of tumorigenesis, this side effect is potentially detrimental and may possibly contribute to tumor recurrence in the wake of chemotherapy (Fig. 5). Indeed, another group reported a similar side effect in mammals in which anticancer drugs induced cells in the microenvironment to express TNF-α, which then fed back to the tumor cells to increase tumorigenesis (41). This result in mammals supports the generality of our findings, showing that the impact of a chemotherapy drug on the stem cell microenvironment is just as important as its impact on the stem cell itself.

Here we have identified a negative consequence of drug effects on the microenvironment; however, we anticipate that positive consequences will be identified as well, as more drugs are studied using whole-animal models.

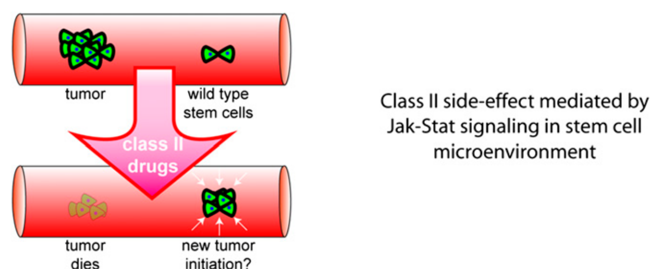

Fig. 5. Model of tumor recurrence. The finding that class II drugs have opposite effects on RAF^{gof} tumors and WT ISCs suggests an additional mechanism for tumor recurrence that includes active signaling from the microenvironment (white arrows) induced by class II drugs.

Materials and Methods

***Drosophila* Stocks.** *Drosophila* stocks were raised on standard cornmeal media at 18–25 °C. Fly genotypes are detailed in *SI Materials and Methods*.

Tumor Transplantation. Transplants were conducted with an Eppendorf FemtoJet Injection System as detailed in *SI Materials and Methods*.

Low-Melt Fly Food. We developed a fly food formula with optimal properties for mixing drugs in low volumes, which we call low-melt fly food. It contains low-melt agarose and standard agarose in place of agar. Low-melt fly food was developed with distilled water containing 2% (wt/vol) autoclaved yeast, 7% (vol/vol) corn syrup, and 1.5% (wt/vol) agarose (composed of 1 part standard agarose to 11 parts low-melt agarose). The food was stored at 4 °C, boiled, and mixed as a liquid with drugs at 37 °C. The resulting food-plus-drug mixtures solidified at 30 °C into soft fly-edible gels. In initial experiments, low-melt fly food was labeled with human-grade red food coloring (McCormics) and mixed with a wide spectrum of known bioactives. Ingestion of the food was visible in the abdomen of flies within 1 h of feeding, indicating that low-melt fly food and likely most drugs are palatable to flies.

Drug Libraries. Two libraries were screened: (*i*): 88 FDA-approved chemotherapy drugs from the Drug Therapeutics Program of the NCI and (*ii*) 6,100 compounds from the Harvard Institute for Chemistry and Cell Biology. Drugs were dissolved in 100% DMSO at a concentration of 10 mM, stored at −20 °C, and screened at a concentration of 100 μM by diluting 1:100 in low-melt fly food.

Drug Screening. The screen was conducted in 96-well plates as detailed in *SI Materials and Methods*.

Fly Homogenates and Luciferase Assay. Flies were homogenized with a 96-well plate multihomogenizer (Burkard Scientific, BAMH-96 1911101). Luciferase assays were conducted as previously described (35) and are detailed in *SI Materials and Methods*.

Immunostaining. Tissues were prepared as previously described (28). Information on antibodies used can be found in *SI Materials and Methods*.

Dose–Response Curves. The IC_{50} values for each drug were calculated by fitting the data to the four-parameter logistic sigmoidal dose–response curve, using the Prism6 software package (GraphPad Software, Inc.). Drugs were tested from 100 to 0.1 μM. The IC_{50} dose was defined as the dose that reduced luciferase by 50% in flies with RAF^{gof} ISC tumors.

ACKNOWLEDGMENTS. We thank Chrysoula Pitsouli and Sara Cherry for invaluable advice; Moritz Matthey, Ben Housden, Laura Quilter, and Kendra Frederick for critical reading of the manuscript; Michael Schnall-Levin, Jennifer Nale, Claire Hu, and Jacob Mayfield for help with statistical analyses; Richard Binari, Djade Soumana, and Laura Holderbaum for technical support; Caroline Shamu and David Wrobel (Harvard Institute of Chemistry and Cell Biology) for assistance with screening; the National Cancer Institute Drug Therapeutics Program for generously providing the set of 88 "approved oncology" drugs; and the Bloomington *Drosophila* Stock Center and the Harvard Transgenic RNAi Project for fly stocks. M.M. received support from the Jane Coffin Childs Memorial Fund, the Charles King Trust, and a Harvard University Research Enabling grant. This work was supported by the National Institutes of Health and the Howard Hughes Medical Institute (HHMI). N.P. is an investigator of the HHMI.

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