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Natural diversity facilitates the discovery of conserved chemotherapeutic response mechanisms

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Organismal fitness depends on adaptation to complex niches where chemical compounds and pathogens are omnipresent. These stresses can lead to the fixation of alleles in both xenobiotic responses and proliferative signaling pathways that promote survival in these niches. However, both xenobiotic responses and proliferative pathways vary within and among species. For example, genetic differences can accumulate within populations because xenobiotic exposures are not constant and selection is variable. Additionally, neutral genetic variation can accumulate in conserved proliferative pathway genes because these systems are robust to genetic perturbations given their essential roles in normal cell-fate specification. For these reasons, sensitizing mutations or chemical perturbations can disrupt pathways and reveal cryptic variation. With this fundamental view of how organisms respond to cytotoxic compounds and cryptic variation in conserved signaling pathways, it is not surprising that human patients have highly variable responses to chemotherapeutic compounds. These different responses result in the low FDA-approval rates for chemotherapeutics and underscore the need for new approaches to understand these diseases and therapeutic interventions. Model organisms, especially the classic invertebrate systems of *Caenorhabditis elegans* and *Drosophila melanogaster*, can be used to combine studies of natural variation across populations with responses to both xenobiotic compounds and chemotherapeutics targeted to conserved proliferative signaling pathways.

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Xenobiotic and targeted chemotherapeutic drug responses vary across natural populations

In their natural habitats, metazoans are exposed to small molecules produced by bacteria, fungi, and plants as defense mechanisms to prevent predation. Modern medicinal chemistry has employed these cytotoxic small molecules to treat human diseases, so that approximately 70% of cancer chemotherapeutics (hereafter chemotherapeutics) developed from 1981 to 2010 were derived originally from natural products [1]. Oftentimes, these small molecules disrupt essential cellular processes and can act as strong selective pressures that reduce genetic diversity [2]. By contrast, the combinations of small molecules in ecological niches change over time and can maintain genetic diversity within a species through balancing selection [3]. In addition to xenobiotic compounds, targeted chemotherapeutics specifically perturb the signaling pathways mutated in human cancers and are often lauded as great successes. However, because these proliferative signaling pathways have evolved mechanisms to withstand the accumulation of genetic variation within populations, chemotherapeutics have variable efficacies across a wide-range of genetically distinct patients [4]. Therefore, for both xenobiotic and targeted chemotherapeutics, it is not surprising that responses to chemotherapeutics are highly variable among the human population [5].

This variability in patient responses to chemotherapeutics can be caused by differences in the drug mechanism of action, absorption, metabolism, and elimination. Additionally, these processes can be impacted by germline variation, rare somatic mutations in the target tumor, environmental factors, and interactions among these factors and others [5]. This complexity results in a narrow range of concentrations that cause maximal tumor clearance among patients (defined as the therapeutic index). Also, chemotherapeutics are the most toxic drugs that are prescribed and cause severe and variable side effects among patient populations, thereby limiting the therapeutic index. In order to tailor treatments to individuals, drug responses must be correlated with genetic variants in specific patients. These data provide markers to broaden the therapeutic index for specific patients. This identification of genetic determinants that contribute to variability in patient responses to chemotherapeutics largely depends on the sample size of the patient population, the allele frequency and effect size of the causative

variant(s), and the reliability of the responses being measured [6]. These factors are limited in clinical oncology because it is extremely difficult to acquire large cohorts of patients that undergo the same therapeutic regimen [7], the high levels of genetic heterogeneity present in tumor [8] and patient populations [9], and the confounding effects of environmental variability [10,11]. As a result, only 6.4% of anti-cancer compounds in phase I clinical trials become FDA-approved chemotherapeutics, which is the lowest of any drug class [12]. Even if these limitations were resolved and genetic markers were associated with variable chemotherapeutic responses, the underlying mechanisms that are affected by the causal genetic variants would remain unknown, limiting clinical applications to recommendations based solely on genetic information.

In this review, we will highlight recent developments using invertebrate model organisms to better understand mechanisms of chemotherapeutic responses and discuss approaches to determine the effects of natural genetic variation on these responses. We contend that quantitative analyses of chemotherapeutic responses across different genetic backgrounds will increase the likelihood that new anti-cancer compounds will receive FDA approval and will augment the efficacies of existing chemotherapeutics.

Chemotherapeutic drug responses are conserved in invertebrate models

The invertebrate model organisms, *C. elegans* and *D. melanogaster*, have long facilitated the discoveries of molecular mechanisms associated with therapeutic responses [13,14]. These systems enable the study of chemotherapeutic effects because xenobiotic-response pathways are highly conserved between invertebrates and humans [15•], including cytochrome P450s [16,17], UDP-glucuronosyltransferases [2], and ABC transporters [18]. Similarly, numerous additional examples of responses to cytotoxic chemotherapeutics conserved between *D. melanogaster* and humans are known [19]. Additionally, the utility of *C. elegans* and *D. melanogaster* can be extended to chemotherapeutics that target cell proliferation pathways often constitutively activated in human cancers [20]. Because most of these pathways were discovered and characterized in studies of *C. elegans* vulval development and *D. melanogaster* compound eye development [21], the relevance of tractable models to understand conserved signaling pathways is long-standing. Cellular overproliferation associated with activating mutations in Ras pathway components have been shown to be conserved among *C. elegans*, *D. melanogaster*, and humans [22,23]. For example, the severities of different activating mutations in the Ras pathway kinase, MEK1, and the suppressive effects of a MEK1 inhibitor have the same rank orders between invertebrates and vertebrates [24•]. Although this highlighted example and others are important for the understanding of cytotoxic and targeted chemotherapeutic responses, most studies have been performed only in a single genetic background without any consideration of natural genetic variation.

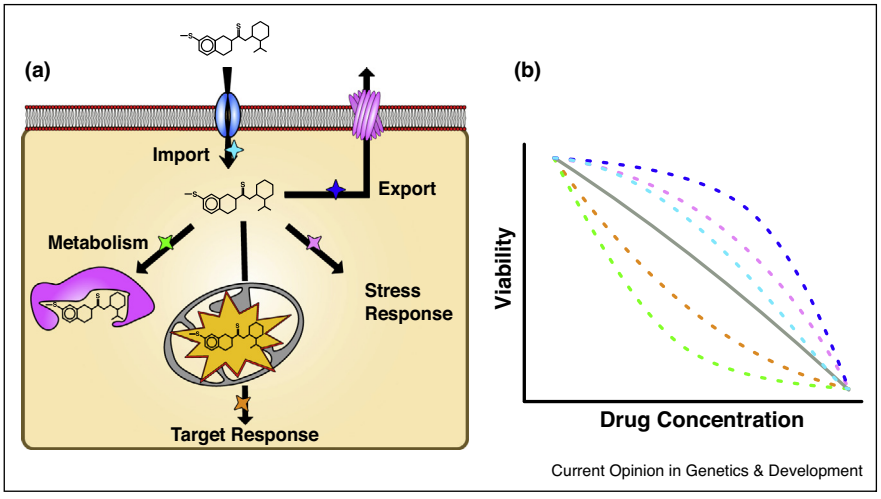
The effect of genetic background on chemotherapeutic drug responses

Individuals across populations harbor seemingly neutral genetic variation that causes phenotypic differences in the presence of chemical perturbations (Figure 1). This cryptic variation can cause large and divergent responses to chemotherapeutic regimens across cancer patient populations. Pharmacogenetics, pharmacogenomics, and genome-wide association studies of patient responses to chemotherapeutics focus on the identification and characterization of this genetic variation, but few broadly applicable results have been obtained [25]. Therefore, new approaches must be taken to understand how physiological responses to chemotherapeutics are affected by the genetic makeup of an individual without the difficulties associated with clinical oncology studies.

The *D. melanogaster* and *C. elegans* communities have developed numerous strain resources with divergent genetic backgrounds, including wild isolates with whole-genome sequence data [26–28] and recombinant inbred lines (RILs) generated by crossing distinct genetic backgrounds [29••,30,31]. Across both species, drug responses generally affect fitness, including offspring production, growth rate, and viability. High-throughput assays have been developed to quantify these traits across a large number of individuals in tightly controlled environmental conditions. When applied to studying the effects of chemotherapeutics on diverse genetic backgrounds, these powerful assays enable the identification of genomic regions (quantitative trait loci or QTL) that vary across the population and are predictive of drug response [19,29••,32] because environmental conditions are strictly controlled, drug responses from large numbers of divergent individuals can be measured, and high levels of replication can be obtained. Additionally, the abundance of genome-editing tools available in both species facilitate the functional validation and molecular characterization of genetic variants associated with chemotherapeutic responses [33,34]. Through these resources, assays, and genetic tools, investigators can rapidly go from a difference in drug response to the variant underlying that phenotypic difference.

In *D. melanogaster*, one collection of genetically divergent wild strains [27,28] and another collection of recombinant inbred strains [31] have been exposed to a variety of abiotic stresses and chemical perturbations. It was found that most responses to chemotherapeutic compounds are highly heritable [35], suggesting that variants controlling drug response differences exist in these populations. However, few examples of drug response QTL have been connected to a causal genetic variant (or QTL in

Figure 1



Natural variation alters cellular responses to a xenobiotic. **(a)** The cellular response to a xenobiotic that affects mitochondrial function and organismal viability. Arrows represent steps in the xenobiotic response and colored stars next to arrows represent possible points where genetic variation can alter the response. **(b)** Organism viability as a function of xenobiotic drug concentration for multiple genetic backgrounds, represented by colored dashed lines (average of backgrounds in gray), is shown. The potential reasons for altered xenobiotic responses are shown as different colors, increased viability results from increased xenobiotic export (dark blue), increased viability results from an increased cellular stress response (pink), increased viability results from decreased xenobiotic import (cyan), decreased viability results from increased target affinity (orange), decreased viability results from reduced metabolism (green). Importantly, the effect of the variant can be altered by the effects of other variants in the genetic background.

general [36]). One notable exception came from studies using the *Drosophila* Synthetic Population Resource [37], where variable responses to methotrexate were mapped to three QTL that each contain candidate genes conserved with humans and previously implicated in methotrexate toxicity. Additionally, responses to tunicamycin were mapped using the *Drosophila* Genetic Reference Panel [28] to a large number of loci but none of these loci were shown to play a direct role in the variable drug response [38]. In a global approach, a large-scale expression study of 80 inbred *D. melanogaster* strains from the *Drosophila* Genetic Reference Panel found 2000 genes with variable expression that can be explained by genetic differences in the panel, referred to expression quantitative trait loci or eQTL [39•]. Interestingly, significant differences in mRNA expression of approximately 20 glutathione S-transferases (GSTs) and cytochrome P450 genes, which have roles in xenobiotic responses, were observed among these strains, suggesting that natural variability to metabolize xenobiotics likely exists among these strains [39•]. As another example, European populations of *D. melanogaster* harbor a deletion in the 3′ UTR of the metallothionein A (*MtnA*) gene that results in a four-fold increase in *MtnA* expression [40]. This increased expression of *MtnA* results in decreased resistance to oxidative stress, which is a defining characteristic of cancer cells [41], as compared to the ancestral population. Expression levels of the human homologs of *MtnA* have been shown to have variable expression levels across different cancer types, and increased expression of metallothionein genes have been associated with resistance to the ROS-inducing chemotherapeutics cisplatin and bleomycin [42]. Though many studies indicate that the *D. melanogaster* species has heritable responses to chemotherapeutics, further investigations into the specific genetic causes of this variability are required to inform conserved drug response mechanisms.

Recently, the molecular mechanism associated with natural differences in *C. elegans* responses to topoisomerase II poisons was identified [43••]. This study leveraged a high-throughput assay to quantify the drug responses in a population of wild strains and recombinant inbred lines. A large-effect QTL was identified, and a causal variant in the *C. elegans* homolog of a topoisomerase II gene was validated using CRISPR/Cas9 genome editing. Furthermore, genome editing of the conserved variant site in human cells recapitulated the results from *C. elegans*, providing a functionally validated model of differential toxicity associated with topoisomerase II poison treatment in cancer patients. This combination of natural variation, high-throughput assays, and genome-editing technologies available only in model organisms enables similar approaches to understand responses to other cytotoxic compounds.

The effect of genetic background on the signaling pathways targeted by chemotherapeutics

Much like in xenobiotic responses, organisms have evolved mechanisms to ensure that phenotypes remain

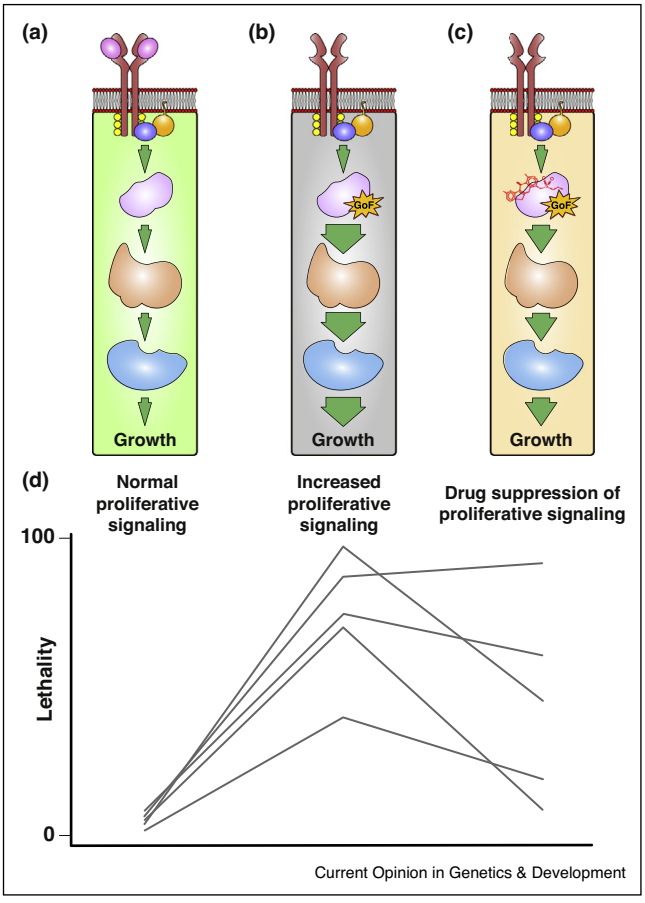
constant in the presence of genetic and environmental perturbations [44]. This robustness is exemplified by similar levels of Ras/MAPK pathway ligand expression found between two genetically divergent species of nematodes, *C. elegans* and *Oscheius tipulae* [45•]. Despite the inherent buffering that these proliferative pathways maintain to reduce the effects of diverse genetic perturbations, cancer-causing mutations disrupt these pathways beyond their suppressive capacities. These disruptive mutations sensitize proliferative pathways to the effects of previously phenotypically silent genetic differences among individuals. To improve the effectiveness of chemotherapeutic regimens, the interactions between genetic background and mutations that cause cancer must be characterized. Currently, it is extremely difficult to identify background variants that modulate the effects of sensitizing mutations and chemotherapeutic responses across diverse human populations because too few patients with variable responses are identified and genotyped. However, by introducing mutations that sensitize proliferative pathways in diverse model organism genetic backgrounds, it is possible to reveal genetic variants that influence both cancer progression and drug responses.

A recent study highlighted how *C. elegans* can be used to identify genetic variants that modify the ectopic proliferation phenotype of an oncogenic Ras mutation [46••]. The authors observed that genetically diverged *C. elegans* strains harboring the same gain-of-function (GoF) allele of the *C. elegans* Ras homolog exhibited variable severity of a vulva hyperproliferation defect. To identify the genetic background differences that could influence Ras pathway activity, the authors quantified vulva hyperproliferation in a collection of recombinant strains constructed from two genetically diverged strains with the same GoF allele. This approach led to the identification of three QTL that modify the expressivity of the vulva hyperproliferation defect. Next, the authors functionally validated the *amx-2* gene, which underlies one of the identified QTL, as an inhibitor of Ras/MAPK signaling in *C. elegans* [46••]. Interestingly, the closest human homolog of AMX-2 has been shown to be downregulated in a wide-range of cancer types and is widely used as an early indicator of cancer [47]. This and similar experiments in *C. elegans* highlight the power of testing the effect of oncogenic mutations in multiple genetic backgrounds [48,49]. However, further insights can be gained by incorporating targeted chemotherapeutic treatments along with cancer-causing mutations. For example, the oncogenic recombinant lines generated in this study can be used to identify the genetic modifiers of targeted Ras and other chemotherapeutic kinase inhibitors. Studying the effects of chemotherapeutics on diverse genetic backgrounds that contain cancer-causing mutations is only feasible in model organisms and is a powerful approach to elucidate the mechanisms associated with variable therapeutic responses among patients.

The transgenic expression of human oncogenic mutations has been used recently in *D. melanogaster* to identify optimal combinations of chemotherapeutics that suppress tumor-related phenotypes [50]. Human genome rearrangements commonly found in papillary thyroid carcinomas fuse the RET receptor tyrosine kinase gene, which promotes cell growth, proliferation, survival, and differentiation through the activation of downstream targets [51], with either *CCDC6* or *NCOA4*. Levinson and Cagan used the *D. melanogaster* GAL4/UAS transcriptional activation system to drive the expression of these fusion proteins [52••] and cause higher levels of activated RET, organism lethality, and cell migration, which is a phenotype associated with epithelial-to-mesenchymal transition [53]. The authors systematically tested each of the genes within the *Drosophila* kinome and identified 15 druggable kinases that suppressed the tumor-related phenotypes caused by fusion protein overexpression. Only two of the downstream kinases suppressed phenotypes caused by both of the RET fusions, which is surprising because the fusions share an identical RET kinase domain. Using these genetic interaction data, the authors identified chemical inhibitors of the downstream kinases that suppress the tumor-related phenotypes in the *Drosophila* model. This study highlights the utility of *Drosophila* cancer models for the characterization of signaling pathways that are disrupted by oncogenes and the optimization of therapeutic interventions to mitigate cancer promotion. Given the variability in patient responses to targeted chemotherapeutics, it would be interesting to see how consistent the effects of the fusion proteins and targeted chemotherapeutics are in the context of different genetic backgrounds.

The two studies described above demonstrate the power of model organisms to study the effects of oncogenic mutations that sensitize proliferative signaling pathways. The *C. elegans* approach taken by Schmid *et al.* identified genetic modifiers of an oncogenic Ras mutation but did not study the effects of targeted chemotherapeutics. The *Drosophila* approach taken by Levinson and Cagan addressed the effects of targeted chemotherapeutics but did not study the effects of genetic background on the response. The principles of studies can be combined to elucidate how genetic background modifies chemotherapeutic drug responses (Figure 2). However, given the large number of genetically distinct *C. elegans* [26] and *D. melanogaster* [28] strains, diversity of cancer-driving mutations in conserved signaling pathways, and panoply of targeted chemotherapeutic drugs, the possible combinations are seemingly endless. To combat this scaling issue, newly created high-throughput methods [19,29••,32] enable the quantification of tumor-related phenotypes across divergent genetic backgrounds, sensitizing pathway mutations, and drugs.

Figure 2



Natural variation modifies effects of sensitizing pathway mutations and chemotherapeutic responses. **(a)** A simplified cellular signaling pathway that results in proliferative growth upon ligand (pink) binding is shown. **(b)** The proliferative signaling pathway from (a) is shown with a gain-of-function (GoF) mutation that results in increased pathway activity and cellular proliferation that is independent of ligand binding. The size of the arrows that connect the steps in the pathway correspond to the amount of pathway activation. **(c)** The sensitized pathway from (b) is treated with a chemotherapeutic to suppress the effects of the GoF mutation. **(d)** The lethality phenotypes associated with each pathway (a–c) are shown for five diverged *Drosophila* genetic backgrounds represented by different gray lines. All five genetic background exhibit little-to-no lethality with normal pathway activity. However, low levels of lethality might occur with normal pathway activity because laboratory growth conditions may not be ideal for diverged genetic backgrounds. Introduction of a sensitizing GoF mutation (gray) results in increased signaling activity, uncontrolled cellular growth, and animal lethality. However, the mutation affects each genetic background differently. This variability can be caused by various modifying variants present in the five strains that have no visible effect with normal signaling activity. Similarly, chemotherapeutic-induced suppression of pathway signaling activity and organism lethality associated with the GoF allele varies among genetic backgrounds. The variable efficacy of the chemotherapeutic to suppress lethality may result from a number of reasons, some of which are discussed in Figure 1.

Where do we go from here?

The novel invertebrate systems discussed here have taken crucial steps toward unraveling the complexity of cancer and responses to associated chemotherapeutic interventions [35,43••,46••,52••,54,55]. However, we contend that the benefit of these invertebrate systems has not been fully realized because drug response measurements and natural variation are rarely combined. Additional large-scale experiments that quantify xenobiotic responses across diverse genetic backgrounds, which have the power to identify variants with no observable fitness consequence in normal conditions [35,38,43••,56], are required to expand our understanding of how conserved pathways accumulate cryptic variation revealed by drug exposure. Similarly, large-scale experiments that look at the effect of genetic background on sensitizing oncogenic mutations and responses to targeted chemotherapeutics, facilitate the simultaneous identification of genetic modifiers of the sensitizing mutation and novel targeted chemotherapeutic combinations. Of course, findings across diverse invertebrate genetic backgrounds might not assure success when translated to human patients. However, we posit that the likelihood of translation will be greater if validated in multiple genetic backgrounds and interesting new discoveries about how genetic diversity influences xenobiotic responses and conserved signaling pathways will undoubtedly be discovered.

Conflict of interest statement

Nothing declared.

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