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Figs. S1 to S7

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REPORTS

A Single P450 Allele Associated with Insecticide Resistance in *Drosophila*

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Insecticide resistance is one of the most widespread genetic changes caused by human activity, but we still understand little about the origins and spread of resistant alleles in global populations of insects. Here, via microarray analysis of all P450s in *Drosophila melanogaster*, we show that *DDT-R*, a gene conferring resistance to DDT, is associated with overtranscription of a single cytochrome P450 gene, *Cyp6g1*. Transgenic analysis of *Cyp6g1* shows that overtranscription of this gene alone is both necessary and sufficient for resistance. Resistance and up-regulation in *Drosophila* populations are associated with a single *Cyp6g1* allele that has spread globally. This allele is characterized by the insertion of an *Accord* transposable element into the 5′ end of the *Cyp6g1* gene.

Insecticide resistance represents an important example of natural selection. Resistance can be mediated either by changes in the sensitivity of insecticide targets in the nervous system or by metabolism of insecticides before they reach these targets (*1*). Insecticide resistance associated with target site insensitivity is well documented within the *para*-encoded voltage-gated sodium channel, the *Rdl*-encoded ligand-gated chloride channel, and the *Ace*-encoded acetylcholinesterase (*1*). However, the up-regulation of metabolic enzymes associated with resistance, such as the cytochrome P450s and glutathione *S*-transferases, remains less well understood (*2*–*7*). We are using *Drosophila melanogaster* as a model insect in which to dissect the genetic basis of metabolic insecticide resistance (*8*, *9*), particularly at the *DDT-R* locus. This locus not only represents insect resistance to DDT (*10*), a compound largely withdrawn but still used in the control of disease vectors (*11*), but also confers cross-resistance to a wide range of other existing and novel insecticides (*12*–*15*). *DDT-R* is a dominant gene that maps to the right arm of chromosome II at 64.5 cM (*16*, *17*). Recently, we have shown that *DDT-R* is associated with overtranscription of the P450 gene *Cyp6g1* in three *D. melanogaster* strains (*18*). In this study, we were interested in answering three questions. First, is *Cyp6g1* overtranscribed in all P450-mediated DDT-resistant *D. melanogaster* strains? Second, if so, is resistance globally associated with a single resistance allele? Third, is overtranscription of *Cyp6g1* alone both necessary and sufficient for resistance?

The genes for the cytochrome P450s are a large family involved in a wide variety of metabolic functions. In insects, these enzymes play roles in key processes ranging from host plant utilization to xenobiotic resistance (*3*). Within the complete genome sequence of *D. melanogaster*, some 90 individual P450 genes have been identified (*19*). To determine the breadth of the correlation between *Cyp6g1* overtranscription and DDT resistance in *D. melanogaster*, we challenged a microarray carrying polymerase chain reaction (PCR) products from all identified P450 open reading frames in the genome. Array analysis of Hikone-R (*20*), a resistant strain established from field collections in the early 1960s (*16*, *21*), shows that only *Cyp6g1* is overtranscribed relative to Canton-S, a susceptible reference strain (Fig. 1A). Similar array analysis of a second DDT-resistant strain, WC2, which was recently collected in the field (U.S.A.), revealed the same result, with only *Cyp6g1* being overtranscribed relative to P450 gene expression in the susceptible standard (Fig. 1B). To measure the level of overtranscription associated with resistance, we performed quantitative reverse transcriptase (RT)–PCR on mRNA from a range of resistant and susceptible strains (*22*), relative to the standard *RP49* (*23*). This analysis confirms the relative overtranscription of *Cyp6g1* in a range of strains and shows 10 to 100 times as much mRNA in resistant strains as in a range of susceptible strains

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(Fig. 1C). To examine a range of insecticides to which *DDT-R* confers cross-resistance, we mapped resistance to a chlorinated hydrocarbon (DDT), two neonicotinoid nicotinic acetylcholine receptor agonists (imidacloprid and nitenpyram), and a novel insect growth regulator (lufenuron). We measured recombination rates against visible genetic markers, *P*-element insertions of known location, and restriction fragment length polymorphisms (RFLPs). Resistance to all three different insecticides maps within the same genetic region encompassing the *Cyp6g1* locus (Fig. 2A), which suggests that a single P450 may be capable of metabolizing a wide range of insecticide structures.

To examine the population genetics of *DDT-R* in global populations of *D. melanogaster*, we completely sequenced two different resistant alleles, Hikone-R and WC2. These alleles have an identical nucleotide sequence, and both carry an insertion in the 5′ end of *Cyp6g1* that shows homology to the terminal direct repeat of an *Accord* transposable element (Fig. 2B). To further examine the similarity of *DDT-R* resistance alleles, we screened with DDT field-collected strains available from *Drosophila* stock centers. We did not find DDT resistance in strains established in the laboratory in the 1930s (Oregon-R-C, Swedish-C, and Canton-S) and collected before DDT usage, but did find resistance in 28 of 75 strains established from different locations across the globe in the 1960s and later (table S1). Although this high frequency (37%) of resistant strains does not strictly represent the past or current frequency of resistance in global populations, as resistance alleles may have been lost by chance in subculturing, it does support two hypotheses. First, resistance to DDT was widespread, as expected, and second, resistance can persist in laboratory strains in the absence of pesticide selection, which suggests that little or no fitness cost is associated with this mechanism. To examine the apparent correlation between the presence of the transposon and resistance, we surveyed resistant and susceptible strains for the presence or absence of the *Accord* element. We also sequenced the first intron of the *Cyp6g1* gene in the same resistant and susceptible strains to determine the relatedness of the *DDT-R* alleles. A diagnostic, using PCR to detect the presence of the transposon and based on the length of the product generated, showed the insertion to be present in all 20 resistant alleles examined (Fig. 3A). The similarity of all the resistant alleles is also supported by a phylogeny of DNA sequences (fig. S1) derived from the first intron of the *Cyp6g1* gene. This shows that the susceptible alleles belong to several different clades of diverse geographic origin. In contrast, the resistant alleles all belong to a single well-supported clade (Fig. 3B), also containing a single susceptible genotype. This genotype, which does not carry the *Accord* transposable element,

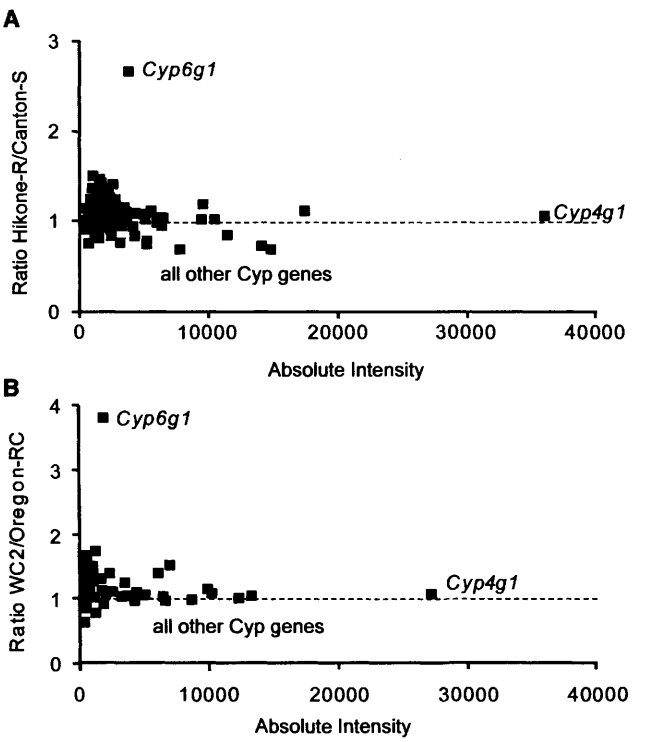

Fig. 1. A single P450 gene, *Cyp6g1*, is overtranscribed in insecticide-resistant *Drosophila*. (**A** and **B**) Microarray analysis of *D. melanogaster* cytochrome P450 genes in two different DDT-resistant strains, Hikone-R (A) and WC2 (B). The relative hybridization ratio of resistant (Hikone-R or WC2) versus susceptible (Canton-S) mRNA to an array containing PCR-derived products of P450 gene open reading frames is shown against absolute intensity (relative transcript abundance) for each gene. Note that *Cyp6g1* is overtranscribed in both resistant strains. (**C**) Measurement of *Cyp6g1* transcription, relative to the standard *RP49*.

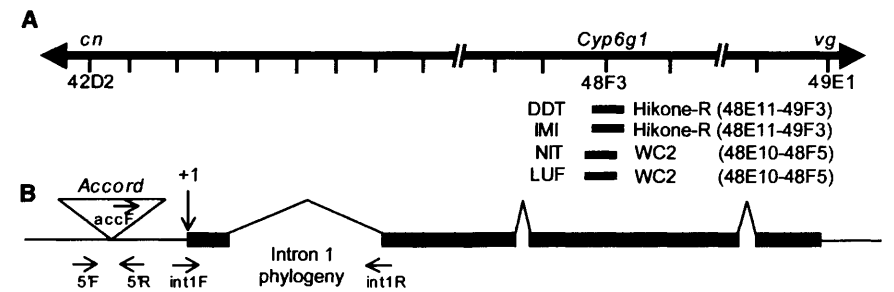

Fig. 2. Resistance to DDT and several novel insecticides maps to the region encompassing the *Cyp6g1* locus. (**A**) Recombinational mapping against both visible mutants (*cn*, cinnabar eyes, and *vg, vestigial* wings), *P*-element insertions of known genomic location, and RFLPs show that resistance to DDT and the novel insecticides imidacloprid (IMI), nitenpyram (NIT), and lufenuron (LUF) maps to a region encompassing *Cyp6g1*. Map estimates for each compound are given (solid bars represent confidence intervals) alongside the predicted cytological regions encompassed (in parentheses). (**B**) Map of the genomic structure of the *Cyp6g1* locus showing the intron-exon organization of the gene and the location of the *Accord* element insertion in the resistant (Hikone-R) allele (GenBank AY131284). Note the location of the PCR primers used in the diagnostic for the presence of the *Accord* element (*22*).

is therefore the putative susceptible progenitor into which the transposable element inserted, thus forming the resistant allele. This allele has spread globally, under the combined influences of insecticide selection and migration (*24*). The observation that the nucleotide sequence around the first intron in *Cyp6g1* (291 bp away from the site of the insertion) is identical in all the resistant alleles supports the concept of this global spread and suggests strong linkage disequilibrium or "hitchhiking" of nucleotide variation with the spread of DDT resistance.

Finally, to verify that overtranscription of *Cyp6g1* alone is responsible for DDT resistance, we produced resistance in transgenic flies carrying an inserted copy of *Cyp6g1* driven by the *GAL4/UAS* system (*25*). After germline transformation mediated by *P*-element transposition of constructs containing *UAS-Cyp6g1*, we were able to overtranscribe the inserted copy of the gene using heat shock–based GAL4 drivers. We again quantified the level of overtranscription from the *Cyp6g1* transgene via quantitative RT-PCR relative to *RP49* transcription (Fig. 4A). These results show that transgenic overtranscription leads to an overabundance of *Cyp6g1* transcript of about 100 times that in susceptible nontransgenic fly strains. Heat-shocked flies were also resistant to a discriminating dose of 10 μg of DDT per vial, whereas controls lacking the GAL4 drivers, and thus lacking overtranscription of the inserted copy of the gene, were susceptible (Fig. 4B). These transgenic experiments demonstrate that overtranscription of *Cyp6g1* alone is both necessary and sufficient for P450-mediated DDT resistance.

Although several studies have implicated the overexpression or alteration of individual P450s in insecticide resistance (*3*, *5*, *7*), our results raise several important new conclusions for the molecular basis and origins of metabolic resistance. First, given the number of P450 genes present in *D. melanogaster* (*19*) and the potential complexity of their interactions (*3*), it was not expected that a single allele of a single gene could be associated with such widespread insecticide resistance. Second, it was also not expected that resistance alleles mapping to the same region as *DDT-R* should show cross-resistance to such a wide range of compounds, including organochlorine, organophosphorus, carbamate, neonicotinoid, and insect growth regulator insecticides. Such broad cross-resistance would make a pest insect difficult to control with available insecticides and, although we have not formally demonstrated that this cross-resistance is strictly correlated with *Cyp6g1* overtranscription, this is now our working hypothesis. In this respect, we note that the insect CYP6 P450 family is phylogenetically related to the mammalian CYP3 family of which CYP3A4, expressed in the human liver, is also noted for its broad substrate specificity (*26*). Third, the possibility that insecticide resistance might involve transposable elements has been raised previously (*27*). However, a previous putative association of a transposable element with a P450 gene thought to be associated with resistance (*28*) was subsequently disproved (*29*). In the current study, although linkage between resistance and the *Accord* element is complete, the causal relation between the el-

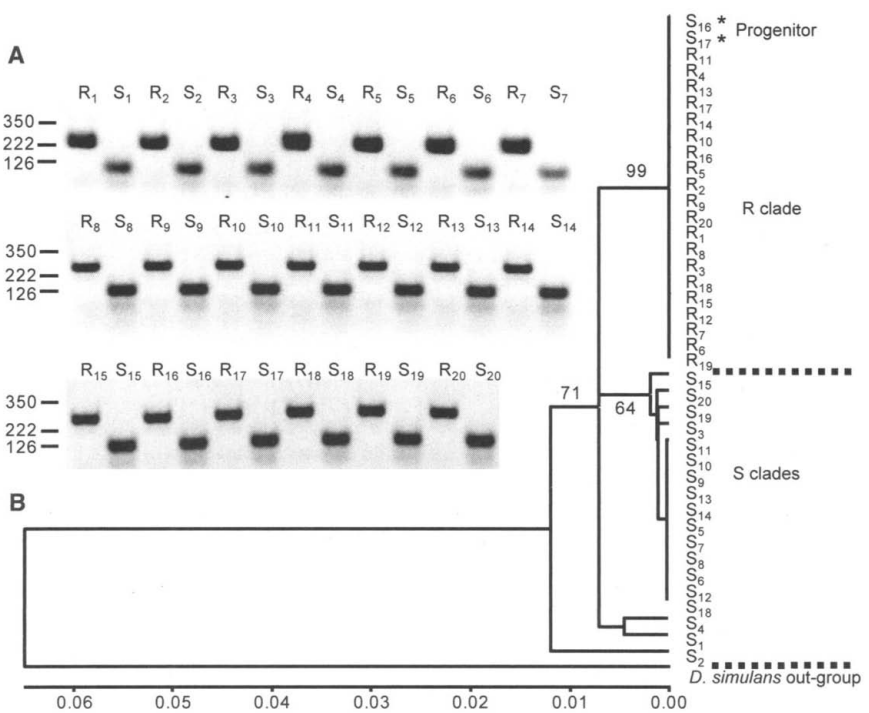

Fig. 3. All resistant *Cyp6g1* alleles carry the *Accord* insertion and show identical nucleotide sequence within the first intron, supporting a single global origin of *DDT-R*. (**A**) Survey of a range of susceptible ($S_{1\text{-}20}$) and resistant ($R_{1\text{-}20}$) fly strains (see table S1 for strain origins) with a PCR-based diagnostic to detect the presence or absence of the *Accord* element in the 5′ end of the *Cyp6g1* gene. Note the perfect correlation between the presence of the element (larger, *Accord* associated 243-bp PCR product) and resistance. The relative positions of PCR primers used in the diagnostic are described in Fig. 2B. (**B**) Phylogeny of a global collection of susceptible ($S_{1\text{-}20}$) and resistant ($R_{1\text{-}20}$) *DDT-R* alleles. Note that the susceptible alleles form seven groups or "clades" and that the eighth clade contains all the resistant alleles and a single susceptible allele found in two different susceptible strains.

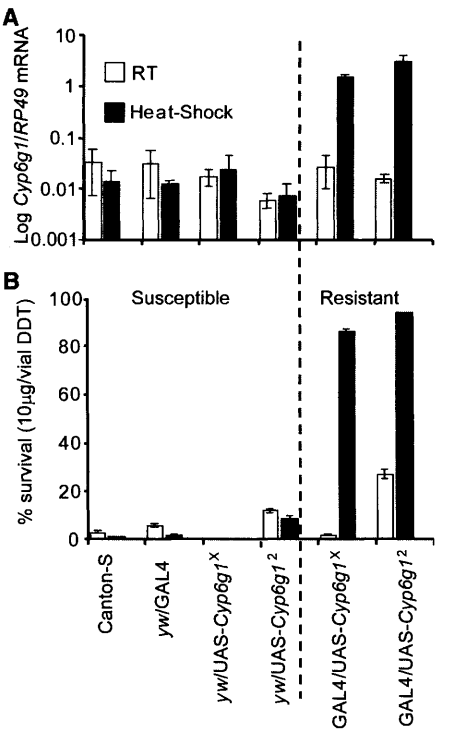

Fig. 4. Heat shock–mediated overtranscription of *Cyp6g1* is both necessary and sufficient for DDT resistance. (**A**) An inserted transgenic copy of *Cyp6g1*, under the control of *GAL4/UAS* with a heat-shock driver, can be overtranscribed after heat shock. Ten 20-min heat shock (37°C) treatments (with a 20-min 25°C recovery period between heat shocks) of two different transgenic fly lines (*GAL4/UAS-Cyp6g1*^{X} and *GAL4/UAS-Cyp6g1*^{2}, on the X and 2nd chromosome, respectively) up-regulated the *Cyp6g1* transcript about 100 times in both strains. (**B**) Heat shock–induced overtranscription of *Cyp6g1* allows the transgenic strains to survive a discriminating dose of 10 μg of DDT per vial. Adult flies were exposed to DDT applied to the inside of 20-ml glass scintillation vials as described previously (*18*).

ement and overtranscription remains to be proven. Fourth, and finally, earlier work on amplified esterase genes in mosquitoes suggested that a single global spread of one specific amplicon accounted for insecticide resistance in global populations of *Culex pipiens* mosquitoes (*30*). Further analysis of mosquito populations, however, showed that numerous different mutational events and their resulting amplicons make up the extant global population of resistance alleles in mosquitoes (*24*, *31*). Our description of an identical resistant allele in 20 DDT-resistant strains of *D. melanogaster* of diverse geographic origin represents the global spread of a single insecticide-resistance allele.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/297/5590/2253/DC1
Materials and Methods
Fig. S1
Table S1

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Little Evidence for Developmental Plasticity of Adult Hematopoietic Stem Cells

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To rigorously test the in vivo cell fate specificity of bone marrow (BM) hematopoietic stem cells (HSCs), we generated chimeric animals by transplantation of a single green fluorescent protein (GFP)–marked HSC into lethally irradiated nontransgenic recipients. Single HSCs robustly reconstituted peripheral blood leukocytes in these animals, but did not contribute appreciably to nonhematopoietic tissues, including brain, kidney, gut, liver, and muscle. Similarly, in GFP$^+$:GFP$^-$ parabiotic mice, we found substantial chimerism of hematopoietic but not nonhematopoietic cells. These data indicate that "transdifferentiation" of circulating HSCs and/or their progeny is an extremely rare event, if it occurs at all.

As many recent reports have suggested that BM HSCs may harbor unexpected developmental plasticity (*1–14*), we set out to test rigorously the cell fate potential of prospectively isolated, long-term reconstituting HSCs (*15–17*) using chimeric animals generated by transplantation of a single GFP$^+$ c-kit$^+$Thy1.1^{lo}Lin$^-$Sca-1$^+$ (KTLS) BM HSC (fig. S1) (*18*). GFP$^+$ HSCs were isolated by fluorescence-activated cell sorting (FACS) from BM of transgenic animals that constitutively express GFP, driven by the β-actin promoter, in all tissues (*19*). About 18% of recipients of single GFP$^+$ KTLS HSCs showed significant levels of long-term, multilineage (both lymphoid and myeloid) hematopoietic engraftment in the peripheral blood (Table 1). Although the hematopoietic contribution from single GFP$^+$ HSCs varied, in some recipients donor-derived contributions reached levels as high as ~70% (Table 1).

To further evaluate the cell fate potential of transplanted HSCs, we analyzed tissues from engrafted recipients 4 to 9 months after transplant for the presence of GFP$^+$ cells by standard and confocal fluorescence microscopy (*18*). Tissues from recipient animals exhibiting multilineage reconstitution of GFP$^+$ blood leukocytes were stained with tissue-specific antibodies and/or with the pan-hematopoietic marker, CD45 (*20–22*). These sections were then analyzed to identify potentially transdifferentiated GFP$^+$ cells, which satisfied one or more of the following criteria: (i) the GFP$^+$ cell stains with tissue-specific markers; (ii) the GFP$^+$ cell does not stain with a monoclonal antibody to CD45; and (iii) the cell exhibits distinctive morphology, indicative

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Table 1. Frequency and degree of reconstitution in single HSC–transplanted mice. The peripheral blood (PB) of single HSC–reconstituted mice was analyzed by flow cytometry 5 and 14 weeks after transplant for the presence of GFP$^+$ donor-derived leukocytes. PB cells were stained for markers of the lymphoid (L) lineage (CD3$^+$,B220$^+$) versus myeloid (M) lineage (Mac-1$^+$, Gr-1$^+$) or separately for B cells (B, B220$^+$), T cells (T, CD3$^+$), and myeloid cells (M, Mac-1$^+$, Gr-1$^+$).

Frequency of reconstitution (reconstituted mice/total)	
5 weeks	14 weeks
7/22 (32%) L + M	4/22 (18%) BTM
5/22 (23%) L only	1/22 (5%) BT
	2/22 (9%) B only
Average reconstitution (% GFP$^+$ PB leukocytes)	
17.6% (range: 0.12–77.6%)	20.2% (range: 0.03–71.6%)