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Mdr65 decreases toxicity of multiple insecticides in *Drosophila melanogaster*

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ABSTRACT

ABC transporters are ubiquitous membrane-bound proteins, present in both prokaryotes and eukaryotes. The major function of eukaryotic ABC transporters is to mediate the efflux of a variety of substrates (including xenobiotics) out of cells. ABC transporters have been widely investigated in humans, particularly for their involvement in multidrug resistance (MDR). Considerably less is known about their roles in transport and/or excretion in insects. ABC transporters are only known to function as exporters in insects. *Drosophila melanogaster* has 56 ABC transporter genes, including eight which are phylogenetically most similar to the human *Mdr* genes (ABCB1 clade). We investigated the role of ABC transporters in the ABCB1 clade in modulating the susceptibility to insecticides. We took advantage of the *GAL4/UAS* system in *D. melanogaster* to knockdown the expression levels of *Mdr65*, *Mdr50*, *Mdr49* and *ABCB6* using transgenic *UAS-RNAi* lines and conditional driver lines. The most notable effects were increased sensitivities to nine different insecticides by silencing of *Mdr65*. Furthermore, a null mutation of *Mdr65* decreased the malathion, malaoxon and fipronil LC_{50} values by a factor of 1.9, 2.1 and 3.9, respectively. Altogether, this data demonstrates the critical role of ABC transporters, particularly *Mdr65*, in altering the toxicity of specific, structurally diverse, insecticides in *D. melanogaster*.

1. Introduction

The ATP-binding cassette (ABC) transporters are one of the largest families of transmembrane proteins that have been characterized in all organisms from microbes to humans (Dassa and Bouige, 2001; Higgins, 1992). ABC proteins not only mediate the efflux of a large diversity of substrates, including amino acids, peptides, sugars, polysaccharides, lipids, inorganic ions, metals, waste compounds and xenobiotics (Dassa and Bouige, 2001; Dermauw and Van Leeuwen, 2014; Porretta et al., 2008; Sarkadi et al., 2006), but also participate in diverse biological processes, such as ribosome biogenesis and translation regulation (Andersen and Leevers, 2007; Paytubi et al., 2009). Due to their ability to transport drugs, some ABC transporters are involved in multidrug resistance (MDR) in cancer cells (Vasiliou et al., 2009), and have been well studied for their roles in the absorption, distribution and excretion of drugs *in vivo* and *in vitro* (Glavinas et al., 2008; Sarkadi et al., 2006; Zhang et al., 2003). A typical ABC transporter consists of two highly conserved nucleotide-binding domains (NBDs), a characteristic feature of this family, and two highly hydrophobic transmembrane domains (TMDs), which are diverse and mediate substrate translocation (Higgins et al., 1986). The NBDs bind and hydrolyze ATP, thus providing energy to transport substrates out of the cell (Hollenstein et al., 2007). There are two types of ABC transporter genes: full transporters (FTs) contain all four domains, while half transporters (HTs) possess only one NBD and one TMD and need to homodimerize or heterodimerize to form a functional unit (Higgins, 1992; Higgins and Linton, 2004). Based on sequence similarity, domain structure and organization, ABC proteins have been divided into eight subfamilies (ABCA to ABCH), with the *Mdr* genes (those commonly found to play a role in transport of xenobiotics, such as drugs) being in the ABCB subfamily (Dean et al., 2001; Dermauw and Van Leeuwen, 2014).

Insecticide poisoning can be divided into five general processes: penetration (through the exoskeleton or the gut epithelium), distribution (movement within the insect), interaction with the target site, metabolism and excretion (Welling, 1977). There have been tremendous advances in understanding how insecticides interact

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with their target sites and in determining the enzymes involved in metabolism. However, comparatively little progress has been made in understanding the processes involved in penetration, distribution and/or excretion. Given the role that ABC transporters play in the movement of xenobiotics, they have the potential to be key regulators of one or more of these processes.

Drosophila melanogaster has 56 ABC transporter genes, including eight (four full transporters and four half transporters (Dermauw and Van Leeuwen, 2014)) that are phylogenetically most similar to the human *Mdr* genes (Dean et al., 2001). *Mdr65* was found to have an important role in preventing drugs and dyes from penetrating the blood-brain barrier of *D. melanogaster* (Mayer et al., 2009). Few studies have looked at the potential of these ABC transporters to act as a barrier to, or alter the distribution of, insecticides. A previous study found that RNAi suppression of *Mdr50* and *Mdr65* resulted in a small (but significant) decrease in the LT_{50} of DDT, while RNAi of *Mdr49* had no effect. (Gellatly et al., 2015).

As a first step towards understanding the role of ABC transporters in insecticide distribution and/or excretion, we focused on the orthologs of the human *Mdr* genes in the ABCB clade (Dean et al., 2001) for which RNAi lines were available. This included three full transporters (*Mdr49*, *Mdr50* and *Mdr65*) and two half transporters (*ABCB6* and *ABCB7*). We first used *in vivo* RNAi and the *Gal4/UAS* system to knock-down the expression of these transporters and then evaluated the resulting impact on sensitivity to insecticides. We determined the mortality of these knocked down flies to nine diverse insecticides and found that *Mdr65* is a significant regulator of toxicity to multiple insecticides in *D. melanogaster*. We further confirmed these results using an *Mdr65* null mutant line showing that *Mdr65* is a key ABC transporter involved in limiting toxicity to some, but not all insecticides, primarily due to effects in the nervous system, Malpighian tubules (MTs) and muscles.

2. Materials and methods

2.1. Fly stocks and rearing

Five transgenic *UAS-RNAi* lines from the DRSC/TRiP collection were employed to target 5 ABCB subfamily member genes. The transgenic *UAS-Mdr65-IR*, *UAS-Mdr50-IR*, *UAS-Mdr49-IR* and *UAS-ABCB7-IR* lines were constructed using the *Attp2* landing site, and the transgenic *UAS-ABCB6-IR* line (also known as *UAS-Hmt-1-IR*) was constructed using the *Attp40* site. For controls, matching *Attp2* and *Attp40* lines were used accordingly. All lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, Bloomington, IN, USA (Table 1). *D. melanogaster* were raised at ~23 °C on standard fly medium (sucrose, cornmeal, yeast, and agar, see https://cornellfly.wordpress.com/protocols/) with a relative humidity of 60–70%, and a photoperiod of 16L: 8D.

Some ABC transporters are required for development (Broehan et al., 2013). Therefore, as a first step we evaluated the viability of flies in which these genes had been suppressed from the start of development, using the ubiquitous *Act5c-Gal4* driver. For that purpose, females from each *UAS-RNAi* line (*UAS-Mdr65-IR*, *UAS-Mdr50-IR*, *UAS-Mdr49-IR*, *UAS-ABCB6-IR* and *UAS-ABCB7-IR*) were crossed to males from the *Act5c-Gal4* line. Ten replicates, with a minimum of ten females and five males, were set up for each cross. Flies were transferred to new vials after 48 h, and egg oviposition was repeated three times. The F_1 progenies were raised until there was complete emergence. Viability of the F_1 progeny was evaluated qualitatively by whether or not adults emerged.

To study the role of ABCB transporters in altering the toxicity of insecticides, we knocked down the expression of *Mdr65*, *Mdr50*, *Mdr49*, and *ABCB6* (*Hmt-1*) by RNAi. Virgin females of the control lines or *UAS-RNAi* lines were crossed to males of the driver lines and F_1 progenies were raised at 23 °C. Proper gene silencing in F_1 progeny was confirmed by RT-qPCR (section 2.2). Five *Gal4* driver lines were used in this study: the *Actin5c-Gal4* driver line (*Act5c-Gal4*) drives expression in all tissues, *elav-Gal4* drives expression in the nervous system, *Malpighian tubule type 1-Gal4* (*MT1-Gal4*) drives expression in MTs, *c564-Gal4* drives expression in fat body and hemocytes and *MEF2-Gal4* drives expression in muscles. The tissue specific drivers were selected based on expression of *Mdr65* in adult *D. melanogaster* (www.flyatlas.org). An *Mdr65-null* mutant line ($Mdr65^{KG08723}$ or $Mdr65^{-/-}$) which contained a single *P-element* inserted within the *Mdr65* gene and a matching background control line (*yw*) were also used.

2.2. RT-qPCR experiments

Total RNA was extracted from adult F_1 females (5-7-day-old) for each of our crosses. Briefly, 10 flies were crushed in 500 μL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with an MP FastPrep 24 bead beater (MP-Biomedicals, Santa Ana, CA, USA) and total RNA was extracted according to manufacturer's instructions. RNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and genomic DNA was eliminated by DNase treatment (TURBO DNA-free kit, Invitrogen). The cDNA was synthesized using 1 μg total RNA using the Promega GoScript™ Reverse Transcription System with random primers according to the manufacturer's instructions. The original solution of cDNAs was diluted 5 times with ddH_2O before proceeding to RT-qPCR.

The gene-specific primer sets for four ABC transporter genes used in the RT-qPCR reactions are shown in Supplementary Table 1. The reaction volume (20 μL) contained 10 μL of 2 × iQ™ SYBR® Green Supermix, 7.4 μL of ddH_2O, 8 μM of each specific primer, and 1 μL of first-strand cDNA template. The program was followed by a typical 2-step RT-qPCR protocol, which included an initial denaturation and enzyme activation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and an automatic dissociation step cycle was added for melting curve analysis. Reactions were performed in a Bio-Rad Real-Time PCR system (Bio-Rad, Hercules, CA, USA). Three biological replicates and two technical replicates were conducted for each treatment. The target gene expression was normalized relative to *ribosomal protein 49* (*RP49*, also named *RpL32*). One-way ANOVA with Tukey's test (overall significance level $P \leq 0.01$) was used to determine the

Table 1
UAS-RNAi lines used for genetic crosses.

Strain	Stock#	Gene targeted	Transporter type[a]	CG#	Genetic background	Length of dsRNA
UAS-Mdr65-IR	28664	*Mdr65*	FT	10181	*Attp2*	509bp
UAS-Mdr50-IR	35034	*Mdr50*	FT	8523	*Attp2*	21bp
UAS-Mdr49-IR	32405	*Mdr49*	FT	3879	*Attp2*	21bp
UAS-ABCB6-IR	53284	*ABCB6*	HT	4225	*Attp40*	21bp
UAS-ABCB7-IR	51696	*ABCB7*	HT	7955	*Attp2*	21bp

[a] Full transporter (FT) or half transporter (HT). See Introduction for details.

significant statistical difference between treatments.

2.3. PCR validation of Mdr65-null mutant line

We confirmed the null mutation in the *Mdr65*$^{-/-}$ line by PCR. DNA from the heads of ten female adult flies was isolated with an alkaline extraction method (Montero-Pau and Muñoz, 2008). Three separate DNA samples were prepared from *Mdr65*$^{-/-}$ and *yw* lines respectively. PCR reactions were carried out using the forward primer (5′-GGAGGTCAGAAACAACGC-3′) and reverse primer (5′-GTGTCCTCAATGCTGTCC-3′). This pair of primers amplified a 341bp product in *Mdr65* wild-type flies. The reaction volume (25 μL) contained 12.5 μL of GoTaq master mix (Promega, Madison, WI, USA), 9.5 μL of ddH_2O, 1 μL of each specific primer, and 1 μL of DNA template. PCR reactions were carried out using an iCycler (Bio-Rad) with the following program: 94 °C, 3 min; 33 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 20 s; 72 °C for 5 min. Each PCR product was verified by electrophoresis on a 1% agarose gel followed by 0.5 μg/mL ethidium bromide staining.

2.4. Insecticides and bioassays

The following insecticides were used: chlorantraniliprole (97.8%, DuPont, Wilmington, DE, USA), chlorpyrifos (100%, Dow AgroSciences, Indianapolis, IN, USA), cypermethrin (99%, Chem Service, West Chester, PA, USA), deltamethrin (100%, Roussel UCLAF, Paris, France), diazinon (99.5%, Chem Service), esfenvalerate (82%, DuPont), fipronil (98%, Chem Service), malaoxon (99.4%, American Cyanamid, Wayne, NJ, USA), malathion (99.2%, Chem Service), oxamyl (99%, Chem Service), parathion (99.3%, Chem Service), permethrin (100%, Chem Service), phenthoate (100%, DuPont), and spinosad (98.6%, Chem Service). These were selected to represent a diverse group of insecticides (structures, mechanisms of action, metabolism, etc.).

Bioassays were carried out by residual contact application. Test insecticides were dissolved in acetone and 0.5–1.0 mL of the dilution was applied evenly to the inside of a scintillation vial (Wheaton Scientific, Millville, NJ, USA) of 38.6 cm^2 and allowed to evaporate on a hot dog rolling machine (Gold Medal Products Co., Cincinnati, OH, USA) for at least 30 min before flies were placed inside. A piece of cotton ball covered with white nylon tulle was used as a stopper and 20% sugar water was applied with a syringe to the stopper. Treated vials, each consisting of 20 female flies (3-7-day-old) were laid on their side and held at 25 °C with a photoperiod of 12L: 12D. Mortality, defined as the number of ataxic flies in the vial, was assessed after 24 h of exposure for all insecticides. F_1 females (3-7-day-old) from each of the crosses were assayed using a single concentration for each insecticide and at least ten replicates. The concentrations of the insecticide were selected to give approximately 50% kill in the *Act5c-Gal4* × background strain progeny (to maximize the chances of seeing either an increase or decrease in toxicity associated with the RNAi). To evaluate the effect of knockdown of each ABC transporter, mortality was compared between the F_1 progeny of the *Act5c-Gal4* crossed with *UAS-RNAi* and the F_1 progeny of the *Act5c-Gal4* crossed with the matching genetic background. To determine in what tissue *Mdr65* is required in the fly for its effect on insecticide tolerance, the mortality of *UAS-Mdr65-IR* line crossed to our multiple tissue specific drivers was compared in response to fipronil. One-way ANOVA with single factor test (overall significance level $P \leq 0.01$) was used to determine the significant statistical difference between treatments. To determine the LC_{50} of the *yw* background control and the *Mdr65*$^{-/-}$ line in response to exposure to malathion, malaoxon and fipronil, at least six replicates per concentration and at least three concentrations (giving partial mortality) were tested for each insecticide. The bioassay data for *yw* and *Mdr65*$^{-/-}$ lines were pooled and analyzed by probit analysis (Finney, 1971), as adapted to personal computer use by Raymond (1985) using Abbott's (Abbott, 1925) correction for control mortality. LC_{50} values were judged as significantly different if their 95% confidence intervals did not overlap.

3. Results

3.1. Viability of RNAi lines

Act5c-Gal4 driven suppression of *ABCB7* did not generate any viable RNAi flies, suggesting that this gene is required for *D. melanogaster* development. All other F_1 progeny developed properly and no aberrant anatomical features were observed. RNAi in the four viable F_1s resulted in specific silencing of their target gene (Fig. 1). Use of the *Act5c-Gal4* driver resulted in significant RNAi-mediated gene suppression, ranging from 74% (*Mdr49*) to 91% (*Mdr50*). As expected, the mRNA levels of the four ABCB subfamily genes were quite similar in the progeny of *Act5c-Gal4* crossed with two control lines (*Attp2* and *Attp40*, Fig. 1). Altogether, our data demonstrate that using the *Act5c-Gal4* driver we can achieve specific and potent suppression for four ABC transporter genes: *Mdr49*, *Mdr50*, *Mdr65*, and *ABCB6*.

3.2. Does RNAi alter toxicity of insecticides?

RNAi mediated suppression of *Mdr65*, *Mdr50*, *Mdr49* and *ABCB6* significantly altered the toxicity of specific insecticides, in a gene specific manner (Table 2), relative to control flies. Flies with decreased *Mdr65* expression (*Act5c-Gal4* × *UAS-Mdr65-IR*) were significantly more susceptible to exposure to seven of the nine insecticides, including all of the pyrethroids. The most notable difference was for malathion in which mortality was 9% in the controls and 97% in the *Mdr65* RNAi flies (Table 2). There was also increased mortality to the other two insecticides (chlorpyrifos and oxamyl), but the differences were not significant. Flies with decreased *ABCB6* expression (*Act5c-Gal4* × *UAS-ABCB6-IR*) showed little change in response to the insecticides, except for a significant increase in mortality in the case of malathion (less of a change than was seen with *Mdr65*). Small but significant decreases in mortality were seen for flies with decreased *Mdr49* (parathion) or *Mdr50* (malathion, esfenvalerate and permethrin) expression (Table 2). These findings indicate that RNAi mediated suppression of both full and half transporters in the ABCB subfamily can alter the toxicity of insecticides, with *Mdr65* having the greatest effect against the greatest number of insecticides.

Given the importance of *Mdr65* in altering toxicity to seven insecticides in our initial experiments, we expanded the characterization of *Mdr65* with five additional insecticides. Two of these (malaoxon and phenthoate) were chosen because they were structural analogs of malathion. Malaoxon is the bioactivated form of malathion. The other three were selected to represent insecticides with different mechanisms of action. Suppression of *Mdr65* (*Act5c-Gal4* × *UAS-Mdr65-IR*) resulted in increased mortality to malaoxon and fipronil, but not to the other three insecticides (Table 3). The increases in toxicity to malaoxon and fipronil were similar in magnitude to those observed for malathion (Table 2). Altogether, our results suggest that *Mdr65* is a major regulator of the toxicity of a high number of insecticides.

3.3. Sensitivity of Mdr65 null mutants

We next sought to confirm the role of Mdr65 in insecticide tolerance, by monitoring the insecticide sensitivity of flies deficient

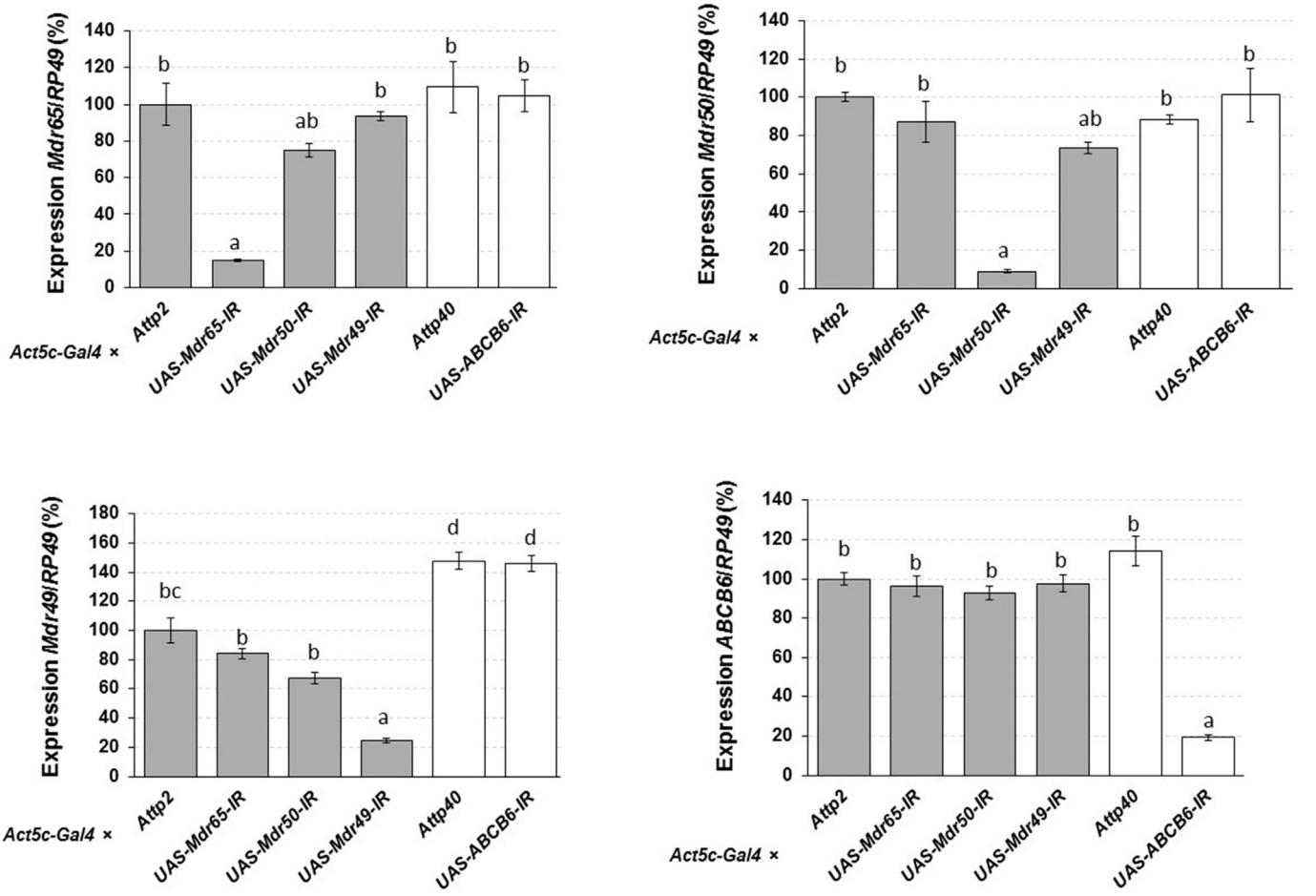

Fig. 1. Validation of the gene expression levels in each progeny of *Act5c-Gal4* × *UAS-RNAi*/control lines. Three biological replicates of 10 F1 females each at 5-7-day-old were used for RT-qPCR analysis. Values shown are the average ± standard error. The letters above the bars indicate significant statistical difference between treatments determined by one-way ANOVA with Tukey's test ($P < 0.01$). Histogram shading denotes flies of common genetic background.

Table 2
Toxicity of nine insecticides to the F_1 progeny of the *Gal4* driver line crossed with the *UAS-RNAi* and its genetic background lines.

Insecticide			Mortality (%) (±SE)					
Class of insecticide[a]	Name	Concentration (ng/cm^2)	*Act5c-Gal4* × *Attp2*	*Act5c-Gal4* × *UAS-Mdr65-IR*	*Act5c-Gal4* × *UAS-Mdr50-IR*	*Act5c-Gal4* × *UAS-Mdr49-IR*	*Act5c-Gal4* × *Attp40*	*Act5c-Gal4* × *UAS-ABCB6-IR*
Group 1-A	Oxamyl	20.7	71(±8)	87(±4)	57(±6)	77(±8)	56(±8)	56(±7)
Group 1-B	Chlorpyrifos	1.09	39(±10)	65(±11)	55(±11)	72(±10)	56(±10)	64(±11)
	Diazinon	2.59	50(±8)	97(±2)▲	34(±10)	62(±9)	52(±8)	37(±7)
	Malathion	3.89	9(±2)	90(±4)▲	1(±1)▼	7(±2)	16(±3)	42(±4)▲
	Parathion	1.62	40(±6)	66(±6)▲	35(±4)	20(±5)▼	44(±5)	52(±6)
Group 3-A	Cypermethrin	25.9	34(±6)	60(±3)▲	21(±5)	30(±5)	44(±5)	34(±6)
	Deltamethrin	9.07	28(±5)	50(±5)▲	16(±3)	44(±6)	56(±6)	38(±5)
	Esfenvalerate	10.4	41(±6)	65(±5)▲	19(±3)▼	30(±4)	76(±5)	68(±8)
	Permethrin	25.9	50(±4)	74(±3)▲	29(±4)▼	64(±5)	73(±4)	61(±5)

▲/▼ Indicates mortality significantly increased/reduced relative to its control flies (F1 progeny of *Act5c-Gal4* × *Attp2*/*Attp40*) at $P \leq 0.01$ level.
[a] Based on the IRAC Mode of Action Classification (Sparks and Nauen, 2015). Group 1-A: carbamates; Group 1-B: organophosphates; Group 3-A: pyrethroids.

in *Mdr65*. We obtained an *Mdr65* null mutant line (*Mdr65*$^{-/-}$) and determined the LC_{50} values for malathion, malaoxon and fipronil. The *Mdr65* mutation was generated in a *yw* background, we therefore used the *yw* line as control to examine the effects of the *Mdr65* deletion. We first verified the *Mdr65*$^{-/-}$ line via diagnostic PCR. A 341 bp product was amplified in all three samples from the *yw* line, and no products were amplified in any *Mdr65*$^{-/-}$ samples, indicating that *Mdr65* was disrupted by the *P-element* in the *Mdr65*$^{-/-}$ line as expected (Fig. S1). Disruption of *Mdr65* resulted in significantly increased mortality to all three insecticides (1.9- to 3.9-fold based on the LC_{50}s, Table 4), consistent with the results of the *Mdr65* RNAi experiments (Tables 2 and 3). *Mdr65* is clearly playing a role in determining the toxicity of malathion, malaoxon and fipronil in *D. melanogaster*.

Table 3
Toxicity of five insecticides to the F_1 progeny of the *Gal4* driver line crossed with the *UAS- Mdr65-IR* and *Attp2* lines.

Insecticide			% Mortality (±SE)	
Classification[#]	Name	Concentration (ng/cm^2)	*Act5c-Gal4* × *Attp2*	*Act5c-Gal4* × *UAS-Mdr65-IR*
Group 1-B	Malaoxon	6.48	6(±2)	92(±3)▲
	Phenthoate	3.24	80(±7)	77(±7)
Group 2-B	Fipronil	3.24	2(±1)	94(±1)▲
Group 5	Spinosad	64.8	31(±6)	39(±4)
Group 28	Chlorantraniliprole	90.7	38(±4)	46(±4)

[#]Based on the IRAC Mode of Action Classification (Sparks and Nauen, 2015). Group 1-B: Organophosphates; Group2-B: phenylpyrazoles; Group 5: spinosyns; Group 28: diamides.
▲Indicates mortality significantly increased relative to its genetic background flies (F1 progeny of *Act5c-Gal4* × *Attp2*) ($P \leq 0.01$).

Table 4
Toxicity of malathion, malaoxon and fipronil against *yw* and *Mdr65-null* (*Mdr65*$^{-/-}$) lines.

Insecticide	Line	LC_{50}[a] (95% CI)	Slope (SE)	n	Ratio[b]
Malathion	*yw*	4.68 (4.53–4.83)	10.8 (0.8)	780	1.9
	Mdr65$^{-/-}$	2.47 (2.39–2.55)	11.2 (0.8)	780	
Malaoxon	*yw*	9.36 (9.05–9.67)	10.6 (0.7)	760	2.1
	Mdr65$^{-/-}$	4.48 (4.32–4.63)	9.6 (0.6)	760	
Fipronil	*yw*	5.16 (4.81–5.53)	3.8 (0.2)	1040	3.9
	Mdr65$^{-/-}$	1.32 (1.25–1.40)	5.3 (0.3)	960	

[a] LC_{50} in ng/cm^2.
[b] LC_{50} *yw*/LC_{50} *Mdr65*$^{-/-}$ (*Mdr65* deletion) strain.

3.4. Tissue specific knock-down of Mdr65

Mdr65 is expressed in the thoracic-abdominal ganglion, brain and MTs, with low expression levels in fat body (www.flyatlas.org). In order to locate the tissues where *Mdr65* modifies insecticide toxicity, four tissue-specific driver lines were chosen to cross with *UAS-Mdr65-IR* and *Attp2* lines: *elav-Gal4* (pan-neuronal), *MT1-Gal4* (MTs), *c564-Gal4* (fat body and hemocytes) and *MEF2-Gal4* (muscles). Single dose assays were performed on the F_1 progeny of each of the crosses using fipronil, because this insecticide had the greatest difference between wild-type and *Mdr65* null flies. Increased mortality was observed in when *Mdr65* was suppressed in neurons (*elav*), MTs (*MT1*) and muscles (MEF2), but not fat body and hemocytes (*c564*) (Fig. 2). Thus, *Mdr65* appears to be modulating the toxicity of fipronil via actions in multiple tissues.

4. Discussion

Altogether, our results demonstrate that *Mdr65* is an important regulator of susceptibility to nine of 14 tested insecticides in *D. melanogaster*. This includes all four pyrethroids, one phenylpyrazole, and four of six organophosphates. The strongest effects were seen for fipronil, malathion and malaoxon. Although the latter two insecticides are structurally very similar, fipronil is not. Furthermore, a relatively small change in structure, from malathion to phenthoate (e.g. substitution of a benzene for the C-C(O)-O-C_2H_5 group) rendered *Mdr65* unable to alter toxicity. In addition, RNAi knock-down of *Mdr65* was previously found to increase DDT toxicity by 12% (Gellatly et al., 2015). Thus, *Mdr65* seems on one hand to have broad specificity for multiple and diverse insecticides (fipronil, malathion, malaoxon, parathion, diazinon, DDT and four pyrethroids), but on the other hand be sensitive to subtle structural modifications (malathion, but not phenthoate). Additional work will be required to clarify the structural limitations that facilitate the ability of *Mdr65* to alter toxicity.

Toxicity of fipronil was enhanced 3.9-fold in flies lacking *Mdr65* (Table 4) and this appears to be due to effects in multiple tissues (the nervous system, MTs and muscles). Fipronil acts on GABA gated chloride channels, so a transporter found in the nervous

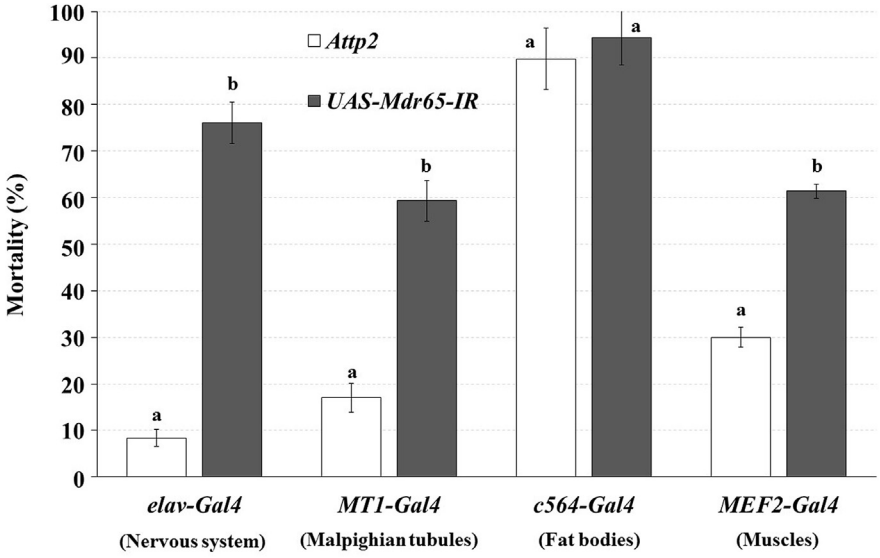

Fig. 2. Tissue specific RNAi alters toxicity to fipronil. The driver strains specific for the nervous system (*elav*), MTs (*Mt1*), fat body (*c564*) and muscles (*Mef2*) (on x-axis) were crossed to *Attp2* or *UAS-Mdr65-IR* and the F_1 tested with a single concentration of fipronil. Values are means of at least 15 replicates ± S.E. A concentration of 3.24 ng/cm^2 was used, except for the F_1s from the *c564-Gal4* crosses where 13 ng/cm2 was used.

system or muscles could conceivably help protect the target site from exposure to the insecticide. Given that *Mdr65* expression in the MTs also influences toxicity suggest that *Mdr65* might be also working in MTs to enhance the excretion of fipronil. Thus, the protective effect of *Mdr65* might be due to both altering distribution (or access) and excretion of the insecticide.

A previous study in *D. melanogaster* using RNAi mediated suppression of *Mdr49* and *Mdr50* found no effect and an increase in DDT toxicity (13%), respectively (Gellatly et al., 2015). These results for *Mdr49* agree with our results in which we found no change in toxicity, except for parathion. In contrast, our results showed that suppression of *Mdr50* did not change the toxicity of six insecticides, and decreased the toxicity of three others.

Given the range of endogenous compounds that ABC transporters can move it might be expected that at least some of these transporters might be required for normal development and physiology. No RNAi adults resulted from the *ABCB7* (*CG7955*) knock-down line, indicating it is necessary for normal development. Similarly, silencing of *TcABCB-5A* in *Tribolium castaneum* prepupae led to severe developmental defects and lethality (Broehan et al., 2013). Evaluating the role of these ABC transporters in modifying insecticide toxicity will require alternative approaches.

One surprising result is that the suppression of two ABC transporters (*Mdr49* and *Mdr50*) decreased mortality to a few insecticides, while suppression of the other two ABC transporters (*Mdr65* and *ABCB6*) increased mortality. This would be possible if some transporters could move insecticides out of cells (or prevent their passage through a membrane) while others move insecticides through the membrane. However, at this point ABC transporters are only known to act as exporters in insects (Merzendorfer, 2014). It is possible that this effect originates in altering the physiology of barrier tissues such as the blood-brain barrier or the excretory machinery and could be involved in reabsorption processes. It is also possible that these transporters indirectly alter the secretion of molecules that interfere or compete with these insecticides. Thus, understanding the reasons for the decreased toxicity of a few insecticides associated with the RNAi mediated suppression of *Mdr49* and *Mdr50* will require further study.

In conclusion, we have identified four ABC transporters that have a role in altering toxicity to one or more insecticides. *Mdr65* had the largest effect and altered the toxicity of the greatest number of insecticides, although the specific relationship between insecticide structure and transport by *Mdr65* is unresolved. Tissue specific RNAi suggests that the effects on insecticide toxicity mediated by *Mdr65* might be to be due to both changes in distribution of the insecticide and changes in excretion.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2017.08.002.

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