

RESEARCH ARTICLE

Genetic knockdown of a single organic anion transporter alters the expression of functionally related genes in Malpighian tubules of *Drosophila melanogaster*

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SUMMARY

Insects excrete a wide variety of toxins *via* the Malpighian (renal) tubules. Previous studies have implicated three transporters in the secretion of the organic anion (OA) methotrexate (MTX) by the *Drosophila* Malpighian tubule: *Drosophila* multidrug resistance-associated protein (dMRP, *CG6214*), a multidrug efflux transporter (MET, *CG30344*), and an organic anion transporting polypeptide 58Dc (OATP58Dc, *CG3380*). RNA interference (RNAi) knockdown and P-element insertion mutation of single OA transporter genes were used to evaluate the importance of these three putative transporters in the secretion of MTX by the Malpighian tubules of *Drosophila melanogaster*. A major finding is that genetic knockdown of a single OA transporter gene leads to reductions in the expression of at least one other OA transporter gene and in secretion of MTX by Malpighian tubules isolated from flies reared on a standard diet. The pattern of changes indicates that decreases in MTX secretion do not correspond to decreases in dMRP expression in all of the RNAi lines. Genetic knockdown of a single OA transporter gene also alters the extent of upregulation of multiple OA transporter genes in the tubules in response to dietary MTX. Knockdown of dMRP is associated with a decrease in MET expression but an increase in OATP expression when flies are reared on MTX-enriched diet. Our results indicate that dMRP and MET are not the dominant MTX transporters in the tubules when flies are reared on MTX-enriched diets. At least one additional transporter, and possibly OATP, are required for MTX secretion. The implications of our results for studies using genetic knockdown techniques to identify OA transporters in whole tissues such as Malpighian tubules are discussed.

Key words: organic anion transport, methotrexate, multidrug resistance-associated protein, MRP, organic anion transporting polypeptide, OATP, RNAi, P-element insertion mutation, remote sensing and signalling hypothesis.

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INTRODUCTION

Organisms use energy-dependent excretory transport as one of the main mechanisms to limit the toxic effects of xenobiotics and endogenous molecules (Miller et al., 1998). The Malpighian tubules are the main excretory organs of insects, and as part of the renal system, they perform the major role in eliminating xenobiotics and wastes (Gaertner et al., 1998; O'Donnell and Spring, 2000; Dow and Davies, 2006). Potentially toxic organic anions (OAs) include endogenous compounds such as folates, bilirubin and prostaglandins. In addition, many plant secondary chemicals or anthropogenic compounds, such as salicylate, indigo carmine and the insecticide metabolites malathion monocarboxylic acid and 3-phenoxybenzoic acid, are OAs that require rapid elimination from the body (O'Donnell and Rheault, 2005; Neufeld et al., 2005).

The *Drosophila* Malpighian tubule transports two types of OAs. Small (<400 Da), univalent and hydrophilic compounds, such as salicylate, fluorescein and para-aminohippuric acid (PAH), are type I OAs. Tubule secretion of type I OAs is Na⁺-dependent and is inhibited by carboxylic acids (Bresler et al., 1990; Linton and O'Donnell, 2000; Ruiz-Sanchez and O'Donnell, 2007). Larger (>400 Da), polyvalent and amphiphilic OAs, such as Texas Red (sulphorhodamine 101 acid chloride) and methotrexate (MTX), are referred to as type II OAs and are transported by Na⁺-independent processes in the Malpighian tubules of fruit flies (Leader and O'Donnell, 2005; Chahine and O'Donnell, 2009).

MTX is a folate analogue that inhibits dihydrofolate reductase (DHFR), a key enzyme for the biosynthesis of thymidylate, purines and several amino acids (Affleck et al., 2006). It reduces DNA synthesis and impairs cellular replication, thus making it a drug of choice for the treatment of a variety of cancers and autoimmune disorders (Affleck et al., 2006). In mammals multidrug resistance-associated proteins (MRPs) play an important role in the excretion of MTX (Hooijberg et al., 1999), and MRP homologues may therefore be involved in the transport of MTX and other antifolates in *Drosophila*. MTX was used in our previous studies of OA transport because it is readily available in tritiated form, allowing its secretion rate to be precisely quantified by liquid scintillation counting (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). These studies showed that MTX is secreted by the Malpighian tubules of both larval and adult *Drosophila*, and chronic exposure of the larvae to dietary MTX increases both the rate of MTX secretion by the tubules and the expression of multiple transporter genes (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). Expression of *Drosophila* multidrug resistance-associated protein (dMRP, *CG6214*), a multidrug efflux transporter (MET, *CG30344*), an organic anion transporting polypeptide (OATP58Dc, *CG3380*) and several other transporters increases significantly in the tubules of flies reared on MTX-enriched diet.

Many of these transporters are members of the ATP binding cassette (ABC) superfamily of membrane transporters (Konig et al., 1999). A single ABC transporter protein may transport several structurally

unrelated compounds. MRPs are in the ABCC subfamily and have been implicated in the transport of xenobiotics, including OAs such as MTX and salicylate (Tarnay et al., 2004; Chahine and O'Donnell, 2009). In vertebrates, OATPs are important membrane transport proteins that mediate the Na⁺-independent transport of a wide range of amphipathic organic compounds, including bile salts, organic dyes, thyroid hormones, anionic oligopeptides, numerous drugs and other xenobiotic substances (Hagenbuch and Meier, 2004). In *Drosophila*, one of the OATPs (OATP58Db) is responsible for the transport of the cardiac glycoside ouabain by the tubules (Torrie et al., 2004).

In the present study, we made use of RNA interference (RNAi) knockdown or P-element insertion mutation of single OA transporter genes to evaluate the importance of putative transporters (dMRP, MET and OATP) in MTX transport across the Malpighian tubules of *Drosophila melanogaster*. We first used flies reared on the standard diet. Given that dietary exposure to MTX alters transporter gene expression, we also examined the effects of RNAi knockdown and P-element insertion mutation on tubules from flies reared on MTX-enriched diet.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster Meigen 1830 stocks were maintained at 22°C on standard artificial diet. Cell type-specific RNAi was used to downregulate the following putative transporters: MRP (*CG6214*), MET (*CG30344*) and OATP (OATP58Dc, *CG3380*). To that end standard genetic crosses were used to target the expression of transporter-specific UAS-dsRNA constructs to the Malpighian tubule principal cells using the *Gal4* enhancer trap line *c42* [FBst0030835 (Rosay et al., 1997; Broderick et al., 2004)]. All RNAi constructs were obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007). The following stocks were used: *UAS-dMRP I-RNAi* (FBst0477246; *UAS-dMRP¹⁰⁵⁴¹⁹-RNAi*), *UAS-MET I-RNAi* (FBst0470674; *UAS-MET⁷³⁷⁸-RNAi*), *UAS-MET II-RNAi* (FBst0470675; *UAS-MET⁷³⁷⁹-RNAi*), *UAS-OATP I-RNAi* (FBst0463041; *UAS-OATP³⁹⁴⁶⁹-RNAi*), and *UAS-OATP II-RNAi* (FBst0463042; *UAS-OATP³⁹⁴⁷⁰-RNAi*). The *c42 Gal4* element was obtained from the Bloomington Stock Center (Indiana University, USA). The progeny of crosses between *c42* and specific RNAi lines were: *c42/UAS-dMRP I-RNAi*, *c42/UAS-MET I-RNAi*, *c42/UAS-MET II-RNAi*, *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*.

We also made use of a dMRP mutation (*dMRP II*), obtained from the Bloomington Stock Center (FBst0020712; *y¹ w^{67c23}; P{EPgy2}MRP^{EY11919}*). This mutation is caused by a P-element insertion 66 nucleotides upstream from the putative start of translation (<http://flybase.org/>). Insertion in the 5'-untranslated region (5'-UTR) may result in the absence of a 5' cap on the mRNA, thus leading to less efficient translation but not a complete loss of function mutation (e.g. Sullivan et al., 2001). Therefore, this P-element insertion created a hypomorphic allele. *yw* (*y¹ w^{67c23}; FBst0006599*) represents the genetic background of the stock carrying the dMRP mutation used in this study and was therefore used as an additional control for *dMRP II*.

Diet preparation

The standard diet was made as described previously (Roberts and Stander, 1998). Solution A consisted of 800 ml of tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH₂PO₄, 0.5 g NaCl, 0.5 g MgCl₂ and 0.5 g CaCl₂. Solution B consisted of 200 ml of tap water and 50 g dry active yeast. Both solutions were autoclaved, combined and stirred. After cooling to 56°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% o-phosphoric acid)

and 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept, Sigma-Alrich, St Louis, MO, USA) dissolved in ethanol were added to the mixture. MTX-enriched diet was prepared by the addition of MTX (0.1 mmol l⁻¹).

Malpighian tubule dissection and Ramsay fluid secretion assay

All experiments were performed with mated adult females, 7 days post-emergence. Ramsay fluid secretion assays were performed with Malpighian tubules dissected under *Drosophila* saline, which contained (in mmol l⁻¹): NaCl (117.5), KCl (20), CaCl₂ (2), MgCl₂ (8.5), NaHCO₃ (10.2), NaH₂PO₄ (4.3), Hepes (8.6), L-glutamine (10) and glucose (20). Saline was titrated with NaOH to pH 7.0.

Ramsay assays were performed as described previously (Dow et al., 1994; O'Donnell and Rheault, 2005). In brief, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet containing [³H]MTX, while the other was wrapped around a steel pin positioned ~1.5 mm away from the bathing droplet. Secreted fluid droplets formed at the ureter and were collected at 60 min intervals with a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (*d*) of the spherical secreted droplet was measured with an ocular micrometer (EfstonScience, North York, ON, Canada), and droplet volume was calculated as (πd^3)/6. Fluid secretion rate (nl min⁻¹) was calculated by dividing the secreted droplet volume by the time over which it formed. The concentration of [³H]MTX in droplets was measured by placing secreted droplets in vials containing 4 ml of scintillation fluid and counting in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, MA, USA). Transepithelial flux of MTX (fmol min⁻¹) was calculated as the product of fluid secretion rate (nl min⁻¹) and MTX concentration (µmol l⁻¹).

RNA extraction and reverse transcriptase PCR amplification

Tissues were dissected from 1-week-old adult female flies raised on standard artificial diet or on diet enriched with 0.1 mmol l⁻¹ MTX (Chahine and O'Donnell, 2010). For each genotype, RNA was isolated from 60 Malpighian tubules using the RNeasy Micro Kit from Qiagen (Valencia, CA, USA), according to the manufacturer's protocol for animal tissue isolation. RNA concentrations were quantified and checked for purity with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. We used 0.3 µg of RNA per sample for cDNA synthesis, after first treating with DNase I (Invitrogen, Carlsbad, CA, USA) to prevent any genomic DNA contamination. First-strand cDNA was synthesised using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at -70°C.

Transporter gene expression (quantitative real-time PCR)

Specific transporter genes were chosen based on their putative function as solute transporters, their enrichment in Malpighian tubules (Chintapalli et al., 2007; Wang et al., 2004) and our previous studies of OA transport (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). The mRNA expression of MET, dMRP and OATP increases >3500-fold, >1200-fold and >23-fold, respectively, in the tubules of adult flies of the Oregon R strain reared on diets containing 0.1 mmol l⁻¹ MTX, relative to the tubules of flies reared on the standard diet (Chahine and O'Donnell, 2009). The primers and GenBank accession numbers for each gene are listed in Table 1.

Table 1. Primer list

| Primer | GenBank accession number | Forward/reverse sequence (5'–3') |
|--------|--------------------------|---|
| GAPDH1 | CG12055 | tgaaggggaatcctgggctac/acccaactcgtgtcgtacc |
| MET | CG30344 | cctgctgacaacttttacgg/gtaatcaaggcgcaagtcc |
| dMRP | CG6214 | actttacgccctgcttgag/tcacgttcagctgttccac |
| OATP | CG3380 | tcgaagcctccaagttctg/catgtgagcagctgcaaat |

dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide; glyceraldehyde 3-phosphate dehydrogenase (GAPDH1).

We first measured mRNA levels of the genes for dMRP, MET and OATP in Malpighian tubules from *c42* and UAS-RNAi parental flies and compared them with their expression in Malpighian tubules from flies carrying both the UAS constructs and *c42-Gal4*. For the P-element insertion line, *dMRP II*, the control line was *yw*. We also measured mRNA levels of the same genes in tubules of RNAi knockdown and P-element insertion mutant flies reared on 0.1 mmol⁻¹ MTX-enriched diet.

mRNA expression was measured by quantitative real-time PCR (qPCR) using the cDNA prepared as described above. Each 20 µl reaction contained 4 µl of cDNA, 6 µl (4 pmol) of each primer and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Amplification reactions were carried out using a Stratagene MX3000P QPCR system (Stratagene, La Jolla, CA, USA), and data were analysed using MxPro QPCR Software v3.00 (Stratagene). The reactions were run at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt-curve analysis verified the production of a single product. Non-reverse-transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. For each gene, a standard curve was performed by serial dilution of one randomly selected experimental sample (Malpighian tubules of flies raised on 0.1 mmol⁻¹ MTX-enriched diet) to ensure that qPCR amplification efficiency was above 95% with its respective primer pair. Specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured the expression of five potential reference genes, including the ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha-tubulin (alphaTub84B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH1). Of these, GAPDH1 had the most stable expression across samples; therefore, it was used as an internal control to calculate relative mRNA expression by the standard curve method.

Chemicals

[³H]MTX (50.8 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Data analysis

Values from all experiments were expressed as means ± s.e.m. for the indicated numbers of samples (*N*). Statistical analyses and curve fitting by non-linear regression analysis were performed using GraphPad InStat and Prism 3.0 (GraphPad software, La Jolla, CA, USA). Significant differences were determined using paired sample or two-sample *t*-tests assuming either equal or unequal variance, according to the outcome of a two-sample *F*-test. Differences were considered significant if *P* < 0.05. One-way ANOVA with Dunnett's *post hoc* multiple comparison was used in experiments in which more than two groups were analysed.

RESULTS

Reduction in mRNA expression of a single OA transporter gene results in decreases in the expression of other OA transporters

The reference gene, GAPDH1, had similar mRNA expression levels in Malpighian tubules from flies with the P-element insertion allele *dMRP II* and flies expressing RNAi knockdown for each of the three putative transporters studied: dMRP, MET and OATP. Experimental levels of mRNA were therefore normalised relative to the expression of GAPDH1 (data not shown).

The levels of mRNA encoded by all three putative ion transporter genes were measured in each genotype. Comparing mRNA expression of dMRP, MET and OATP in the Malpighian tubules indicated which of these three genes showed changes in expression in response to RNAi knockdown or P-element insertion mutation of putative transporters in flies. An unexpected finding was that reduction in the expression of a single OA transporter gene by RNAi or the P-element insertion mutation resulted in a decrease in expression not only of that specific gene but also in the expression of one or more other OA transporter genes in the tubules. mRNA levels of dMRP decreased significantly in tubules of *c42/UAS-dMRP I-RNAi* flies in comparison with control flies (*UAS-dMRP I-RNAi* and *c42*; Fig. 1A). The dMRP P-element insertion mutant (*dMRP II*) also showed a significant decrease in dMRP gene expression in comparison with tubules of control flies (*yw*; Fig. 1B). Unexpectedly, there was a significant reduction in the expression not only of dMRP but also of MET and OATP in tubules of these flies, as well as in the RNAi knockdown line described in Fig. 1A.

A similar pattern was seen when mRNA expression of either MET or OATP was reduced by RNAi. The mRNA expression of both MET and OATP decreased significantly, and dMRP expression was unchanged in tubules of *c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* flies in comparison with control flies (*UAS-MET I-RNAi*, *UAS-MET II-RNAi* and *c42*; Fig. 2A,B). Similarly, mRNA expression of both MET and OATP decreased significantly, and dMRP expression was unchanged in *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* in comparison with control flies (*UAS-OATP I-RNAi*, *UAS-OATP II-RNAi* and *c42*; Fig. 3A,B).

Reduced expression of the putative ion transporters, dMRP, MET and OATP reduces active transport of MTX by the Malpighian tubules

Malpighian tubules from flies reared on the standard diet were set up in the Ramsay assay. In all experiments, fluid secretion rates, MTX concentration and transepithelial MTX flux were measured for tubules isolated from experimental and control flies and bathed in saline containing the same concentration (100 µmol⁻¹) of MTX. Fluid secretion rates in control flies (*yw* and *c42*) were compared with *UAS-RNAi* transgenic flies crossed to *c42* and to the P-element insertion line, *dMRP II*. For tubules of each genotype, the fluid secretion rate was not significantly different from that of control flies (Fig. 4A). In contrast, the concentration of MTX in fluid

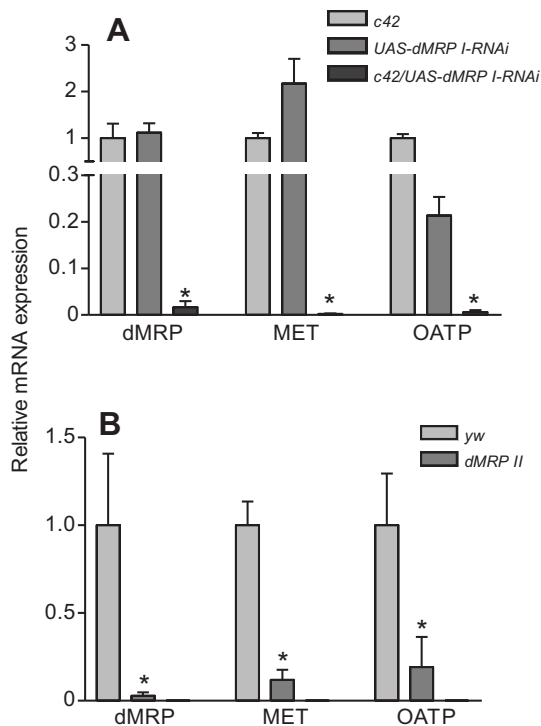


Fig. 1. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of control and experimental groups of adult flies reared on the standard diet. (A) The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. mRNA expression of *c42/UAS-dMRP I-RNAi* was measured in comparison with *c42* and *UAS-dMRP I-RNAi*. (B) The expression of the three transporter genes in *yw* was set as the baseline expression of these genes and given a value of 1. mRNA expression of *dMRP II* was measured in comparison with *yw*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6-7$). Error bars are +s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide.

secreted by tubules from flies displaying reduced expression levels of dMRP, MET and OATP was significantly decreased relative to fluid secreted by the tubules of the controls (Fig. 4B). There was a decrease of ~60% in MTX concentration in the fluid secreted by the tubules from *dMRP II*, *c42/UAS-dMRP I-RNAi*, *c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* flies, and a decrease of ~80% in MTX concentration in *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* in comparison with control flies (*c42* and *yw*, Fig. 4B). As a result, secretion of MTX by tubules with reduced expression of dMRP, MET and OATP decreased significantly relative to the controls (Fig. 4C). Transepithelial flux of MTX measured in isolated tubules of *dMRP II*, *c42/UAS-dMRP I-RNAi*, *c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* decreased by ~60%, relative to tubules of the controls (*yw* and *c42*). For tubules isolated from *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*, flux decreased by ~80%, relative to controls.

Chronic exposure to MTX in the diet affects the expression of OATP, MET and dMRP genes

Previous studies have shown dramatic increases in the expression of dMRP, MET and OATP in tubules of the Oregon R strain of *D. melanogaster* when flies are chronically exposed to dietary MTX

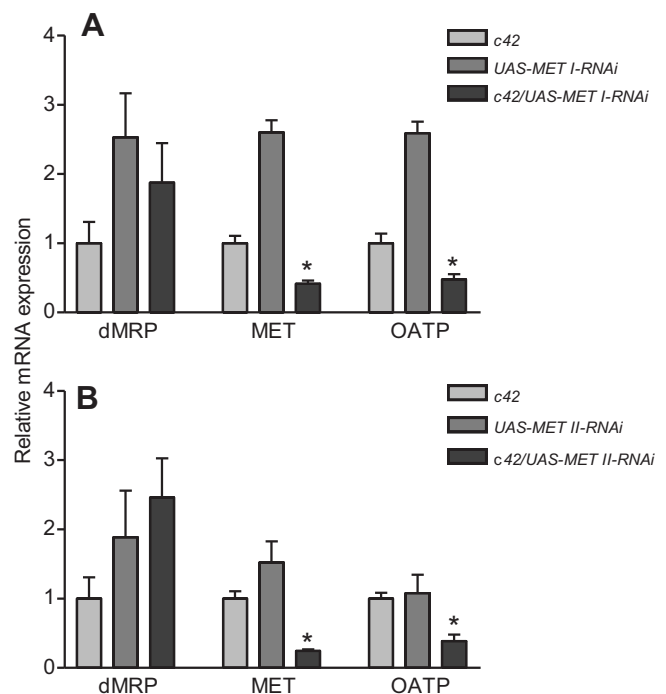


Fig. 2. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. Tubules were isolated from adult flies reared on the standard diet. The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. (A) mRNA expression of *c42/UAS-MET I-RNAi* in comparison with *c42* and *UAS-MET I-RNAi*. (B) mRNA expression of *c42/UAS-MET II-RNAi* in comparison with *c42* and *UAS-MET II-RNAi*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6-8$). Error bars are +s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide.

(Chahine and O'Donnell, 2009). We wished to confirm that a similar pattern was seen when the control lines (*c42* and *yw*) used in this study were exposed chronically (7 days) to 0.1 mmol^{-1} dietary MTX. In tubules of *c42* flies exposed chronically to MTX-enriched diet, dMRP, MET and OATP mRNA expression increased 2000-fold, 240-fold and 25-fold, respectively, above the levels seen in tubules of flies reared on the standard diet (Fig. 5A). In tubules of *yw* flies exposed chronically to MTX-enriched diet, expression of dMRP, MET and OATP increased more than 2000-fold, 1.7-fold and 20-fold, respectively, above the levels seen in tubules of flies reared on the standard diet (Fig. 5B).

Next, we measured mRNA levels of dMRP, MET and OATP in tubules of flies that had been exposed chronically to MTX-enriched diet and in which the expression of a single OA transporter gene was reduced. In both *c42/UAS-dMRP I-RNAi* and *dMRP II* flies, there were significant decreases in mRNA levels of not only dMRP but also MET, similar to the pattern observed in tubules of flies reared on the standard diet (Fig. 6). Fig. 6 also shows that mRNA expression levels of OATP in tubules of *c42/UAS-dMRP I-RNAi* flies increased 23-fold above the levels of the *c42* controls (Fig. 6A). Similarly, mRNA expression levels of OATP in tubules of *dMRP II* flies increased 3-fold above the levels of the *yw* controls (Fig. 6B).

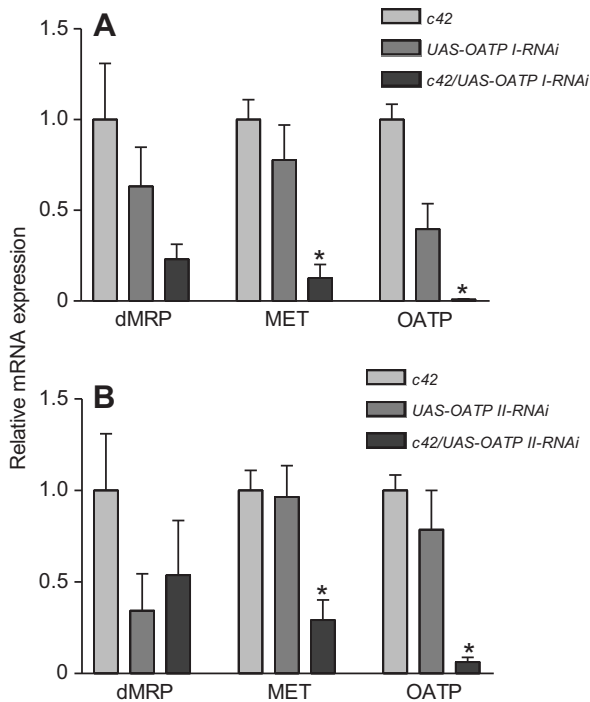


Fig. 3. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. Tubules were isolated from adult flies reared on the standard diet. The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. (A) mRNA expression of *c42/UAS-OATP I-RNAi* in comparison with *c42* and *UAS-OATP I-RNAi*. (B) mRNA expression of *c42/UAS-OATP II-RNAi* in comparison with *c42* and *UAS-OATP II-RNAi*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6-8$). Error bars are \pm s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide.

It is worth noting that whereas the expression of *dMRP II* was only 3% of that of the *yw* flies when reared on the control diet (Fig. 1B), *dMRP II* expression was 18% of that of the *yw* flies when both were reared on MTX-enriched diet (Fig. 6B). These comparisons indicate that *dMRP II* is a hypomorph rather than a null mutation, as discussed below.

In tubules of MET RNAi knockdown flies (*c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi*) chronically exposed to MTX in the diet, there was a significant reduction in mRNA levels of both MET and dMRP, relative to the levels seen in tubules of *c42* flies. Although levels of OATP mRNA trended toward an increase, relative to the levels seen in tubules of *c42* flies, the difference was not statistically significant (Fig. 7).

Similarly, in tubules of OATP RNAi knockdown flies (*c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*) chronically exposed to MTX in the diet, there was a significant reduction in mRNA levels of both MET and dMRP, relative to the levels seen in tubules of *c42* flies (Fig. 8). There was no change in OATP levels in tubules of *c42/UAS-OATP I-RNAi* flies, relative to the levels seen in tubules of *c42* flies, and there was a trend towards an increase ($P = 0.054$, two-tailed *t*-test) in OATP levels in tubules of *c42/UAS-OATP II-RNAi* flies, relative to tubules of *c42* flies. A comparison of Figs 6–8 reveals that flies with dMRP P-element insertion mutation or dMRP, MET or OATP RNAi knockdown fail to upregulate mRNA levels

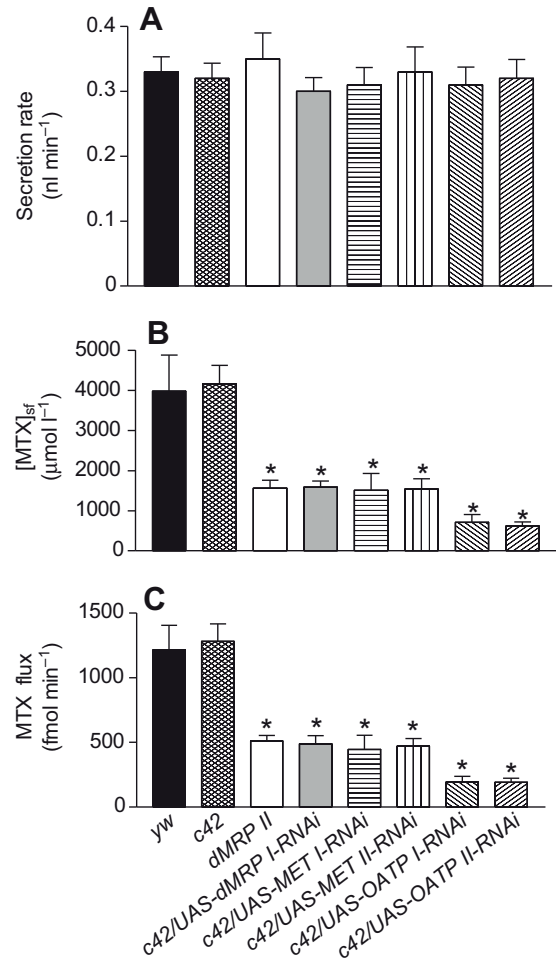


Fig. 4. The effects of the P-element insertion mutation or RNA interference (RNAi) knockdown of putative transporters on (A) Malpighian tubule fluid secretion rate, (B) the concentration of methotrexate (MTX) in the secreted fluid ($[MTX]_{sr}$), and (C) transepithelial flux of MTX. Isolated Malpighian tubules ($N = 6-13$) were set up in a Ramsay assay containing $100 \mu\text{mol l}^{-1}$ $[^3\text{H}]$ MTX in the bathing saline. Secreted droplets were collected after 60 min. Significant differences relative to the tubules of control flies (*yw* and *c42*) reared on standard diet are indicated by asterisks ($P < 0.05$). Error bars are \pm s.e.m.

of dMRP and MET in the tubules to the same extent as that seen in control flies (*yw* and *c42*) in response to MTX in the diet. By contrast, levels of OATP in tubules of all six experimental lines were at levels equal to or greater than those seen in control flies.

Dietary exposure to MTX alters rates of fluid and MTX secretion in RNAi knockdown flies and in a P-element insertion allele for dMRP

Malpighian tubules from flies exposed chronically to MTX in the diet were set up in the Ramsay assay. Fluid secretion rates, MTX concentration and transepithelial MTX flux were measured for tubules isolated from experimental and control flies and bathed in saline containing $100 \mu\text{mol l}^{-1}$ MTX. Fluid secretion rates of tubules isolated from *yw* and *c42* control flies reared on MTX-enriched diet were well above those of the tubules of the same group reared on the standard diet (Fig. 9A vs Fig. 4A). Fluid secretion rates in tubules isolated from flies expressing the RNAi knockdowns of the three putative ion transporters were similar to those of the control flies (*yw* and *c42*). However, there was a decrease in fluid secretion rate for

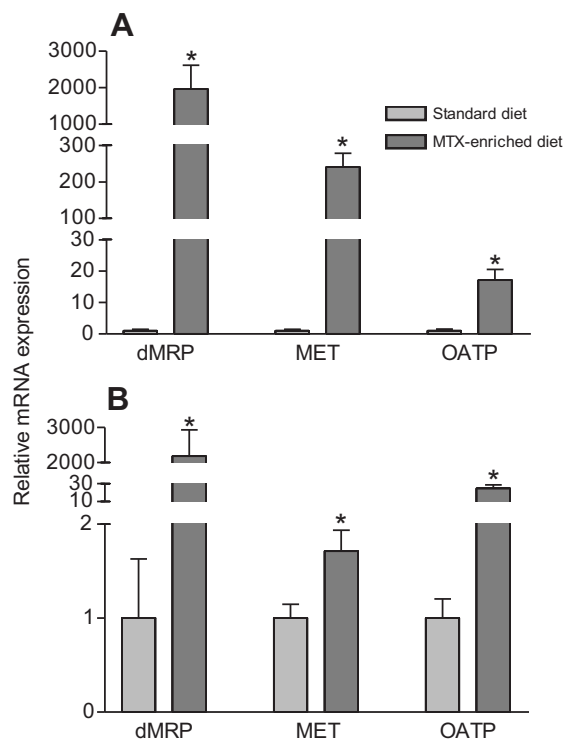


Fig. 5. mRNA expression of putative transporter genes relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of control flies exposed to 0.1 mmol l^{-1} methotrexate (MTX)-enriched diet. (A) mRNA expression in tubules of *c42* flies in MTX-enriched diet in comparison with standard diet. (B) mRNA expression in tubules of *yw* flies in MTX-enriched diet in comparison with standard diet. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6$). Error bars are +s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide.

tubules from the P-element insertion line, *dMRP II* chronically exposed to MTX-enriched diet relative to the controls (*yw* and *c42*; Fig. 9A). Secreted fluid MTX concentration and MTX secretion rates of tubules isolated from *dMRP II* flies or the dMRP or MET RNAi knockdown lines were similar to those of the control groups. However, MTX concentration in the fluid secreted by the tubules from OATP RNAi knockdown flies (*c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*) was significantly lower than in fluid secreted by tubules of control flies (Fig. 9B). As a consequence of the decrease in secreted fluid MTX concentration, tubules from OATP RNAi knockdown flies chronically exposed to MTX-enriched diet secreted MTX at less than half the rate of the corresponding *yw* and *c42* controls (Fig. 9C).

DISCUSSION

A major finding of this study is that a reduction in the expression of a single OA transporter gene leads to reductions both in the expression of at least one other OA transporter gene and in the secretion of the OA MTX by isolated Malpighian tubules of flies reared on the standard diet. When flies are exposed to dietary MTX, rates of MTX secretion and the expression of dMRP, MET and OATP increase in tubules of control flies (*yw* and *c42*), as seen previously in tubules of the Oregon R strain (Chahine and O'Donnell, 2009). Genetic knockdown of a single transporter when flies are reared on MTX-enriched diet results in a decrease in the mRNA expression level of the targeted OA transporter gene and

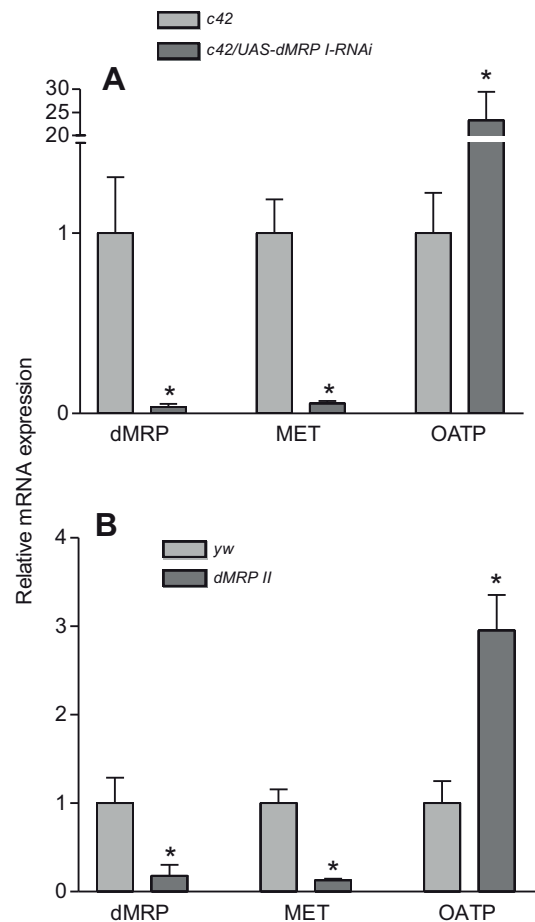


Fig. 6. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of control and experimental groups of adult flies exposed to 0.1 mmol l^{-1} methotrexate (MTX)-enriched diet. (A) The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. mRNA expression of *c42/UAS-dMRP I-RNAi* was measured in comparison with *c42*. (B) The expression of the three transporter genes in *yw* was set as the baseline expression of these genes and given a value of 1. mRNA expression of *dMRP II* was measured in comparison with *yw*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6$). Error bars are +s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter; OATP, organic anion transporting polypeptide.

either an increase or a decrease in the level of at least one additional functionally related gene. Tubules from flies reared on MTX-enriched diet and in which dMRP or MET expression is reduced by genetic knockdown secrete MTX at rates comparable to tubules of wild-type flies. In contrast, tubules from flies with RNAi knockdown of OATP secrete MTX at much reduced rates, irrespective of the diet on which the flies are reared.

Expression of multiple OA transporter genes is reduced by knockdown or P-element insertion mutation of a single OA transporter gene

In an attempt to determine the contributions of the putative transporters in transporting MTX across Malpighian tubules, we used multiple transgenic *Drosophila* lines and also flies carrying a mutation in one of the genes to reduce expression of dMRP, MET

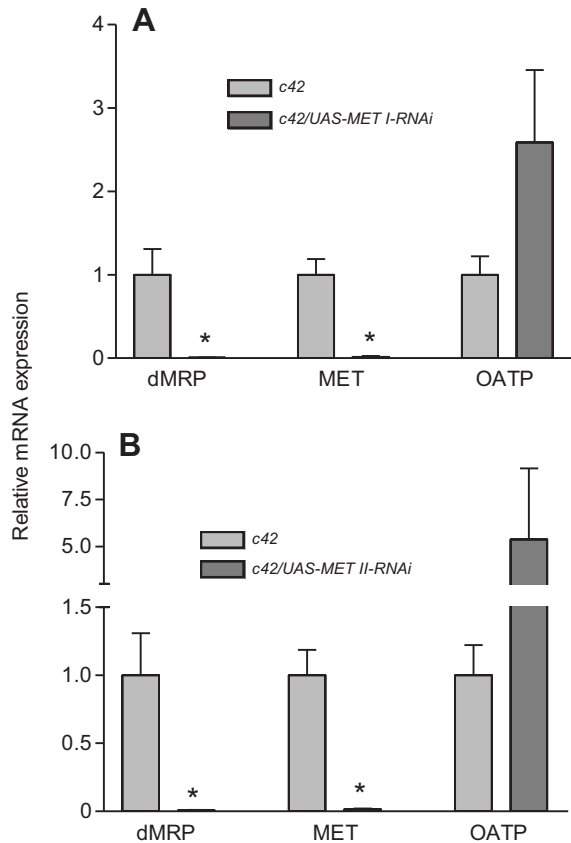


Fig. 7. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of *c42* and multidrug efflux transporter (MET) knockdown flies exposed to 0.1 mmol l^{-1} methotrexate (MTX)-enriched diet. (A) mRNA expression of *c42/UAS-MET I-RNAi* in comparison with *c42*. (B) mRNA expression of *c42/UAS-MET II-RNAi* in comparison with *c42*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6-7$). Error bars are \pm s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; OATP, organic anion transporting polypeptide.

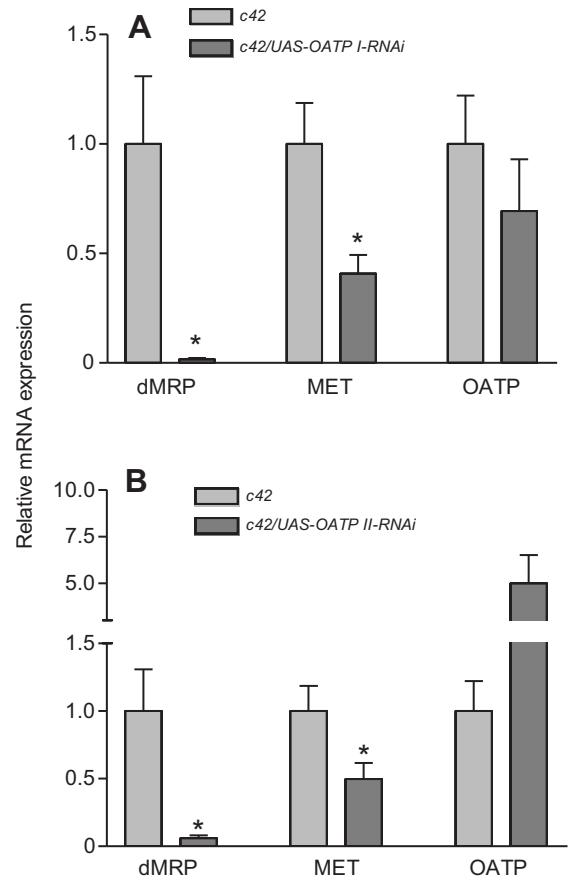


Fig. 8. mRNA expression of three putative transporters relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) expression in Malpighian tubules of *c42* and organic anion-transporting polypeptide (OATP) knockdown flies exposed to 0.1 mmol l^{-1} methotrexate (MTX)-enriched diet. (A) mRNA expression of *c42/UAS-OATP I-RNAi* in comparison with *c42*. (B) mRNA expression of *c42/UAS-OATP II-RNAi* in comparison with *c42*. Significant differences between means for control and experimental groups are indicated by asterisks ($P < 0.05$, $N = 6-7$). Error bars are \pm s.e.m. dMRP, *Drosophila* multidrug resistance-associated protein; MET, multidrug efflux transporter.

and OATP. In the P-element insertion mutant *dMRP II*, the mRNA level of dMRP is reduced in all cells in the fly. By contrast, in the transgenic lines, regulated expression of dsRNA knocks down the expression of putative transporters only within the principal cells of the Malpighian tubule.

To determine the efficacy of the RNAi knockdown and P-element insertion mutation of putative transporters, qPCR was used to measure the level of mRNA expression in Malpighian tubules. mRNA level of the putative transporters decreased significantly (Figs 1–3), indicating that RNAi knockdown of dMRP, MET or OATP or a P-element insertion mutation for dMRP was effective. The P-element is inserted in the 5' UTR of the dMRP gene and the *dMRP II* flies showed a significant decrease (97%) in the level of mRNA expression in comparison with wild-type flies (*yw*; Fig. 1B). We conclude that the insertion, while severely disrupting gene expression, did not completely abolish it. This may also account for the finding that dMRP expression increased in tubules of *dMRP II* flies reared on MTX-enriched diet. The increase was small relative to that measured in tubules of the *yw* controls, but nonetheless measurable,

indicating that the insertion of P-element did not completely prevent upregulation of MRP by dietary exposure to MTX. Our observations are thus consistent with the idea that this P-element insertion does not lead to a complete loss of function.

Knockdown of one transporter was always accompanied by downregulation of one or more other transporters when flies were reared on the standard diet. For example, RNAi knockdown of dMRP leads to a reduction in gene expression not only of dMRP but also of MET and OATP (Fig. 1A). Flies with a P-element insertion mutation in the dMRP gene were also characterised by the decreased expression of dMRP, MET and OATP genes (Fig. 1B). In addition, in tubules of flies with MET knockdown, there was a downregulation of OATP gene expression and *vice versa* (Figs 2, 3). These experiments indicate that P-element insertion mutation or RNAi knockdown of dMRP, MET or OATP is sufficient to downregulate expression of other putative transporters in *Drosophila*. The use of both multiple RNAi lines and a P-element insertion mutation of dMRP rules out the possibility of experimental artifacts associated with off-target RNAi effects and interference by genetic background.

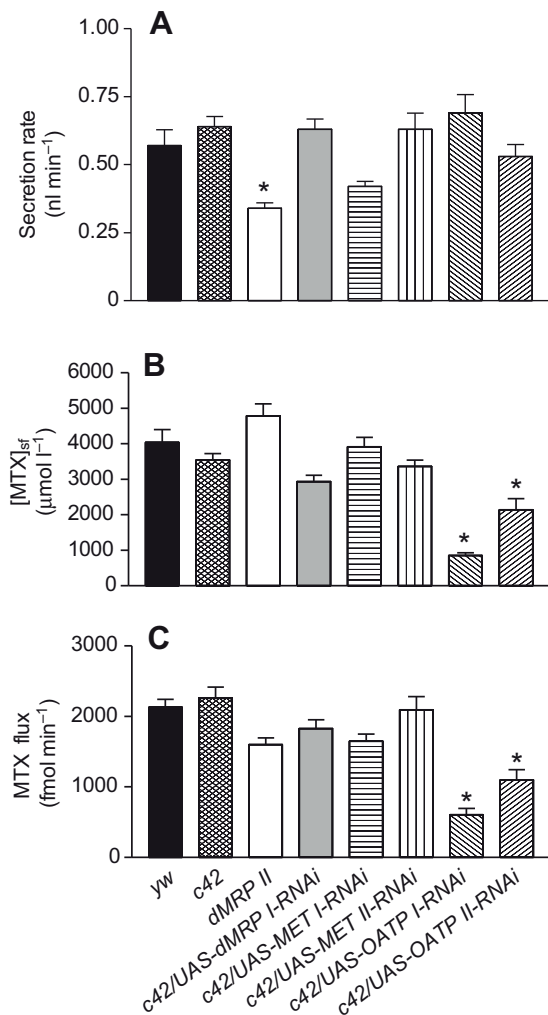


Fig. 9. The effects of chronic exposure to dietary methotrexate (MTX) (0.1 mmol l^{-1}) on (A) Malpighian tubule fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[\text{MTX}]_{st}$), and (C) transepithelial flux of MTX. Tubules were isolated from control flies (*yw* and *c42*) or from flies with the P-element insertion mutation or RNA interference (RNAi) knockdown of putative transporters. Tubules ($N=6-11$) were set up in a Ramsay assay containing $100 \mu\text{mol l}^{-1}$ [^3H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*yw* and *c42*) exposed to MTX-enriched diet are indicated by asterisks ($P<0.05$). Error bars are \pm s.e.m.

Changes in multiple OA transporters in response to genetic knockdown of a single OA transporter in the tubules may represent the first insect example of the remote sensing and signalling hypothesis (Ahn and Nigam, 2009; Wu et al., 2011). In mammalian tissues, this hypothesis has been proposed to account for interactions between OA transporters with overlapping substrate specificities. Transporters such as the OA transporters and MRPs are involved in sensing and signalling in response to cellular injury or alterations in substrate levels. It is proposed that carrier proteins such as OA transporters and MRPs not only transport substrates across an epithelial barrier, but that they also 'sense' related transporters in the same or other tissues. Impaired clearance of substrates by the OA transporters as a result of exposure to toxins, ischemia or competitive inhibition by other substrates can disrupt OA transporter function and perturb homeostasis. The hypothesis is based on parallel changes in OA transporter gene expression and also by evidence

that loss of function of OA transporters is compensated by enhanced expression and/or function of other OA transporters on the transcriptional, translational or post-translational level in either the injured tissues or other tissues, thereby restoring homeostasis. In mice, for example, expression of OAT1 and OAT3 is co-ordinately regulated; deletion of either one results in reduced renal expression of the other. Parallel reductions in expression of both genes during ischemic kidney injury are followed by parallel increases in their expression during recovery of renal function during reperfusion. Increases in the levels of uremic toxins, such as indoxyl sulphate, also lead to alterations in the expression of OA transporters, OATPs and MRPs (Naud et al., 2008). Transporters involved in drug absorption decrease and those involved in drug extrusion increase. Upregulation of OA transporters at the transcriptional or translational level in intact proximal tubule cells may compensate for the loss of OA transporters in damaged cells in the same proximal tubule.

MTX secretion in tubules of flies reared on the standard diet

Reduction in the expression of a dMRP, MET or OATP transporter gene is associated with decreased MTX secretion relative to tubules of the control flies (Fig. 4). These results suggest that these transporters may be involved in tubule secretion of MTX. However, our finding that RNAi knockdown or P-element insertion mutation of a single gene results in changes to other transporter genes makes it impossible to assign a rate-limiting role in MTX secretion to a single transporter. Moreover, given that genetic knockdown of dMRP is associated with reductions in both MET and OATP, it seems probable that mRNA expression levels of other OA transporters are also reduced.

The findings that MTX secretion declines by 60–80% in all four of the MET and OATP RNAi lines (Fig. 4) but that mRNA expression levels of dMRP remain at control levels (Figs 2, 3) suggest that dMRP is not involved in MTX transport. However, MTX secretion by tubules of *dMRP II* or *c42/UAS-dMRP I-RNAi* flies remains at 40% of the control level in spite of dramatic reductions in the mRNA expression of all three genes (dMRP, MET and OATP). The latter finding suggests the contributions of other transporters that can transport MTX across the Malpighian tubules. Organic solute transporters are heavily represented in the tubule transcriptome (Wang et al., 2004), so there are other potential candidate transporters that have not been examined in this study. The tubule is highly enriched in several classes of broad specificity transporters including OA transporters and ABC transporters (Wang et al., 2004). Eight *Drosophila* OATPs are expressed in the Malpighian tubule and one of these (OATP58Db) plays a pivotal role in the secretion of ouabain (Torrie et al., 2004).

Interaction between dietary MTX and RNAi knockdown or P-element insertion mutation of putative transporters in flies

Our results suggest that the level of expression of OA transporter genes is set by two opposing factors in our experiments. RNAi knockdown or P-element insertion mutation tends to reduce transporter gene expression whereas dietary exposure to MTX tends to increase the expression of these genes. Thus, when flies are reared on MTX-enriched diet it is even more difficult than with flies reared on standard diet to ascribe the transport of MTX to a single transporter in the Malpighian tubules.

The rate of fluid secretion rate also increases in tubules of flies reared on MTX-enriched diet, suggesting that genes for the inorganic ion transporters that drive osmotic water flow increase in response to MTX. Previous studies have shown that acute or chronic exposure to the type I OAs salicylate and fluorescein or chronic exposure to

the type II OA MTX is associated with increases in the secretion rates of both fluid and MTX (Chahine and O'Donnell, 2010). An increase in fluid secretion rate in tubules of flies with knockdown of putative transporters indicates that dietary exposure to OAs must also lead to increases in rates of transport of inorganic ions (Na^+ , K^+ and Cl^-), which drive the flow of osmotically obliged water. Exposure to dietary OAs may thus act as a general signal, leading to increases both in the expression of multiple OA transporter genes (dMRP, MET, OATP and others) and in the expression of genes related to the transport of inorganic ions. We suggest that an increase in dietary toxins may result in a remodelling of the epithelium so that more and/or different transporters for both inorganic ions and toxins are expressed. Because mRNA levels of all three of the transporters that we selected increased in response to dietary MTX, it seems probable that the levels of other OA transporters also increased.

Although mRNA levels of dMRP and MET are reduced in tubules of all six experimental lines, relative to tubules of the *yw* and *c42* controls, secretion of MTX was maintained at control levels in four of the lines. In flies reared on MTX-enriched diet, therefore, it appears that dMRP and MET are not involved in MTX secretion by the tubules. Given that OATP mRNA expression levels in tubules of all six experimental lines remain at or above the levels of the controls, OATP is a candidate MTX transporter. The increases in OATP mRNA expression in tubules from flies of the *dMRP II* and *dMRP I-RNAi* lines further suggest that increases in the expression of one OA transporter may provide some degree of compensation for the reduction in dMRP expression. However, the decline of MTX secretion by tubules isolated from flies from either of the two OATP RNAi knockdown lines indicates that other transporters must also be involved in MTX transport. This conclusion follows from the drop in MTX secretion in spite of the evidence that mRNA expression levels of OATP in the two OATP RNAi knockdown lines were equal to or above the levels in tubules of the controls when the flies were reared on MTX-enriched diet. Taken together, our experiments indicate that dMRP and MET are unlikely to play a major role in MTX transport by tubules of flies reared on MTX-enriched diet. OATP may play a role but at least one other transporter, as yet unidentified, must also be involved.

Studies of mammalian tissues, for example, show that MTX and folate are also substrates of SLC19A1 (the reduced folate carrier, RFC) and SLC46A1 [the proton-coupled folate transporter, PCFT (Zhao et al., 2011)]. Although FlyAtlas (Chintapalli et al., 2007) shows no homologues to SLC19A1 or SLC46A1 in the tubules of adult *D. melanogaster*, there are three close matches to another member of the SLC19A family (SLC19A3) that are enriched 5.6–23-fold (*CG6574*, *CG14694*, *CG17036*). However, SLC19A3 transports thiamine but neither folate nor MTX (Rajgopal et al., 2001). MTX is also a substrate of several OATPs (A, B, C and 8) as well as MRPs 1, 2, 3, 4 and 5 in mammals (Zhao et al., 2011).

Although this and previous studies (e.g. Chahine and O'Donnell, 2010) have indicated clear regulation of tubule MTX transport in response to dietary OAs, it is important to note that changes in absorption across the gut could affect the levels of MTX in the haemolymph. In the RNAi lines, the *Gal4* enhancer trap line *c42* drives expression of transporter-specific UAS-dsRNA in the principal cells of the tubules. We would expect, *a priori*, that changes in gene expression would be restricted, therefore, to these cells. However, studies of mammalian tissues indicate that changes in circulating OAs may lead to alterations of OA transporter expression in multiple tissues such as the kidney and liver (Ahn and Nigam, 2009). Changes in gut absorption in the different fly lines used in

this study could alter haemolymph concentrations of MTX. The tubules may be thus exposed to different levels of MTX during the 7-day exposure and there could be corresponding differences in transporter gene expression in the tubules.

Identification of OA transporters in whole tissues: a caveat

Multiple pieces of evidence from previous studies suggested that one or more of three transporters examined in this study (dMRP, MET, OATP) were involved in the secretion of MTX by the Malpighian tubules. Tubule secretion of MTX is inhibited by known blockers or competitive inhibitors of MRPs, such as MK-571, probenecid and Texas Red (Chahine and O'Donnell, 2009). Chronic exposure to dietary MTX results in increases in the expression of these three genes and in the rates of MTX secretion by isolated tubules (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). MRPs are known to transport MTX in mammalian cells (Hooijberg et al., 1999). In addition, treatments known to increase expression of specific detoxification enzymes, such as the P450 monooxygenases (P450s) and the glutathione S-transferases (GSTs), also lead to an increase in expression of dMRP and MET as well as to increased secretion of MTX by the tubules (Chahine and O'Donnell, 2011). The latter finding suggests a coordinated response to toxin exposure, so that when detoxification pathways are increased there is a corresponding increase in the capacity for elimination of the products of P450 and GST enzymes. MTX, for example, is known to be metabolised by the tubules (Chahine and O'Donnell, 2011) and presumably by other tissues such as the fat body.

In the present study, we assessed the functional importance of each of the three transporters by RNAi knockdown or P-element insertion mutation of a single transporter gene. Had we simply correlated the mRNA expression level of the single target gene with the secretion of MTX, we would have concluded that each of these transporters is involved in MTX secretion. A reduction in mRNA expression level of the target gene was clearly correlated with reduced secretion of MTX by tubules of flies reared on the standard diet. However, by using qPCR to measure the levels of all three genes in each of the six experimental lines and the controls, we discovered that RNAi knockdown or P-element insertion mutation of a single gene leads to alterations in the mRNA expression levels of multiple, functionally related genes. In spite of previous evidence implicating dMRP and MET in MTX secretion, our studies of tubules from flies reared on MTX-enriched diet indicate that these two genes are unlikely to play a major role in MTX secretion. The role of OATP is ambiguous; it may contribute to MTX secretion in tubules of flies from the dMRP and MET RNAi knockdown lines or the *dMRP II* line.

It has always been difficult to unequivocally assign transport of a particular OA to a specific OA transporter. Tissues such as the Malpighian tubule and vertebrate kidney are characterised by the presence of multiple OA transporters with overlapping substrate specificities (Ahn et al., 2009), so knocking down a single transporter does not lead to complete inhibition of transport. At the very least, two transporters are required for secretion across the basolateral and apical membranes of the tubule and into the tubule lumen. An elegant study of ouabain transport (Torrie et al., 2004), for example, showed that knocking down OATP58Db reduces active uptake of ouabain by the *Drosophila* tubule by ~50%. The authors conclude that the data show the importance of OATP58Db in ouabain excretion but pointed out that the residual transport activity may reflect residual OATP58Db protein or additional contributions from other transporters (Torrie et al., 2004).

Results of the present study add two further layers of complexity to the study of OA transport by whole tissues such as the Malpighian tubule. First, genetic knockdown of a single OA transporter is inevitably associated with alterations in the expression of other, functionally related transporters. Second, dietary exposure to OAs, such as methotrexate, alters the changes in gene expression produced by genetic knockdown. We initiated this study in the belief that the combined use of RNAi knockdowns and P-element insertion mutation would provide something of a 'magic bullet' for inhibiting specific transporters without the off-target effects of pharmacological transport blockers. In fact, our results provide a cautionary tale regarding the use of genetic knockdown approaches in studies designed to identify the contributions of specific transporters to the transport of a specific OA in intact epithelia. Precise characterisation of putative MTX transporters such as OATP will thus require the use of heterologous expression systems (e.g. *Xenopus* oocytes), which allow the study of a single transporter in isolation.

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