

# **The nuclear hormone receptor DHR96 mediates the resistance to xenobiotics but not the increased lifespan of insulin-mutant *Drosophila***

Sonita Afschar<sup>a,1</sup>, Janne M. Toivonen<sup>c,1</sup>, Julia M. Hoffmann<sup>a</sup>, Luke S. Tain<sup>a</sup>, Daniela Wieser<sup>d</sup>, Andrew J. Finlayson<sup>a</sup>, Yasmine Driege<sup>e,f</sup>, Nazif Alic<sup>b</sup>, Sahar Emran<sup>b</sup>, Julia Stinn<sup>a</sup>, Jenny Froehlich<sup>a</sup>, Matthew D.W. Piper<sup>b,2</sup>, Linda Partridge<sup>a,b,2</sup>

<sup>1</sup>Equal contribution

## **Affiliations**

<sup>a</sup> Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Strasse 9b, 50931, Cologne, Germany

<sup>b</sup> UCL Institute of Healthy Ageing, and GEE, University College London, Darwin Building, Gower St, London, WC1E 6BT, UK

<sup>c</sup> LAGENBIO, Departamento de Anatomía, Embriología y Genética Animal, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2), IIS Aragón, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

<sup>d</sup> European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SD, UK

<sup>e</sup> Department of Molecular Biomedical Research, Unit of Molecular Signal Transduction in Inflammation, VIB, Zwijnaarde, Ghent, Belgium

<sup>f</sup> Department of Biomedical Molecular Biology, Ghent University, Zwijnaarde, Ghent, Belgium

<sup>2</sup>Correspondence during submission:

Linda Partridge

Max Planck Institute for Biology of Ageing

Joseph-Stelzmann-Strasse 9b

50931 Cologne

Germany

Email: Partridge@age.mpg.de

Phone number MPI: +49 (0)221 37970600

## Abstract

Lifespan of laboratory animals can be increased by genetic, pharmacological and dietary interventions. Increased expression of genes involved in xenobiotic metabolism, together with resistance to xenobiotics, are frequent correlates of lifespan extension in the nematode worm *C. elegans*, the fruit fly *Drosophila* and mice. The Green Theory of Aging suggests that this association is causal, with the ability of cells to rid themselves of lipophilic toxins limiting normal lifespan. To test this idea, we experimentally increased resistance of *Drosophila* to the xenobiotic DDT, by artificial selection or by transgenic expression of a gene encoding a cytochrome P450. Although both interventions increased DDT resistance, neither increased lifespan. Furthermore, dietary restriction increased lifespan without increasing xenobiotic resistance, confirming that the two traits can be uncoupled. Reduced activity of the insulin/Igf signalling (IIS) pathway increases resistance to xenobiotics and extends lifespan in *Drosophila*, and can also increase longevity in *C. elegans*, mice and, possibly, humans. We identified a nuclear hormone receptor DHR96 as an essential mediator of the increased xenobiotic resistance of IIS mutant flies. However, the IIS mutants remained long-lived in the absence of *DHR96* and the xenobiotic resistance that it conferred. Thus, in *Drosophila* IIS mutants, increased xenobiotic resistance and enhanced longevity are not causally connected. The frequent co-occurrence of the two traits may instead have evolved because in nature lowered IIS can signal the presence of pathogens. It will be important to determine if enhanced xenobiotic metabolism is also a correlated, rather than a causal, trait in long-lived mice.

## **Significance**

Lifespan of animals can be extended by genetic and environmental interventions, which often also induce resistance to toxins. This association has given rise to the Green Theory of Aging, which suggests that the ability to remove toxins is limiting for lifespan. To test this idea, we genetically increased resistance to toxins in *Drosophila*, but found no consequent increase in lifespan. Furthermore, we could block the xenobiotic resistance of genetically long-lived flies without reducing their lifespan. It will be important to understand if the xenobiotic resistance of long-lived mice is also a correlated, rather than a causal, trait, and to understand the functional significance of the common increase in xenobiotic resistance in long-lived animals.

## Introduction

The aging process can be ameliorated by genetic and environmental interventions, which can also delay or prevent age-related loss of function and pathology (1-4). Notably, the lifespans of the nematode worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*) and the mouse (*Mus musculus*) can be extended by reduced activity of the insulin/insulin like growth factor signalling (IIS) network (1-4), which may also be important in human aging (5). This evolutionary conservation indicates that at least some aspects of mammalian aging can be understood by work with invertebrates, with their short lifespans and ease of genetic manipulation.

In *C. elegans* and *Drosophila*, the single Forkhead Box O (FOXO) transcription factor is essential for the increased lifespan upon reduced IIS (6-8), suggesting that altered transcription of the direct or indirect targets of dFOXO mediates the changes in physiology required for longer life. In *Drosophila*, most of the pleiotropic traits induced by lowered IIS are merely correlated with, rather than causal for, extension of lifespan, because they are still present in the absence of dFOXO (7). Only extended lifespan and increased resistance to xenobiotics of IIS mutants have been demonstrated to require the presence of dFOXO (6-8), suggesting that lowered IIS may extend lifespan through increased detoxification of endo- and xenobiotic compounds.

The metabolism of xenobiotics is divided into three phases: 1) modification, 2) conjugation, and 3) excretion. Genome-wide transcript profiles from long-lived animals, including IIS mutant worms and flies (9, 10), long-lived mutant Ames and Little dwarf mice (11), and mice from crowded litters, subjected to dietary restriction or treated with rapamycin (12) all show increased expression of genes involved in Phase 1 and 2 drug and xenobiotic metabolism (13). Little mice are also resistant to toxicity from xenobiotic compounds (14), indicating that the gene expression profiles are physiologically relevant. The link between increased lifespan and xenobiotic metabolism has led to the “Green Theory”, which suggests that aging results from an accumulation of xenobiotic and endobiotic toxicity as a consequence of a declining detoxification response with age (15).

We have found that, in *Drosophila*, aging and xenobiotic metabolism are independently controlled. We identified a nuclear hormone receptor, DHR96, as

required for the increased xenobiotic resistance of long-lived IIS mutants. However, IIS mutants that lack *DHR96* are equally long-lived without enhanced resistance to xenobiotics, demonstrating that the association between increased lifespan and xenobiotic metabolism is not causal.

## Results

### Increased resistance to the insecticide DDT does not increase lifespan

In *Drosophila*, increased lifespan from reduced IIS is consistently associated with resistance to the insecticide DDT, and both traits require the presence of dFOXO (7). We first investigated if enhanced resistance to DDT would extend lifespan, by using artificial selection or over-expression of a cytochrome P450-encoding gene that enhances resistance to DDT (16).

Two large populations of *Drosophila* (*sel-A* and *sel-B*) were artificially selected for resistance to DDT, and both showed a response to selection (Fig. 1A). However, in the absence of DDT the DDT-resistant lines were short-lived compared to controls (Fig. 1B). Detoxification enzymes expressed in the insect excretory Malpighian tubules play an important role in xenobiotic metabolism (17). DDT resistance was induced by over-expression of the cytochrome P450-encoding *Cyp6g1* in the Malpighian tubules (Fig. 1C). However, the lifespan of the flies in the absence of DDT was unaffected (Fig. 1D). Hence, resistance to DDT *per se* is not sufficient to extend lifespan.

Dietary restriction (DR) increases lifespan in diverse organisms, including *Drosophila* (4) where the increased longevity from DR is dFOXO-independent (18). Interestingly, we found that flies subjected to DR were not resistant to DDT (Fig. S1A). This result cannot be explained by increased consumption of the DDT-dosed food by the DR flies, because DR flies do not differ from fully fed flies in food intake (19, 20). This finding demonstrates that DDT resistance is not necessary for increased longevity and is associated only with particular interventions that extend lifespan.

### Transcriptional signatures of long-lived IIS mutants identify DHR96 as mediating xenobiotic resistance

If IIS mutants are long-lived due to enhanced xenobiotic metabolism, a broader

spectrum of detoxification activities than those induced by either artificial selection to one xenobiotic compound or *Cyp6g1* over-expression may be necessary. To address this, we identified candidate transcription factors that could mediate the increased resistance to xenobiotics of long-lived IIS mutant flies. We profiled transcripts from flies of two different IIS mutants: (1) ablation of median neurosecretory cells (mNSC) in the brain that produce insulin-like ligands (21) and (2) heterozygous loss of the insulin receptor substrate *chico* (22). Both of these mutants exhibited increased resistance to DDT (Fig. S1B). Genes that were down-regulated in the long-lived mutants were enriched for functions in growth (including nucleic acid biosynthesis and translation), development, and reproduction including gametogenesis (Fig. S2). Genes with increased expression were enriched for functions in energy metabolism (including amino acid, carbohydrate and lipid catabolism), protein turnover (numerous peptidases), and transmembrane transport and defence, including metabolism of toxic compounds (Fig. 2). These changes in gene expression correlate well with the phenotypes of IIS mutants (7). Within the enriched defence category, 72 up-regulated genes met our significance cut off and were associated to metabolism of toxic compounds (Dataset S1). The majority of these genes were regulated in response to heterozygous loss of *chico* (55 in total) with the remainder regulated in mNSC-ablated flies. In concordance with previous comparative studies (13) we detected clear differences between the transcriptional profiles (Figure S7), although the overlap between them was significant.

Using the program Clover (23), we identified over-represented transcription factor binding sites (Table S1) in the promoters of genes with altered expression. Most of the putative, cognate transcription factors have documented roles in development, but only a few have known roles in adult flies. Despite this, transcripts of all but two of the genes encoding these transcription factors (*CG10348* and *Grn*) were expressed at reliably detectable levels during adulthood. Of these, two groups are involved in immunity (the GATA-binding and AP-1 transcription factors), in accordance with the enriched GO category in the IIS mutants and the resistance to bacterial infections of *chico*<sup>1</sup> mutant flies (24). We also identified a binding site corresponding to the sequence bound by mammalian pregnane X receptor (PXR) (25, 26), a nuclear receptor that regulates multiple genes involved in the metabolism of endo- and xenobiotic toxins (27). This PXR binding site was enriched near genes with higher expression in both long-lived IIS mutants, including those genes with a proposed role in toxin metabolism (Dataset S2).

PXR is phylogenetically related to *Drosophila* DHR96, one of 18 nuclear receptors in flies (28). Interestingly, null mutation in *DHR96* causes flies to become lean and sensitive to treatment with xenobiotic toxins (29, 30). *DHR96* is also a direct target of dFOXO, which is required for basal transcript levels of *DHR96* (10). We validated the previously published dFOXO chromatin immunoprecipitation (ChIP) binding data by quantitative PCR (qPCR) and found, compared with U6 control (a non polyA - transcribed gene), a significant enrichment of DNA neighbouring *DHR96* in samples immunoprecipitated with a dFOXO antibody (Fig. 3A) Thus, the loss of resistance to xenobiotics in IIS mutant flies lacking dFOXO could be attributable to loss of normal expression of *DHR96*. DHR96 was thus selected as a candidate for mediating the enhanced xenobiotic resistance of IIS mutants.

### **DHR96 mediates xenobiotic resistance of IIS mutants**

We first investigated the role of DHR96 in xenobiotic resistance of adult flies. We subjected mutant *DHR96* null flies (29) to treatment with DDT and found them to be sensitive (Fig. S3). In contrast, removal of *DHR96* caused only a mild reduction in lifespan under non-stressed conditions (Fig. 3B). Ubiquitous over-expression of *DHR96* resulted in developmental lethality (Fig. S4), but over-expression in the Malpighian tubules increased resistance to DDT (Fig. 3C), without affecting lifespan (Fig. 3D), again showing that an increase in DDT resistance does not necessarily increase longevity. DHR96 thus has an important role in xenobiotic metabolism of adult flies.

To test if DHR96 mediates the xenobiotic resistance of IIS mutant flies, we introduced a *DHR96* null mutant into two IIS mutants: over-expression of *dFOXO* in muscle (31) or targeted deletion of the mNSC cells (32). Over-expression of *dFOXO* (Fig. 4A, repeated experiment in Fig. S5) and targeted ablation of the insulin-like peptide-producing mNSC cells (Fig. 4B, repeated experiment in Fig. S6) both significantly increased resistance to the xenobiotics DDT, phenobarbital (PB), and malathion. Strikingly, this resistance to all three xenobiotics was lost in a *DHR96* null background (see Table S2 for Cox Proportional Hazards statistics). *DHR96* is thus a key mediator of the enhanced xenobiotic resistance of long-lived IIS mutants.

If *DHR96* mediates xenobiotic resistance of IIS mutant flies, then it should regulate expression of genes directly involved in xenobiotic metabolism in the tissues responsible for detoxification. With the help of the software tool FIMO (33), we identified the putative binding motif of DHR96 six times in the flanking region of the

glutathione S transferase gene *GstE1* (region 2 kb upstream and 2 kb downstream of the gene,  $p \leq 0.00096$ ) and ten times in the flanking region of the cytochrome P450 gene *Cyp6g1* ( $p \leq 0.00096$ ). Furthermore, *GstE1* and *Cyp6g1* expression is induced by phenobarbital (PB) (29). We therefore investigated the role of IIS and DHR96 in regulating their expression in gut and Malpighian tubules. *GstE1* and *Cyp6g1* were both up-regulated in mNSC-ablated flies but not in dFOXO over-expressors (Fig. S7A). The up-regulation of *GstE1* and *Cyp6g1* in MNC-ablated flies was lost in a *DHR96* null background, suggesting the response was *DHR96*-dependent ( $p=0.027$  for *GstE1* and  $p=0.011$  for *Cyp6g1*, Two-way ANOVA, Fig. 5A-B). *DHR96* thus mediated the increased expression of both detoxification genes.

To further investigate the differences in expression of genes involved in xenobiotic metabolism in different IIS mutants, we re-interrogated our *chico/+* and mNSC-ablated array data. In total 72 genes associated to xenobiotic response were regulated in at least one array dataset, with the majority of those genes being upregulated (Fig. S7B), indicating a common functional response across different models. However, the two models show overlapping, but distinct transcriptional profiles, 55 genes were regulated in the heterozygous *chico* flies, and 17 in the mNSC-ablated flies, with only 8 being regulated in both (Fig S7C). Two-way ANOVA of these common genes confirmed a significant ( $p < 0.0001$ ) interaction, showing a mutant-specific response to reduced IIS. Our qPCR data, together with the statistical analysis of the microarray data, thus demonstrate that reduced IIS can induce cellular detoxification by regulation of both common and distinct sets of genes, as is also the case for IIS mutants in different model organisms (13).

### **DHR96 does not mediate the increased lifespan of IIS mutant flies**

To determine if the increased lifespan of IIS mutant flies was mediated by *DHR96*, we measured adult survival of flies with *dFOXO* over-expression in muscle or ablation of the mNSC, in the presence or the absence of *DHR96*. Consistent with published data (31), muscle-specific over-expression of *dFOXO* significantly extended lifespan when compared to controls (Fig. 6A; see Table S2 for Cox Proportional Hazards statistics). However, this lifespan extension was unaffected by null mutation of *DHR96* (Fig. 6B). Lifespan was also significantly increased by the ablation of mNSC cells (Fig. 6C, repeated experiment in Fig. S8A) and, again, this extension was unaffected by the absence of *DHR96* (Fig. 6D, repeated experiment in Fig. S8B). *DHR96* thus played no role in the extension of lifespan by reduced IIS.



## Discussion

The IIS mutants used in this study showed both enhanced expression of genes involved in xenobiotic metabolism and resistance to xenobiotics. Cognate observations have led to the proposal that enhanced detoxification processes could act as an evolutionarily conserved mechanism for lifespan extension (12, 13, 15, 34). Indeed, there is evidence from both worms and flies that enhanced expression of glutathione-S-transferase (GST)-encoding genes can increase longevity (35, 36). These findings led us to investigate if experimentally enhancing xenobiotic detoxification could also promote longevity. However, although artificial selection for DDT-resistance and over-expression of the cytochrome P450 *Cyp6g1* in a key detoxification tissue, the Malpighian tubule, both increased DDT resistance, neither intervention increased lifespan and, indeed, artificial selection even decreased lifespan. Such costs of selection-induced insecticide resistance have been previously reported (37). On the other hand, dietary restriction increased fly lifespan but not DDT resistance. Thus xenobiotic resistance and lifespan could clearly be uncoupled from each other.

A search for binding motifs of transcription factors differentially regulated in IIS mutants revealed a significantly enriched sequence corresponding to the binding site of mammalian PXR (Pregnane X receptor), the homolog of *Drosophila* DHR96. We also confirmed *DHR96* as a direct target of dFOXO, which is required for basal transcript representation of *DHR96*. We confirmed the sensitivity to xenobiotics of *DHR96* null mutant flies, and showed that they are also short-lived, both characteristics shared by *dFOXO* null mutants. Over-expression of *DHR96* in the Malpighian tubules increased DDT resistance, demonstrating the role of DHR96 in mediating xenobiotic resistance in adult flies. Interestingly, however, *DHR96* over-expression did not increase lifespan, again showing that the two traits can be uncoupled. We showed that DHR96 mediates the resistance of IIS mutants to the xenobiotics that we tested, because this resistance was completely lost when DHR96 was absent. Furthermore, we demonstrated, using microarray data, that detoxification genes are upregulated in two different models of reduced IIS and that up-regulation of two of these genes in mNSC-ablated flies is dependent on DHR96. Interestingly, the up-regulated genes were model-specific, but coalesced into a protective response evident in the resistance to the three xenobiotics that we tested. These model-specific differences agree with previously published studies which have

led to the proposal that enhanced detoxification processes could act as an evolutionarily conserved mechanism for lifespan extension (12, 13, 15, 34). Interestingly, the mammalian *DHR96* homologues *CAR* (constitutive androstane receptor) and *PXR* are also key regulators of phenobarbital-induced xenobiotic response (38, 39), but it is not yet known if they function downstream of IIS. It will be important to investigate if the increased expression of genes involved in xenobiotic metabolism and xenobiotic resistance of long-lived mammals is causal in their increased lifespan.

Importantly, we showed that, at least for the three xenobiotics that we tested, the increased xenobiotic resistance and lifespan of IIS mutants are independently mediated traits with no direct, causal connection between them. Increased expression of genes involved in xenobiotic metabolism together with xenobiotic resistance are, nonetheless, common correlates of lifespan-extension (7, 10, 11, 13-15), raising the question of why this association is so frequent. Interestingly, genes involved in xenobiotic metabolism are indirectly activated by toxic by-products of microbes and pathogens, through the surveillance-activated detoxification and defence (cSADD) system (40), which senses xenobiotics through the dysfunction in cellular processes that they cause, including decreased host translation and altered metabolism (41). Importantly, microbes and pathogens can alter metabolism in the gut, resulting in lower IIS (42). Organisms may hence have evolved systems to sense lowered IIS as an indirect signal of the presence of pathogens, and mount cSADD as a defence response, thus inducing a form of hormesis. Many of the interventions that can increase lifespan involve altered signal transduction of pathways linked to metabolism, and activation of cSADD could provide a common mechanism.

## **Material and Methods**

### **Fly Strains and Maintenance**

The control *white* Dahomey (*wDah*) was derived by backcrossing *w1118* into the outbred, wild type Dahomey background. All transgenic lines were maintained with periodic backcrossing into *wDah*, and are summarized in Table S3. The *DHR96* null mutant was a generous donation by Dr. Carl Thummel. Generation of mNSC-ablated flies, construction of transgenic lines and of DDT selection lines is described in Supporting Information S1-S3.

### **Lifespan measurement**

Lifespan measurements were performed as previously described in Bass *et al.* (43). Lifespan experiments included 100 - 200 female flies per genotype which were allowed to mate for 48h prior to the start of the experiment and transferred to fresh food three times weekly. Experiments were performed at least twice with the exception of the *dFOXO* over-expression epistasis experiment (Fig. 6) which was performed only once. Lifespan measurements and statistical analyses are described in Supporting Information S4.

### **Stress assays**

Flies for stress assays were prepared in the same way as for lifespan experiments. At least 100 females from each cross were sorted into wide plastic vials, 20 flies per vial containing 1 x SYA food, and transferred to fresh food 3 times a week. Stress resistance was assayed at age 10 days. Stock solutions of DDT (Dichlordiphenyltrichlorethan; Greyhound), and phenobarbital (Sigma Aldrich) were dissolved in ethanol, and stock solution of malathion (FLUKA) was dissolved in isopropanol. Final concentration was 175 mg/L or 275 mg/L for DDT (see Supplement S5 for details), 5% (w/v) for Phenobarbital and 7.5  $\mu$ M for malathion. Nearly all stress assays were performed twice, independent repeats of the experiments are in the Supporting Information.

### **Microarrays**

In total, cRNA derived from 5 biological replicates of each IIS mutant genotype and control (Dahomey, *chico*<sup>1/+</sup>, UAS-*rpr*/<sup>+</sup> and UAS-*rpr/dilp2*-Gal4) were hybridized to Quintuplicate Affymetrix Dros2 microarrays. We chose a q-value <0.15 as significance cut-off to consider a gene to be differentially regulated. A detailed description of the microarray experimental procedures and data analysis is summarised in Supplement Materials and Methods S6.

### **Chromatin immunoprecipitation**

ChIP was performed on 3 biological repeats of chromatin as described in (10, 13, 22)

and DNA quantified by qPCR using the primers Hr96 56 (CAAAGAGAGCATATTTAGGATACCAAG) with Hr96 36 (CACAGAACCCAC GCTTCCAAG).

### **Quantitative real-time PCR**

For the gene expression analysis of *GSTE1* and *Cyp6d5* guts including Malpighian tubules of 10 - 15 female flies per sample were dissected and expression was quantified by qPCR using Taqman probes (Applied Biosystems) for *GstE1* (# Dm01826984), *Cyp6g1* (# Dm01819889), *Actin5C* (# Dm02361909) and *Rpl32* (# Dm02151827) using the  $\Delta\Delta\text{Ct}$  method,  $n \geq 3$  for all experiments.

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## Figure legends

### Figure 1. Enhancing DDT resistance by artificial selection or over-expression of *Cyp6g1* in Malpighian tubules did not extend fly lifespan.

(A) Both selection lines (Selection A and Selection B) showed significant DDT resistance compared with three control populations (Control line X, Y and Z) that had been maintained in parallel under non-selection conditions. (B) Lifespans of the same lines as in (A), in the absence of DDT. The DDT-selected lines were shorter-lived than controls ( $p < 0.005$  in all comparisons of selection vs. control populations, Log Rank Test). (C-D) Uo-GAL4 drove expression of *Cyp6g1* in Malpighian tubules. This intervention increased resistance to DDT ( $p = 0.040$  for comparison with Uo-GAL4/+ and  $p = 0.001$  for comparison with UAS-*Cyp6g1-8a*/+, Log Rank Test) (C) but did not affect longevity (D) ( $p > 0.3$  for all experimental lines vs. controls, Log Rank Test).

### Figure 2. Functionally related changes in gene expression in IIS mutants.

Microarray data from *chico*<sup>1</sup> and mNSC-ablated females were analyzed using CATMAP, which retrieves significant changes in functionally-related groups of genes (44). The p-values for genes with increased expression in common between the two mutants are plotted ( $p < 0.1$ , *chico*<sup>1</sup> compared to wild type Dahomey control, mNSC-ablated flies compared to UAS-*rpr* control), where one data point represents a single functionally related gene, and the genes are labelled with the higher-level categories shown in the legend. P-values from the *chico*<sup>1</sup> comparison are plotted on the x-axis, those from the mNSC-ablation comparison on the y-axis. The equivalent data for genes with lower expression in common in the two mutants are shown in Figure S2.

### Figure 3. *DHR96* is a direct target of dFOXO and required for normal xenobiotic response and lifespan

(A) Relative enrichment of chromatin immunoprecipitated with a dFOXO-specific antibody. Higher levels in the precipitate of DNA neighboring *DHR96* versus *U6*, a non-pollI transcribed gene, indicate direct binding of dFOXO to DNA adjacent to the gene ( $p < 0.001$ , Welch T-test). Relative enrichment was calculated as proportion of chromatin recovered in the IP for each region divided by the average of the two regions (*DHR96* and *U6*) for each chromatin (arbitrary scale). (B) Genetic deletion of *DHR96* modestly decreased lifespan of female flies ( $p < 0.0001$ , Log Rank Test). (C-D) Tissue-specific over-expression of *DHR96* in the Malpighian tubules (Uo-GAL4 driver) increased DDT resistance (C,  $p < 0.005$ , Log Rank Test), but did not affect lifespan (D).



**Figure 4. Analysis of the effects of DHR96 on the xenobiotic resistance of two IIS mutants.** (A) Muscle-specific over-expression of *dFOXO* significantly enhanced resistance to DDT, phenobarbital, and malathion when compared to control lines (upper graphs, Log Rank Test, p-values for all comparisons with the matching driver and UAS lines  $<0.001$ , except for comparison of DDT resistance of *dFOXO* over-expressors with the MHC-GAL4 line,  $p=0.61$ ). Enhanced resistance was lost, when *dFOXO* was over-expressed in a *DHR96* null background (lower graphs; p-values for all comparisons with the matching driver and UAS lines  $>0.05$ ). Cox proportional hazards (CPH) was used to test for a statistical interaction between the effects of *dFOXO* over-expression and genomic deletion of *DHR96*, and revealed that each significantly affected stress resistance, with a significant interaction between them ( $p<0.01$ , Table S1).

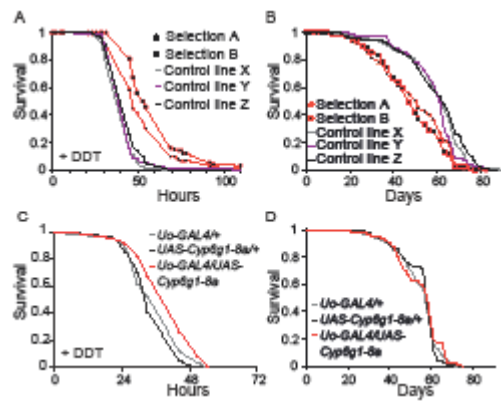
(B) Deletion of the mNSC cells significantly enhanced resistance to the three xenobiotics (upper graphs, Log Rank Test, p-values for all comparisons with the matching driver and UAS lines  $<0.001$ ), and this was lost in a *DHR96* null background (p-values for all comparisons with the matching driver and UAS lines  $>0.05$ ). CPH analysis revealed a significant interaction between the effect of mNSC ablation and genomic *DHR96* deletion, indicating that xenobiotic resistance was significantly blocked by the genomic deletion of *DHR96* (CPH,  $p<0.001$ , Table S1).

**Figure 5. DHR96 mediates the increased expression of detoxification genes in IIS mutants.** mRNA expression of *GstE1* (A) and *Cyp6g1* (B) in the gut of mNSC-ablated flies was assessed by qRT-PCR to determine if it was regulated by IIS or *DHR96*. Results represent fold changes in mRNA levels relative to the *InsP3*-GAL4 control (mean  $\pm$  SEM). *GstE1* and *Cyp6g1* were significantly up-regulated in mNSC-ablated flies in a wild type but not a *DHR96* null background. Two-way ANOVA revealed a significant interaction term ( $p=0.027$  for *GstE1* and  $p=0.011$  for *Cyp6g1*) with the response of both genes in the mNSC-ablated flies being entirely dependent on *DHR96* ( $n \geq 4$ ). Individual pair-wise comparisons used Tukey's multiple comparisons test (\*,  $p<0.05$ , \*\*,  $p<0.01$ , \*\*\*,  $p<0.001$ ).

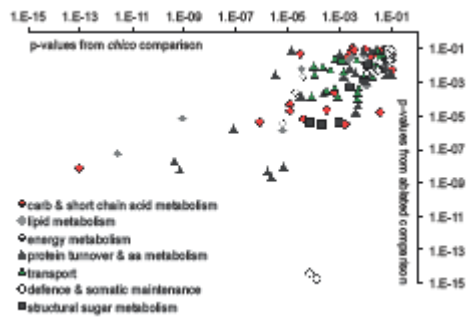
**Figure 6. Lifespan extension by lowered IIS is independent of DHR96.** Lifespan of females was significantly increased by muscle-specific over-expression of *dFOXO* or by targeted ablation of mNSC cells in both a wild type (A and C, respectively) and

a *DHR96* null background (B and D, respectively) (p-values for all comparisons with the matching driver and UAS lines  $<0.001$ , Log Rank Test). CPH analysis revealed that genomic *DHR96* and over-expression of *dFOXO* or ablation of mNSC each significantly affected lifespan, but these effects did not show a significant interaction (Table S1).

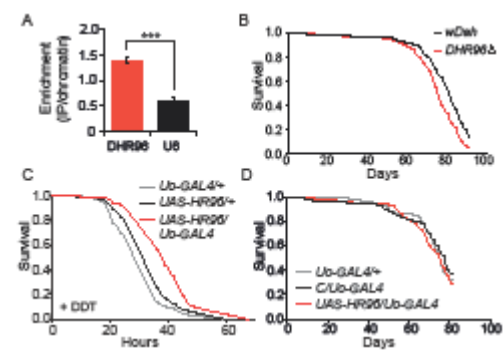
**Figure 1**



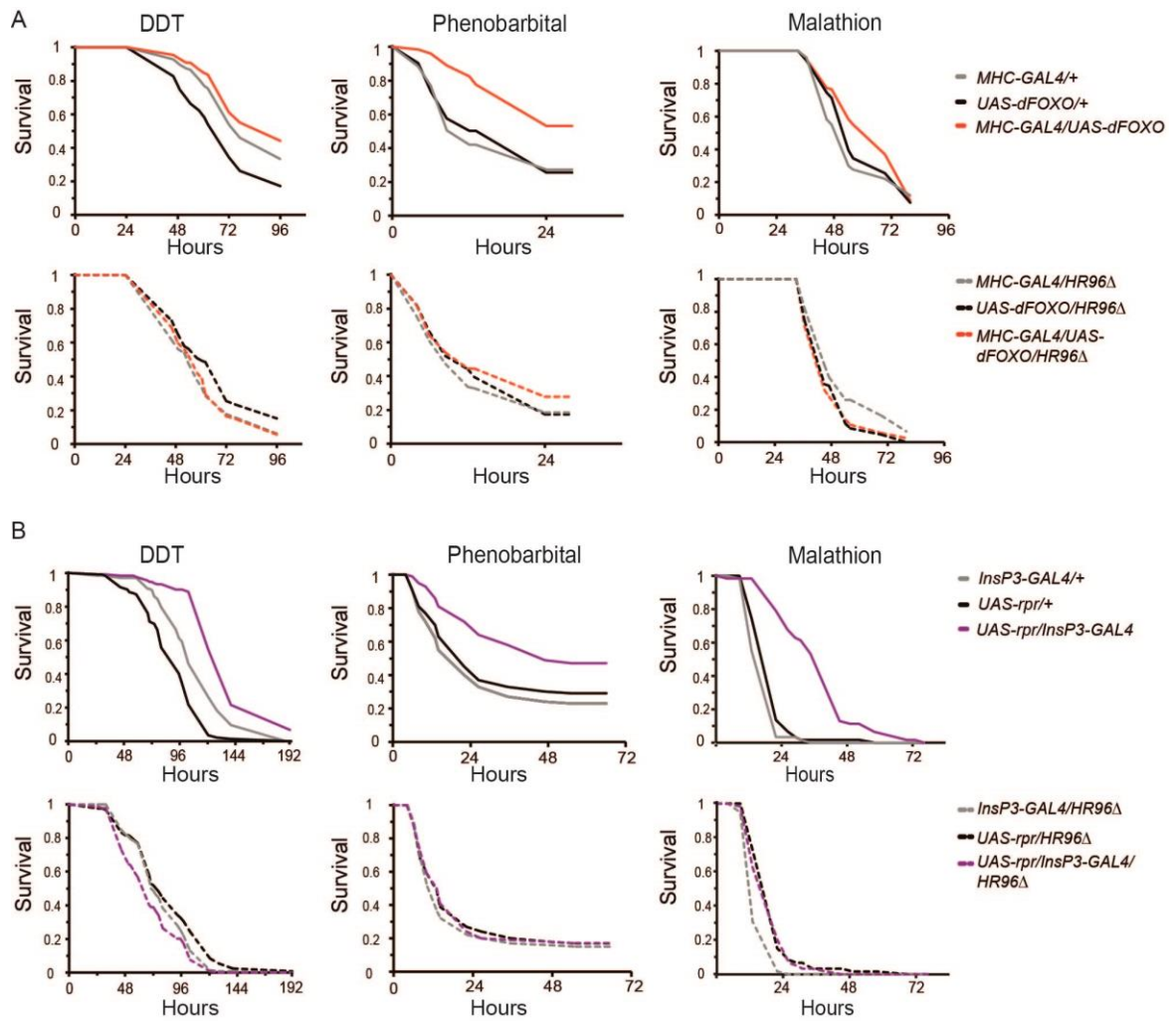
**Figure 2**



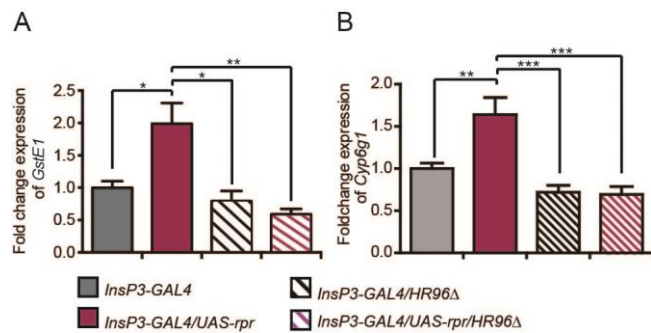
**Figure 3**



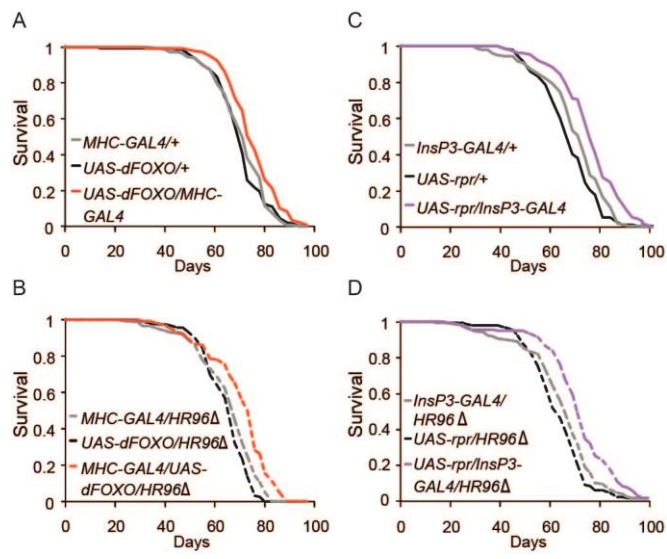
**Figure 4**



**Figure 5**



**Figure 6**



## SI Materials and Methods

### S1 Fly Strains and Maintenance

Flies were kept in glass bottles (13.5 cm x 6 cm diameter) on a standard 1x SYA medium in a controlled temperature room with a 12:12 light:dark cycle, 65% humidity and a temperature of 18°C for stock maintenance and 25 °C for experiments. mNSC-ablated flies were generated by crossing UAS-*reaper* to *dilp2*-GAL4 (21) for the microarray experiments (Fig. 2) and stress assays (Fig. S1) or by crossing UAS-*reaper* to *InsP3*-GAL4 (32) for the qPCR (Fig. 5), stress assays (Fig. 4, S5, S6) and lifespan measurements (Fig. 6). See Table S3 for a list of fly stocks used in this study.

### S2 Construction of transgenic lines

#### Construction of UAS-*DHR96*

Cloned *DHR96* coding sequence (kind gift from Tony Southall) was used as a template to PCR amplify the wild type *DHR96* coding sequence (*HR96*), using the following primers:

Hr96-51- *NotI* (ACGCGGCCGCATGTCGCCGCCGAAGAAC)

Hr96-31Stop-*XbaI* (GTCTAGACTAGTGATTTTTCAAATCGAATATTTTC)

PCR product was inserted into the pUAST vector via the restriction sites *NotI* and *XbaI*. pUAST-*DHR96* was injected into *Drosophila* embryos and resultant UAS-*DHR96* transgenics were backcrossed for at least eight generations into the *wDah wolbachia*<sup>+</sup> background.

#### Generation of UAS-*dFOXO* and of MHC-GAL4 in a *DHR96* null background

UAS-*dFOXO* is inserted at the *attp40* locus on the second chromosome, and flies are marked with the mini white gene and balanced over *CyO*. The deletion in *DHR96* null flies is located on the third chromosome and mutants are white-eyed, but marked with GFP-expressing eyes (29) and balanced over *TM3Sb*. Positive UAS-*dFOXO*; *DHR96* null were identified by orange, GFP expressing eyes and were crossed to homozygosity.

Both MHC-GAL4 and *DHR96* null are on the third chromosome, and were recombined. Both were balanced over *TM3Sb* before recombining them. After screening for GFP, positive +; MHC-GAL4/*DHR96* null were crossed to homozygosity.

### **Generation of UAS-reaper and *InsP3*-GAL4 in a *DHR96* null background**

The crossing for *InsP3*-GAL4 in a *DHR96* null background was performed as for the MHC-GAL4; *DHR96* null, as the driver is inserted on the third chromosome. UAS-*reaper* is integrated into the X-Chromosome and was maintained over FM6.

### **S3 Construction of DDT selection lines**

From our large population cages containing *wDah wolbachia*<sup>+</sup>, six groups of several hundred flies were removed and randomly assigned to one of six new population cages. Every week, three bottles containing 30 ml of fresh food (1xSYA Brewer's) were introduced into the cages and the three oldest bottles removed. This was continued throughout selection so that at all times each cage contained 11 bottles of different ages: three within one week old, three between one and two weeks old, three between two and three weeks old and two between three and four weeks old. Three control cages were always fed normal food while the three selection cages were fed food containing 1SYBrewer's containing DDT at increasing concentrations over time. During the course of five months, the DDT dose was incremented in the following steps (w/v food): 0.001%, 0.0025%, 0.005%, 0.006%, 0.008%, 0.01%, 0.012%, 0.015%, 0.018%, and 0.021%. During the transition from 0.018% to 0.021%, one of the treatment populations died out.

### **S4 Lifespan experiments**

Experimental flies were raised at a density of 200-300 flies per bottle containing 70ml 1xSYA medium. Upon emergence, flies were transferred to fresh bottles for 48h to standardize mating status. Subsequently, females were counted for experiments under light CO<sub>2</sub> anesthesia and transferred to glass vials, 10 flies per vial, and transferred to fresh food three times weekly. Statistics were performed using JMP statistical software (SAS Institute). Differences in death rates at all ages were assessed by Log Rank test, and significance for values of maximum lifespan (final surviving 10% for each population) was assessed by the non-parametric median test. Cox Proportional Hazards was performed in JMP (SAS) to test for an interaction between IIS and *DHR96*. The model included two covariates in all analyses: the status of reduced IIS (*dFOXO* over-expression or mNSC ablation status versus controls) and *DHR96* status (wild type *DHR96* versus *DHR96* null).

### **S5 Stress Assays**

All xenobiotics were added to 1x SYA food after cooling it down to 55°C. Flies

exposed to drugs were not tipped into new vials because they died within few days and no progeny developed. Dead flies were counted every 4-8 hours. Measurement was stopped when flies were dead or response to xenobiotic ceased.

**Note:**

In the first stress assay where flies were treated with DDT (Fig. S5), we used two different concentrations for dFOXO over-expressors in a wildtype background (275 mg/L) and dFOXO over-expressors in a *DHR96* null background (175 mg/L). For *DHR96* mutant flies a lower DDT concentration was used because they were known to be sensitive (29) and we were afraid that we would not see differences between dFOXO over-expressors in a *DHR96* null background and the un-induced controls in a *DHR96* null background. But even with this low concentration we were unable to detect a protective effect of dFOXO over-expression and therefore decided to stick with the standard DDT concentration (275 mg/L).

## **S6 Microarray data analyses**

### Experimental procedure

For sampling, flies were snap-frozen in liquid nitrogen at 3 pm on day 7 after eclosion. For each array, RNA from 20 to 30 whole flies was extracted using TRIzol (Gibco, Paisley, UK) and purified with RNeasy columns (Qiagen, West Sussex, UK) following the manufacturer's instructions. The quality and concentration of RNA was confirmed using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, US), and further procedures followed the standard Affymetrix protocol. All samples were hybridized to the *Drosophila* Genome 2.0 Genechip in quintuplicates.

### Data analysis

All individual probes were mapped against all known and predicted transcripts of the *D. melanogaster* genome release version 5.4. This mapping allowed for up to one alignment error for either perfect match or mismatch of each individual probe, and a composite score was calculated for each probe set. This allowed each probe set to be assigned a qualitative category: perfect (all probes match a single target gene with no mismatches), promiscuous (some or all probes within a probe set map to more than one gene in the genome), weak (the probe set maps to a single gene, but some probes may have mismatches or may not map to the gene), or orphan (no probes in the probe set map to any known or predicted gene in the genome). Both



promiscuous and orphan probe sets were excluded from further analysis. FlyBase gene ids were mapped to GO ids (version 1.107).

Raw data (cel files) were processed to correct for probe-sequence biases, and R's implementation of the Affymetrix's MicroArray Suite 5.0 software was used to determine present target transcripts (46). A transcript was considered present if the p-value was  $< 0.111$ , and absent otherwise. The data was normalized by eight different methods (47) and the statistical analysis of each normalization was combined to identify a robust set of differentially expressed genes. The R code from (46) was altered to exclude absent probe sets prior to the final Loess normalization to reduce the number of false-positives associated with the absent probe sets.

Since lowered IIS can extend lifespan without reduction of fertility (48), we removed ovary-specific transcripts after the first round of normalization. Ovary-specific transcripts were identified as follows. Tissue-specific Affymetrix array data from 11 tissues dissected from the adult fly were downloaded from the FlyAtlas webpage (49). As above, the raw data were preprocessed to correct for probe-sequence biases, and R's implementation of the Affymetrix's MicroArray Suite 5.0 software was used to determine present target transcripts (46). A probeset was considered to be ovary specific if it was called present in ovary but not in any of the other tissues. The microarray data for *chico*<sup>1</sup> heterozygotes have previously been reported in (13), but were re-analysed here to account for software updates. The data for the mNSC-ablated flies was generated for this study. We also analyzed *chico*<sup>1</sup> homozygous flies, which show a great lifespan-extension than do heterozygotes, but the effect of the mutant on the transcriptome was so large that the array data could not be normalized adequately for comparison with any of the other groups.

For functional analysis using all expressed genes, we used the Wilcoxon rank sum test in CATMAP (44). Ranks of genes were based on the Bayes t-statistic for differential expression and, for a given functional category, the significance of the rank sum for all genes in the category was calculated analytically based on a random gene-rank distribution.

The Clover program (23) was used to identify over-representation of TRANSFAC (26) motifs in the 1000 bp upstream of the transcriptional start site, as defined by Ensembl (50).

## Supplemental Tables

**Table S1.** Transcription factor binding sites found over-represented in the promoters of genes with higher expression in the long-lived IIS mutant flies (see also dataset S2).

TRANSFAC	<i>Drosophila</i> TF binding to site	Function summary
AP-1	Jra Kayak	Cytoskeletal re-arrangement in development, immune response, wound healing
DR1	PPAR orthologue unknown in flies	Control aspects of fat tissue formation and metabolism in mammals
Evi-1	putatively CG10348 & Hamlet	Neuronal development
GATA	Pnr, Srp, Grn, GATA <sup>d</sup> GATA <sup>e</sup>	Hematopoiesis, cardiac development, endoderm development and adult immunity
HNF4	Hnf4	CNS development
Pbx	Extradenticle	Developmental leg patterning
PXR	DHR96	Req for normal regulation of detox enzymes
TFAM	mtTF A	mitDNA replication and maintenance
TTF1	putatively Vnd	Ventral nerve system development
Zeste	Zeste	Regulation of homeotic genes

<sup>1</sup> known proteins or predicted orthologues of proteins binding to DNA element

**Table S.2 Cox Proportional Hazard statistics.** Interaction was tested between the effect of reduced IIS (*dFOXO* over-expression (oe) status or mNSC ablation status) and the effect of *DHR96* gene deletion (*DHR96* status). The estimate of the coefficient states the natural log of the hazard ratio. A beneficial effect on survival is displayed by a negative value. “>” Indicates that interaction between two status was tested.

Relevant Figure	Experiment	Coefficient	Estimate	SE	p-value
Fig. 4A	Stress assay DDT	<i>dFOXO</i> oe status	0.0967	0.0433	0.0237
		<i>DHR96</i> status	0.4358	0.0441	<.0001
		<i>DHR96</i> status > <i>dFOXO</i> oe status	- 0.1329	0.0433	<b>0.0021</b>
	Stress assay Phenobarbital	<i>dFOXO</i> oe status	0.2411	0.0490	<.0001
		<i>DHR96</i> status	0.2622	0.0490	<.0001
		<i>DHR96</i> status > <i>dFOXO</i> oe status	- 0.1377	0.0489	<b>0.0046</b>
	Stress assay Malathion	<i>dFOXO</i> oe status	0.0846	0.0506	0.0902
		<i>DHR96</i> status	0.4224	0.0511	<.0001
		<i>DHR96</i> status > <i>dFOXO</i> oe status	- 0.1816	0.0507	<b>0.0003</b>
Fig. 4B	Stress assay DDT	mNSC ablation status	-0.425	0.0727	<.0001
		<i>DHR96</i> status	0.9092	0.0730	<.0001
		<i>DHR96</i> status > mNSC status	0.5854	0.0726	<b>&lt;.0001</b>
	Stress assay Phenobarbital	mNSC ablation status	-0.2000	0.0521	<.0001
		<i>DHR96</i> status	0.4428	0.053	<.0001
		<i>DHR96</i> status > mNSC status	0.2014	0.0508	<b>&lt;.0001</b>
	Stress assay Malathion	mNSC ablation status	-0.3484	0.0607	<.0001
		<i>DHR96</i> status	0.3035	0.0586	<.0001
		<i>DHR96</i> status > mNSC status	0.2694	0.0599	<b>&lt;.0001</b>
Fig. 6A	Lifespan assay	<i>dfoxo</i> oe status	0.2583	0.0362	<0.001
		<i>DHR96</i> status	0.2009	0.0362	<0.001
		<i>DHR96</i> status > <i>dfoxo</i> oe status	0.0662	0.0356	<b>0.0909</b>
Fig. 6B	Lifespan assay	mNSC ablation status	-0.309	0.0383	<0.001
		<i>DHR96</i> status	0.1518	0.0369	<0.001
		<i>DHR96</i> status > mNSC status	-0.0377	0.0368	<b>0.3055</b>

**Table S3 *Drosophila* strains and transgenic lines.**

<b>Wild type, balancer and mutant flies</b>		
<b>Fly strain</b>	<b>Reference</b>	<b>Details</b>
White Dahomey <i>wolbachia</i> plus ( $w^{Dah} w^+$ )	Grönke et al., 2010 (12)	Wild type <i>Drosophila</i> stock
$w^{Dah} w^+$ ; <i>CyO</i>	Bloomington <i>Drosophila</i> Stock Center	Balancer fly on the 2 <sup>nd</sup> Chromosome, homozygous lethal, Curly wings
$w^{Dah} w^+;; TM3Sb$	Bloomington <i>Drosophila</i> Stock Center	Balancer fly on the 3 <sup>rd</sup> Chromosome, homozygous lethal
$w^{Dah} w^+$ ; <i>chico</i> <sup>1</sup>	Clancy et al., 2001 (13)	A <i>Drosophila</i> insulin receptor substrate protein
$w^{Dah} w^+;; DHR96^{\Delta}$	King-Jones et al., 2006 (1)	<i>DHR96</i> null mutation on the 3 <sup>rd</sup> Chromosome
<b>GAL4 driver lines</b>		
<b>Fly strain</b>	<b>Reference</b>	<b>Details</b>
$w^{Dah} w^+;; mhc$ -GAL4	Bloomington <i>Drosophila</i> Stock Center	Muscle-specific driver, Chromosome 3
$w^{Dah} w^+;; mhc$ -Gal4/ <i>DHR96</i> <sup>Δ</sup>	This study	Muscle-specific driver in a <i>DHR96</i> null background
$w^{Dah} w^+$ ; <i>dilp2</i> -GAL4	Broughton et al., 2005 (14)	mNSC-specific driver (median neurosecretory cell)
$w^{Dah} w^+;; InsP3$ -GAL4	Buch et al, 2008 (15)	mNSC-specific driver (median neurosecretory cell)
$w^{Dah} w^+;; InsP3$ -GAL4/ <i>DHR96</i> <sup>Δ</sup>	This study	mNSC-specific driver (median neurosecretory cell) in a <i>DHR96</i> null background
$w^{Dah} w^+$ ; <i>Uo</i> -GAL4	Terhzaz et al., 2010 (16)	Malpighian Tubule-specific driver
<b>UAS-responder lines</b>		
<b>Fly strain</b>	<b>Reference</b>	<b>Details</b>
$w^{Dah} w^+$ ; UAS- <i>Cyp6g1-8a</i>	Yang et al., 2007 (17)	Cytochrome P450 6g1
$w^{Dah} w^+$ ; UAS- <i>DHR96</i> -WT	This study	Wild type UAS- <i>DHR96</i> line on Chromosome 2
$w^{Dah} w^+$ ; UAS- <i>dFOXO</i>	Giannakou et al., 2004 (18)	<i>dFOXO</i> inserted through attp40 sites into the 2 <sup>nd</sup> Chromosome
$w^{Dah} w^+$ ; UAS- <i>dFOXO</i> ; <i>DHR96</i> <sup>Δ</sup>	This study	UAS- <i>dFOXO</i> in a <i>DHR96</i> null background
$w^{Dah} w^+$ ; UAS- <i>rpr</i>	Broughton et al., 2005 (14)	UAS- <i>reaper</i>
$w^{Dah} w^+$ ; UAS- <i>rpr</i> ;; <i>DHR96</i> <sup>Δ</sup>	This study	UAS- <i>reaper</i> in a <i>DHR96</i> null background

## Supplemental Figure legends

**Figure S1. DR flies were not DDT-resistant whereas IIS mutant flies showed increased DDT resistance.** (A) Long-lived, dietarily restricted flies were not resistant to DDT. Age-synchronized female flies were maintained under dietary restriction (DR) or fully-fed (FF) conditions as described in Grandison et al., 2009 (45). On day 7 of adult life, flies were transferred to the same food containing DDT. FF flies were significantly longer lived than DR flies under DDT stress ( $p < 0.001$  in both trials, Log Rank Test). (B) Long-lived *chico*<sup>1</sup>-heterozygote and insulin-producing mNSC-ablated flies were resistant to DDT (for any comparison of mutant versus control in either trial of resistance to either compound,  $p < 0.03$ , Log Rank Test).

**Figure S2. CATMAP categories from microarray data for *chico* and MNC-ablated flies.** Similar functional groups of genes identified by CAPMAP (44) were down-regulated in both long-lived IIS mutants. The p-values for functional group changes that were found in common between the two mutants are plotted ( $p < 0.1$ ), for *chico*<sup>1</sup> on the x-axis and the mNSC-ablation on the y-axis.

**Figure S3. *Cyp6g1-8a* and *DHR96* are important mediators of the response to DDT** (A) Flies overexpressing *Cyp6g1-8a* in the Malpighian tubules were resistant to DDT compared to driver control ( $p < 0.05$ , Log Rank Test; repeat of the experiment shown in Fig. 1C). (B) Flies with genetic deletion of the *DHR96* gene were sensitive to DDT when compared to control wild type flies (*wDah*) ( $p < 0.05$ , Log Rank Test).

**Figure S4. Constitutive over-expression of *DHR96* in the whole body caused developmental lethality.** Over-expression of *DHR96* using the *daughterless*-GAL4 driver resulted in lethality in different stages of *Drosophila* development, and few survivors. Flies reared at 18°C showed increased survival.

**Figure S5. Repeat xenobiotic stress assays with *dFOXO* over-expressing flies in wild type and *DHR96* null background.** (A) *dFOXO* over-expressing flies were resistant to DDT (left panel, 275mg/L DDT, p-values for all comparisons with the matching driver and UAS lines  $< 0.001$ , Log Rank Test), whereas *dFOXO* over-expression in a *DHR96* null background did not increase DDT resistance (right panel, 175mg/L DDT, p-values for all comparisons with the matching driver and UAS lines  $> 0.05$ ). (B) *dFOXO* over-expressing flies in a wild type or *DHR96* null background were exposed to phenobarbital (PB). *dFOXO* over-expression increased PB

resistance, which was entirely dependent on the presence of *DHR96*. Two-way ANOVA revealed a significant interaction term ( $p=0.016$  for Two-way ANOVA against the driver control and  $p=0.0005$  against the UAS control). Individual pair-wise comparisons used Tukey's multiple comparisons test (\*,  $p<0.05$ , \*\*,  $p<0.01$ , \*\*\*,  $p<0.001$ ).

**Figure S6. Repeat xenobiotic stress assay with mNSC-ablated flies in wild type and *DHR96* null background.** Ablation of mNSCs enhanced DDT resistance but this was lost when mNSCs were ablated in a *DHR96* null background as Two-way ANOVA revealed a significant interaction term ( $p=0.0004$  for Two-way ANOVA against the driver control and  $p<0.0001$  against the UAS control). Individual pair-wise comparisons used Tukey's multiple comparisons test (\*,  $p<0.05$ , \*\*,  $p<0.01$ , \*\*\*,  $p<0.001$ ).

**Figure S7. Regulation of detoxification genes by IIS is both common and model-specific.** (A) Fold changes in mRNA expression of *GstE1* and *Cyp6g1* in guts and Malpighian tubules was assessed by qRT-PCR in *dFOXO* over-expressing flies and driver controls. *dFOXO* over-expression did not affect mRNA expression of either gene ( $p>0.05$  for both *GstE1* and *Cyp6g1*, Student's t test). (B) Correlation of fold changes in expression of genes within the GO term Defence in *chico/+* and mNSC-ablated flies. 55 genes were differentially regulated in *chico/+* (green) and 17 in mNSC-ablated flies (red) with 8 being regulated in both datasets (yellow) with a significant overlap between them ( $p=0.0085$ , Fisher's exact test). (C) Differentially expressed genes within the GO term Defence common to both *chico/+* and mNSC-ablated flies were generally up-regulated in both mutants ( $p<0.0001$  for both mutants, One-sample t-test). Expression changes were significantly different for specific genes in the two mutants as revealed by Sidak's multiple comparison test ( $p<0.01$ ). Two-way ANOVA revealed a significant interaction term between differentially regulated genes and the mutant genotype ( $p<0.0001$ ), showing that the two mutants produced different changes in expression of genes within the GO term Defence. \*Although only differently regulated in mNSC-ablated flies, data for *Cyp6g1* are included in this figure because this gene enhanced xenobiotic resistance when over-expressed in the Malpighian tubules (Fig. 1C and S3).

**Figure S8. Repeat of lifespan experiment with mNSC-ablated flies in wildtype and *DHR96* null background.** Ablation of mNSCs significantly increased lifespan in

a wild type background (A, p-values for all comparisons with the matching driver and UAS lines  $<0.0001$ , Log Rank Test) and this lifespan extension was not affected by DHR96 null mutation (B,  $p=0.017$  when compared to driver control *InsP3-GAL3/HR96 $\Delta$*  and  $p < 0.001$  when compared to UAS control *UAS-rpr/HR96 $\Delta$* , Log Rank Test).

## Supplemental Figures

Figure S1

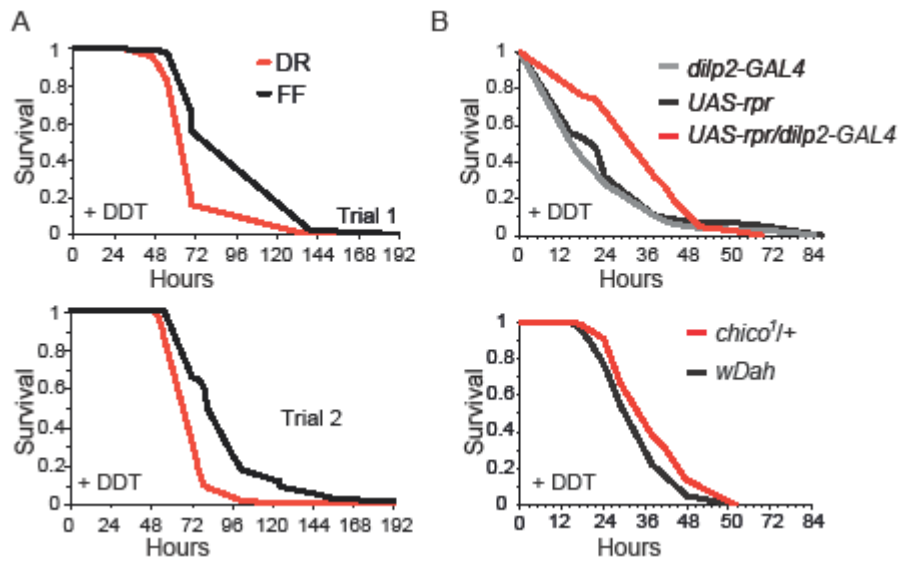
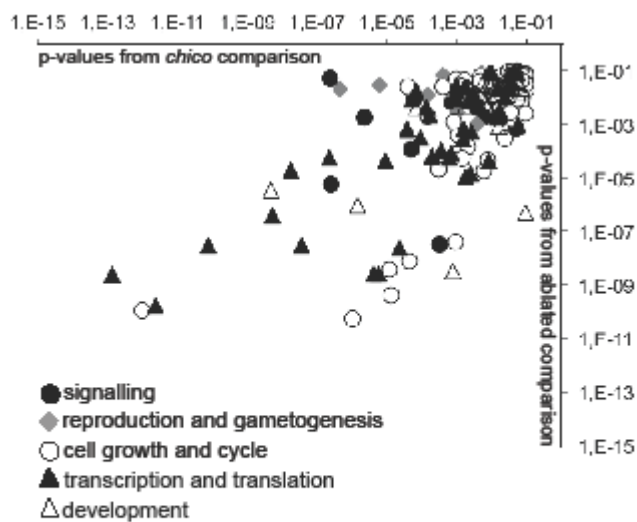
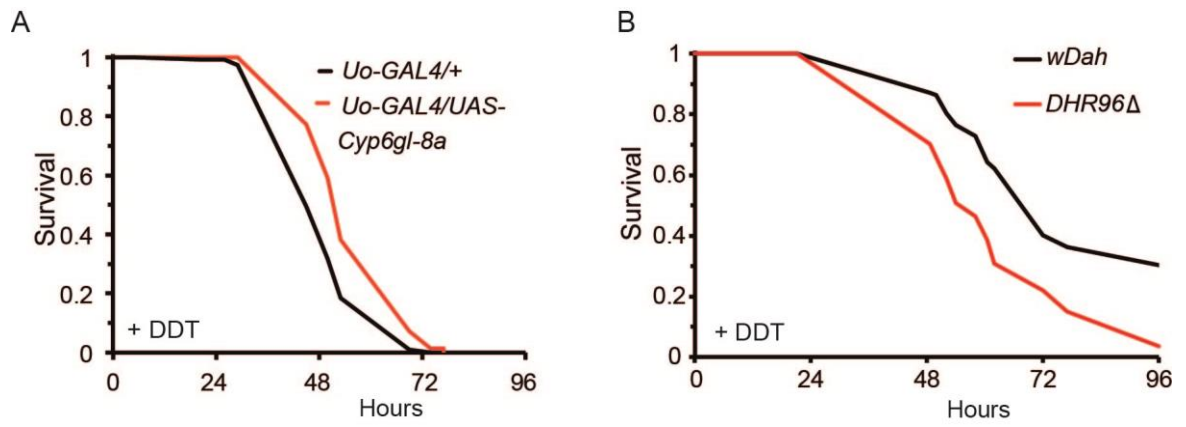


Figure S2





**Figure S3**



**Figure S4**

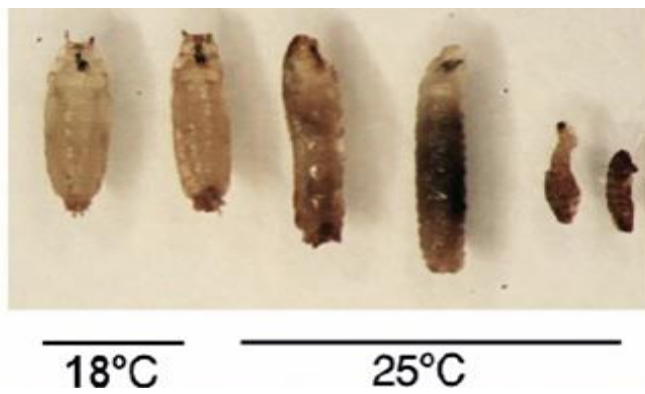
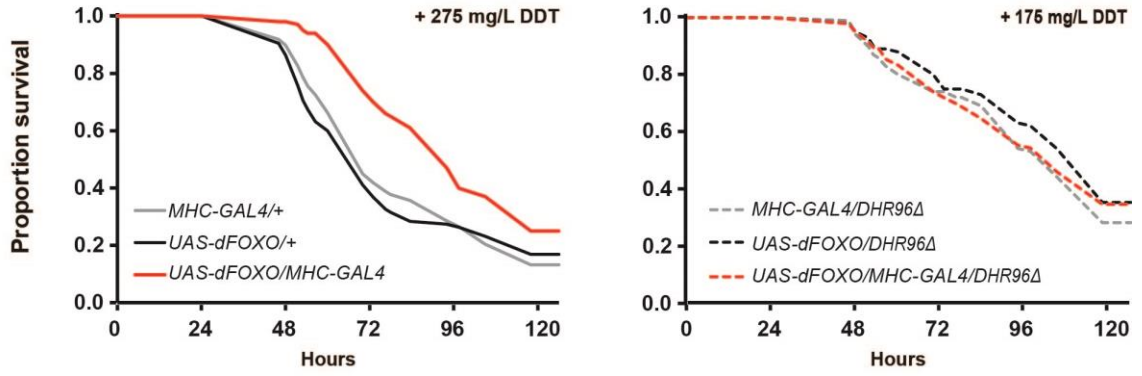


Figure S5

A



B

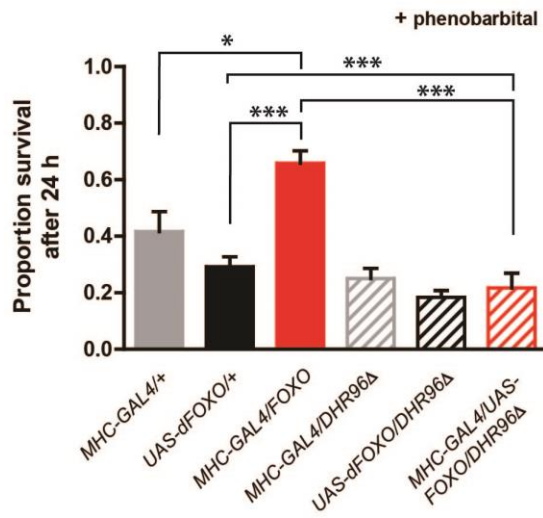


Figure S6

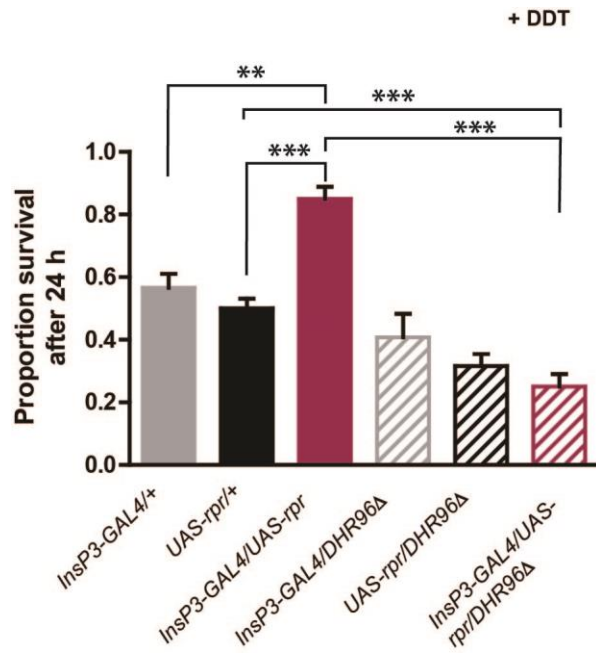


Figure S7

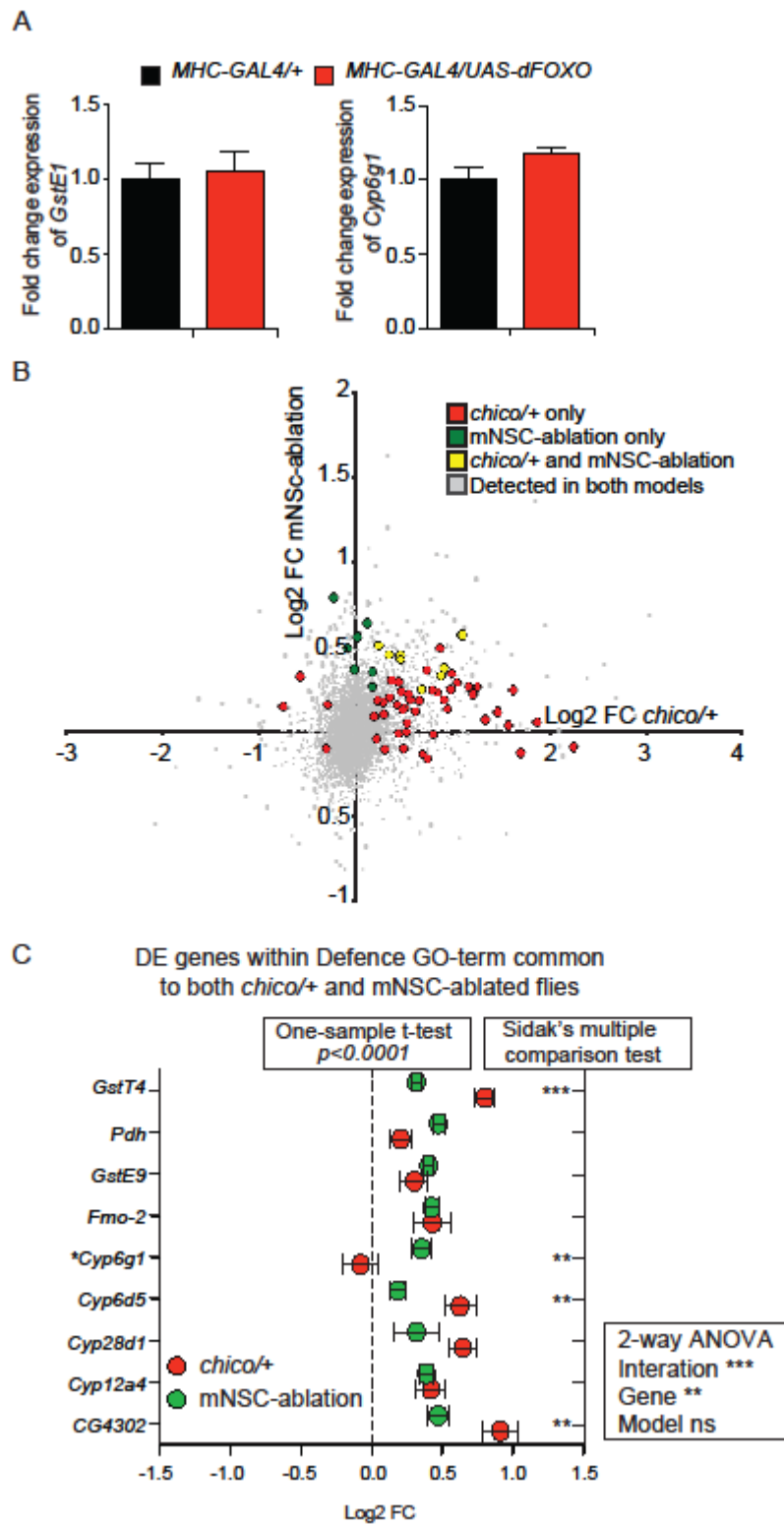
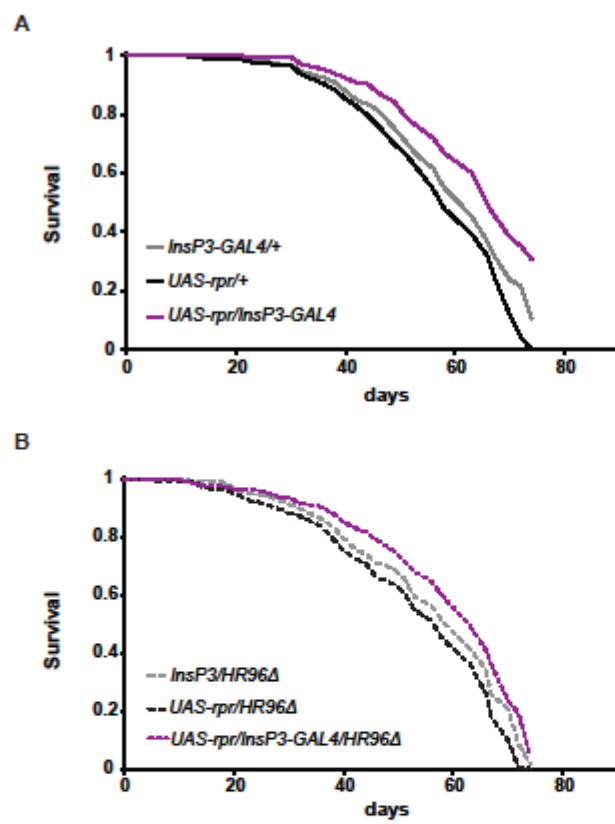


Figure S8



## **Supplemental Datasets**

### **Dataset S1 (located in excel workbook)**

Summary of annotated phase I and phase II detoxification genes, their significance and fold change values for each of the two IIS mutants versus controls.

### **Dataset S2 (located in excel workbook)**

List of genes with higher expression in both long-lived IIS mutants than their respective controls. Q-value, fold change and occurrence of PXR binding site in the promoter is indicated for each gene.