The Hormone Nuclear Receptor DHR96 and its Role in Ageing

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Medicus curat, natura sanat.

Der Arzt behandelt, die Natur heilt.

Table of Contents

Abbreviations

AMP	Antimicrobial peptide
bp	base pairs
C. elegans	*Caenorhabditis elegans*
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CT	Controlled temperature
CYP	Cytochrome P450
CyO	Curly of Oster
TM3Sb	TM3 stubble
Da-GS	*Daughterless* Gene-Switch
DDT	dichlorodiphenyltrichloroethane
dFOXO	*Drosophila* Forkhead box protein
DAF	Dauer formation
DHR96	*Drosophila* Hormone Nuclear Receptor 96
DILP	*Drosophila* insulin-like peptide
DNA	Deoxyribonucleic acid
DR	Dietary Restrictrion
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl Methanesulfonate
ER	Ecdysone Receptor
EtOH	Ethanol
et al.	*Et alii*
E. coli	*Escherichia coli*
e.g.	*exempli gratia*
FC	Fold change
FOXO	Forkhead box protein
g	Gram
Gal4	Yeast transcription activator protein Gal4
GFP	Green Fluorescent Protein
GS	Gene-Switch
HCl	Hydrogen chloride
HNR	Hormone Nuclear Receptor
HSP	Heat Shock Protein
HSF	Heat Shock Factor
h	Hour
H_2O	Water
H_2O_2	Hydrogene peroxide
IGF	Insulin-like growth factor
IIS	Insulin/IGF-like signaling
InR	Insulin receptor
InR^{DN}	Insulin receptor dominant negative mutant
IRS	Insulin receptor substrate

Abbreviations

JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
Lnk	Link adaptor protein
LB	Lysogeny broth
M	Molar
mA	Milliampere
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHC	Myosin heavy chain
min	Minute
ml	Milliliter
mM	Milli molar
MNCs	Median neurosecretory cells
mRNA	Messenger ribonucleic acid
MS	Mass Spectometry
Mtk	Metchnikowin
u	Unit
µg	Microgram
µl	Microliter
µM	Micromolar
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
Na_2HPO_4	Disodium phophate
NaOH	Sodium hydroxide
ng	Nanogram
nt	Nucleotides
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
pH-value	Hydrogen ion concentration
PI3K	Phosphoinositid-3-Kinase
PKB	Protein Kinase B
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
qRT-PCR	Quantitative real-time reverse transcription-PCR
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
rpm	Rotations per minute
ROS	Reactive oxygen Species
RU486	Mifepristone
SDS	Sodium dodecyl sulfate
sec	Seconds
SIR	Sirtuin
SYA	Sugar yeast agar

S6K	S6 kinase
TAE	Tris-acetate-EDTA-buffer
TBE	Tris-borate-EDTA-buffer
TF	Transcription Factor
Tm	Melting temperature
TOR	Target of Rapamycin
Tris	2-amino-2-(hydroxymethyl)1,3-propandiol
Tubulin GS	Tubulin Gene-Switch
U	Units
UAS	Upstream Activation Sequence
UAS-*rpr*	UAS-*reaper*
UPS	Ubiquitin-Proteasome System
V	Volume
w^{Dah}	White Dahomey
WB	Western Blot
wt	Wild type
w/v	Weight per volume
2nd	Second
3rd	Third
20E	20-Hydroxyecdysone
°C	Degree celsius

Abstract

Hormone Nuclear Receptors are transcription factors that can respond to ligands, and modulate diverse biological functions, including development, metabolism, energy homeostasis, and reproduction. The Hormone Nuclear Receptor (HNR) DHR96 is a key regulator of the xenobiotic response, and is involved in cholesterol and Triacylglycerol metabolism. In humans, the Steroid X Receptor (SXR), the Constitutive Androstane Receptor (CAR) and the Vitamin D Receptor (VDR) are closely related to DHR96, and CAR and SXR share conserved function with DHR96 by modulating xenobiotic response and energy homeostasis.

In *C. elegans*, DAF-12 and NHR-8 are the DHR96 orthologs. Interestingly, DAF-12 is a key regulator of lifespan. The identification of the DAF-12 ligand, the dafachonic acids, and EMS generated *daf-12* mutants made a great contribution to DAF-12 longevity studies. In adult worms ligand-insensitive *daf-12* alleles are long-lived. Although DHR96 was shown to bind to cholesterol, the natural ligand for DHR96 is unknown, thus DHR96 is classified as an orphan nuclear receptor as is the case for most HNRs in *Drosophila*.

Interestingly, *dhr96* has been identified as a target gene of the transcription factor dFOXO (Forkhead bOX-containing protein, subfamily O), a key regulator in the insulin/insulin-like growth factor signalling (IIS) pathway. IIS signalling is a highly conserved, nutrient-responsive pathway, and reduced IIS signalling increases lifespan across species and retards many age-related phenotypes. Insulin signalling blocks dFOXO transcriptional activity by nuclear exclusion, whereas reduced IIS signalling induces dFOXO transcriptional regulation of target genes.

Reduced insulin signalling also affects fecundity, stress response, growth and metabolism. In *D. melanogaster*, extension of lifespan and increased xenobiotic resistance in IIS-reduced mutants are dependent upon dFOXO activity, while body size, fecundity and oxidative stress resistance are not affected. As many long-lived mutants are accompanied by an increased cytoprotective response,

we hypothesis that the improved ability to metabolize toxic compounds might lead to the dFOXO mediated lifespan extension. Indeed, RNA transcript profiles of long-lived IIS-reduced flies indicate that many genes involved in detoxification processes are up-regulated. Altered expression of detoxification genes has been also shown in long-lived IIS mouse and worm mutants.

According to the DHR96 function in xenobiotic response and the DAF-12 function in longevity, I investigated the role of DHR96 in ageing by using genetic tools in *Drosophila*. In addition, I studied the relationship between DHR96 and IIS signalling by epistatic analysis.

My results show that ubiquitous over-expression of wild type as well as ligand-insensitive *dhr96*, which carries the equivalent mutation as in ligand-insensitive *daf-12* mutants, extended lifespan, increased stress resistance, and reduced fecundity. Moreover, I could dissect a role of DHR96 in the xenobiotic response downstream of the IIS pathways, while lifespan effects were independent between DHR96 and IIS signalling.
I could also show that the innate immunity in long-lived *dhr96-lbd* over-expressing flies was suppressed. This topic is of major interest, as suppressive effects on immunity are linked to aging in *Drosophila*.

Kurzzusammenfassung

Kernrezeptoren sind Transkriptionsfaktoren, die durch die Bindung eines Liganden aktiviert werden und unterschiedlichste biologische Funktionen ausüben. Dazu gehören Entwicklung, Energie Homöostase, Stoffwechselprozesse und Reproduktion. Der Kernrezeptor DHR96 ist ein wichtiger Regulator im Prozess der Verstoffwechselung von Fremdstoffen (xenobiotischer Stoffwechsel). Des Weiteren ist DHR96 an Cholesterin und Triacylglycerin Stoffwechselvorgängen beteiligt. Im Menschen sind der Steroid X Rezeptor (SXR), der Konstitutive Androstane Rezeptor (CAR), und der Vitamin D Rezeptor (VDR) eng verwandt mit DHR96. Die Funktionen zwischen CAR, SXR und DHR96 sind konserviert, da diese den xenobiotischen Stoffwechsel und die Energie Homöostase modulieren.

In C. elegans sind DAF-12 und NHR-8 ortholog zu DHR96. Interessanterweise ist DAF-12 ein wichtiger Regulator im Alterungsprozess. Die Identifizierung des DAF-12 Liganden, den sogenannten Dafachronic Acid (DA), sowie EMS generierte *daf-12* Mutanten haben einen bedeutenden Beitrag zu DAF-12 Langlebigkeitsstudien geleistet. In adulten Würmern sind liganden-insensitive *daf-12* Mutante langlebig. Auch wenn Interaktionsstudien gezeigt haben, dass DHR96 an Cholesterin binden kann, ist ein natürlicher Ligand von DHR96 noch unbekannt. Daher wird dieser wie auch die meisten anderen *Drosophila* Kernrezeptoren als „Orphan“ klassifiziert.

Interessanterweise ist *dhr96* ein Ziel Gen des Transkriptionsfaktors dFOXO (Forkhead-Box-Proteine), welcher eine Schlüsselkomponente des Insulin/Insulin-ähnlichen Wachstum Faktor Signalweges (IIS) darstellt. Der IIS-Signalweg ist ein hochkonservierter Mechanismus, der durch die Ernährung moduliert wird. Eine reduzierte Signal Übertragung des IIS-Signalweges erhöht die Lebensspanne unterschiedlicher Spezies und verzögert zahlreiche Alterungsmerkmale. Die Insulin Signalfolge hemmt die transkriptionelle Aktivität des dFOXO Proteins, wohingegen ein reduzierter IIS-Signalweg die transkriptionelle dFOXO Regulation induziert.

Zusammenfassung

Eine reduzierte Insulinsignalübertragung wirkt sich ebenso auf Fortpflanzung, Stressresistenz, Wachstum und Stoffwechselprozesse aus. Bei Fliegen Mutanten mit reduzierten IIS sind die erhöhte Lebensspanne und eine verbesserte xenobiotische Verstoffwechselung Effekte welche die Aktivität des dFOXO Proteins erfordern. Dagegen sind Effekte in Körpergröße, Fruchtbarkeit und oxidative Stressresistenz nicht von dFOXO beeinflusst. Da viele langlebige Mutanten von einer verstärkten zellschützenden Fähigkeit begleitet werden, ist eine Hypothese, dass die verbesserte Leistung toxische Stoffe abzubauen zu einer verlängerter Lebensspanne führt. Tatsächlich weisen RNA Transkriptionsprofile von langlebigen IIS-reduzierten Fliegenmutanten eine Hochregulation von zahlreichen Genen auf, die im Entgiftungsprozess involviert sind. Eine alterierte Expression von Entgiftungsgenen wurde ebenso in Langlebigkeitsstudien von IIS genetisch veränderten Mäusen und Würmern nachgewiesen.

Aufgrund der Funktion des DHR96 Proteins in xenobiotischen Vorgängen und der Funktion des DAF-12 Proteins in der Regulation der Lebensdauer, habe ich unter Verwendung von genetischen Studien die Rolle von DHR96 im Alterungsprozess untersucht. Des Weiteren, habe ich den Zusammenhang zwischen DHR96 und dem IIS signalweg durch epistatische Analysen geprüft.
Meine Resultate zeigen, dass eine ubiquitäre Überexpression des Wildtyp *dhr96* sowie einer liganden-insensitive *dhr96* Form die Lebenspanne und Stressresistenz erhöht, die Reproduktion verringert, und sich auf die Immunität auswirkt. Außerdem konnte ich eine Rolle von DHR96 in xenobiotischen Reaktionen innerhalb des IIS Signalweges nachweisen, wohingegen die jeweiligen Effekte in der Lebensdauer keinen direkten Zusammenhang aufweisen.
Um die Regulation des DHR96 Proteins besser zu verstehen, habe ich in Kooperation mit Dan Magner und Shruti Chreti versucht den DHR96 Liganden zu identifizieren. Trotz der positiven Kontrolle, dass das DHR96 Liganden Sensor System funktioniert, war uns die Identifizierung des natürlichen Liganden nicht gelungen.

Chapter 1 General Introduction

1.1 Ageing

Ageing is a process that concerns almost all living organisms and is defined as a progressive decline in physiological function (Young, 1997). It is caused by an accumulation of internal damage and accompanied by a higher risk for diverse diseases like cancer, diabetes or neurodegenerative diseases (Lopez-Otin et al., 2013). Internal damage can arise from different sources and contribute cooperatively to ageing. Cellular and molecular phenomenon promoting ageing are reviewed in nine “ageing hallmarks” stemming from a broad range of studies in diverse organisms. Ageing hallmarks fulfil different criteria. (1) One criterion is to show a characteristic force during natural ageing, whereas the other two criteria include experimental modulation to (2) promote or ideally also to (3) delay ageing. The nine hallmarks include genomic instability, telomere attrition, epigenetic drift, defective proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al., 2013) (figure 1.1).

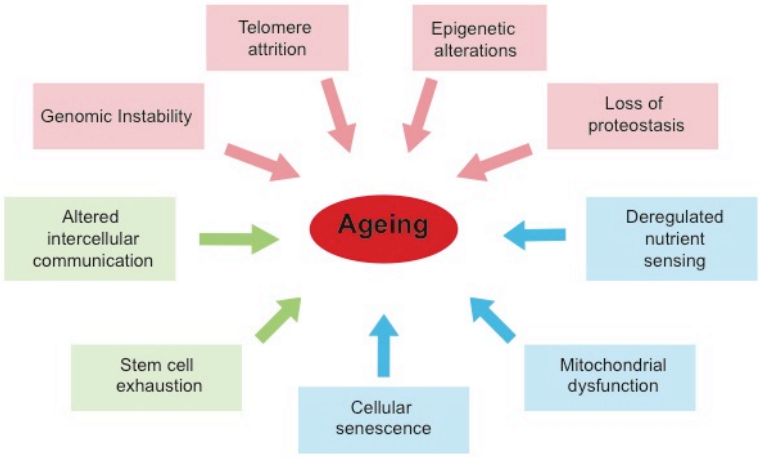


Figure 1.1 Ageing hallmarks (modified from Lopez-Otin et al. (2013)). Proposed ageing hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteosasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Primary hallmarks (red) have all negative effects, antagonistic hallmarks (blue) effects are dependent on their intensity, integrative hallmarks (green) are initiated by damages stemming from the other hallmarks.

(1) Damaging effects of nuclear DNA, mitochondrial DNA, or nuclear architecture induce genomic instability (Lopez-Otin et al., 2013). Deficits in DNA repair mechanisms, which cannot efficiently compensate for DNA mutations or alterations, shorten lifespan (Gregg et al., 2012). In contrast, experimental modulation providing correct chromosome segregation reveals beneficial effects for ageing and health (Baker et al., 2013).

(2) Telomere shortening occurs due to gradual DNA damage at the chromosome ends, and the lack of the enzyme telomerase in the somatic cells, which would be able to replicate these ends (Lopez-Otin et al., 2013). Experimental studies extending the length of telomere or inducing the activity of telomerase in mice increases lifespan (Bernardes de Jesus et al., 2012; Bodnar et al., 1998).

(3) Epigenetic alterations include histone modification, DNA methylation, chromatin remodelling or transcriptional remodelling (Lopez-Otin et al., 2013). One example of longevity modulators are the sirtuins that possess deacylase or mono-ADP-ribosyltransferase activity (Guarente, 2011). SIRT6 loss of function decreases lifespan, whereas induced activity increases lifespan in mice (Mostoslavsky et al., 2006).

(4) The word proteostasis arises from the words protein and homeostasis, and dysfunction in protein homeostasis is associated with numerous diseases (Hartl et al., 2011). Quality control mechanisms evolved to ensure proteostasis. Main components are chaperone proteins that provide correct protein folding and the degradation of misfolded proteins via proteasomal (ubiquitin-proteasome system, UPS) or lysosmal (chaperon-mediated autophagy) pathways to prevent proteotoxicity of aggregated proteins. These quality control systems decline with ageing, leading to many (neurodegenerative) disorders (Morimoto and Cuervo, 2014). Modulation of key regulators, such as of heat shock factor 1 (HSF-1), a transcription factor that induces the expression of heat shock proteins (*Hsps*) in response to stress, affects ageing. Over-expression of *Hsf-1* in worms extends lifespan (Hsu et al., 2003).

(5) Caloric intake and nutrition have a significant impact upon lifespan and a key nutrient responsive pathway is the insulin/insulin-like growth factor signalling (IIS) pathway (Ribaric, 2012). Downstream targets of this pathway are

FOXO (Forkhead bOX-containing protein, subfamily O) transcription factors and mTOR signalling. Reduced insulin signalling extends lifespan across species and can retard many age-related phenotypes (Bonkowski et al., 2006; Cohen et al., 2006; Giannakou and Partridge, 2007; Kenyon, 2005; Selman et al., 2008; Tatar et al., 2003; Wessells et al., 2009).

(6) Mitochondrial dysfunction includes effects from mitochondrial ROS, and reduced mitochondrial function (Lopez-Otin et al., 2013). The free radical theory postulated that age increases the production of mitochondrial free radicals, which cause damaging effects (HARMAN, 1992). Recent ROS studies propose contrary outcomes (Hekimi et al., 2011), showing lifespan beneficial effects for increased ROS levels in *C. elegans* and *S. cerevisae*, or showing no effects in mice where ROS levels were increased by genetic manipulation. New roles of ROS are proposed, in which ROS is induced in response to stress conditions to sustain survival, but excessively high levels might induce toxic effects (Lopez-Otin et al., 2013).

(7) Cellular senescence describes a type of cellular arrest and is more frequent in aged than in young mice. Telomere shortening, DNA damage of certain gene loci, mitotic, or oncogenic modifications can cause cellular senescence (Lopez-Otin et al., 2013). Prominent oncogenes are *p16* ^{INK4a} and *p53*, two tumour suppressors regulating cell cycle. *P16*^{INK4a} levels correlate with age, thus increased *P16*^{INK4a} levels prohibit ageing (Krishnamurthy et al., 2004). However, mild increases of *P16*^{INK4a} or *P53* levels shows anti-ageing effects (Matheu et al., 2009; 2007). Thus opposing effects might occur dependent on conditions.

(8) The potential of stem cells to regenerate declines with age, and can be caused by different damages such as DNA damage or telomere attrition. Mice with Progeroid syndromes – a disease mimicking ageing- show extended lifespan upon transplantation with muscle-derived stem cells from young mice (Lavasani et al., 2012).

(9) Intercellular communication of endocrine or neuronal signals alter with age, affecting a broad range of signalling pathways that might be accompanied with changes in the immune response, known as “inflammaging” (Salminen et al., 2012). Aged organisms show chronic inflammation and a deregulation of the

immune response that might emerge due to effects in other ageing hallmarks (e.g. stem cell exhaustion) (Lopez-Otin et al., 2013). Inhibition of chronic inflammation using pharmacological inhibitors that repress the activity of the immune response regulator NF-κB extends lifespan in *Drosophila* (Shaposhnikov et al., 2011). Tissue-tissue communication is another issue of this hallmark that can lead to indirect ageing effects (Lopez-Otin et al., 2013).

1.2 Insulin/insulin-like growth factor signalling (IIS) pathway

The IIS pathway is a nutrient responsive mechanism to maintain nutrient homeostasis, growth, and survival and is induced by insulin, which is secreted from ß-cells of the pancreas, in response to increased blood glucose (Saltiel and Kahn, 2001). Insulin induces the uptake of glucose in adipose tissue and muscle for use and storage, and blocks the production of glucose in the liver. Moreover, it promotes cell growth and differentiation.
Diabetes is becoming a chronic healthcare issue due to increasing obesity in developed, and increasingly, developing countries. Diabetes is a consequence of high blood sugar caused by the failure to produce insulin (Diabetes I) or by insulin resistance (Diabetes II) (Salsali and Nathan, 2006).

The transduction of insulin signalling is initiated by the binding of insulin to the alpha-subunit of the insulin receptor, a homodimeric tyrosine kinase (Patti and Kahn, 1998). Ligand binding results in auto phosphorylation of the IR beta-subunit, and subsequently in tyrosine phosphorylation of cellular proteins, including insulin receptor substrates 1-4 (IRS1, IRS2, IRS3, IRS4) (White, 1998), SHC (Src homology 2 domain containing protein), and CBL (Casitas b-lymphoma) (Pessin and Saltiel, 2000). Phosphorylated tyrosines of substrates display docking sites to the SRC homology 2 (SH2) domain of signalling partners, leading to the induction of diverse signalling cascade. Ras and MAP kinase signalling results in mitogenic and cell growth response (Boulton et al., 1991). Another cascade is initiated by phosphoinositide 3-kinase (PI3K) through its SRC homology 2 (SH2) domain (Myers et al., 1992). PI3K phosphorylates

the membrane phosphorinositides (PIP_2) that turns into phosphatidylinositol-3-phosphates (PIP_3). PIP_3 can induce multiple signals; among them it activates together with phosphoinositide-dependent kinase 1 (PDK1) the serine kinase PKB/Akt (Alessi, 2001; Alessi et al., 1997). Glycogen synthase kinases 3 (GSK-3), Forkhead family transcription factors (FOXO), and S6-kinase/mammalian target of rapamycin (mTOR) pathway are key downstream components of PKB/Akt and affect gene expression, glucose metabolism, and glycogen/lipid/protein synthesis (figure 1.2) (Nakae et al., 1999; Saltiel and Kahn, 2001) .

Akt inactivates glycogen synthase kinase 3 (GSK-3) by phosphorylation resulting in the induction of glycogen synthase and thus glycogen synthesis (Cross et al., 1995). Akt also induces GLUT-4 translocation to the cell membrane to enhance glucose uptake (Czech, 1995). Protein synthesis is up-regulated via mTOR/S6-kinase signalling (Marygold and Leevers, 2002). FOXO transcription factors regulate diverse mechanism like metabolism, cellular proliferation, stress response and apoptosis (Tzivion et al., 2011a).

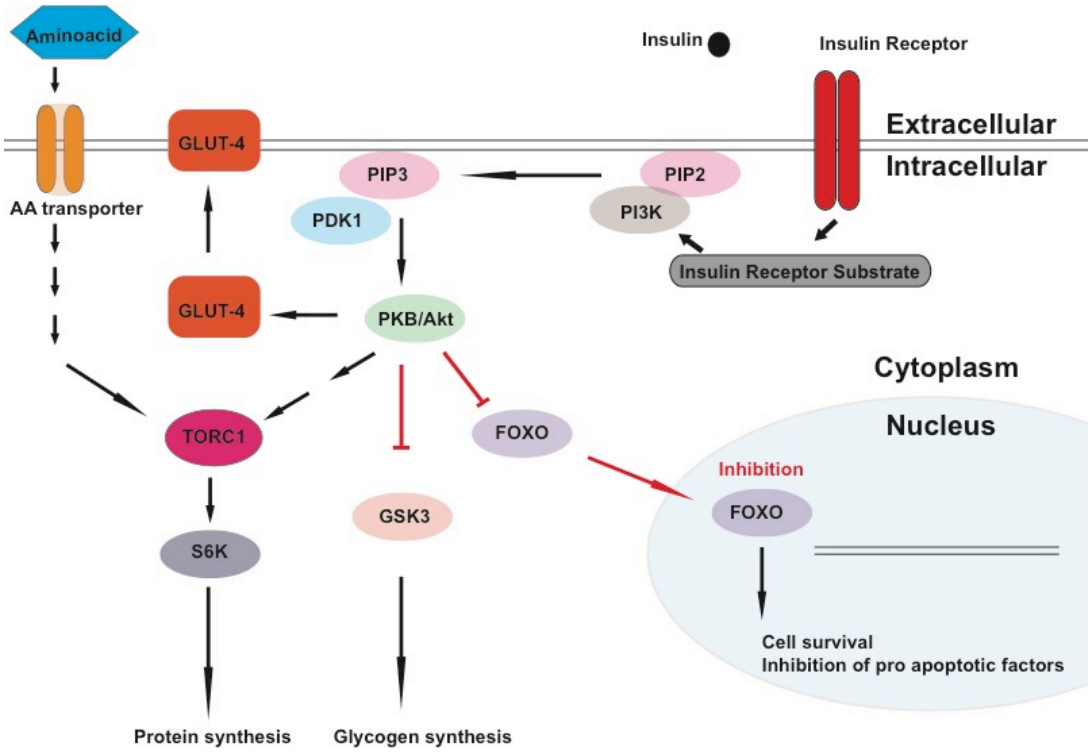


Figure 1.2 The IIS/TOR signalling pathway (modified from Saltiel and Kahn (2001) and Partridge et al. (2011)). Inulin initiates insulin signalling through auto phosphorylation of the insulin receptor. PKB cascade is induced by the insulin receptor substrate via PI3K, PIP3 and PDK1. PKB inhibits FOXO nuclear translocation and GSK3, resulting in glycogen synthesis and cell survival. Enhanced protein synthesis is mediated via TOR/S6K pathway.

The IIS pathway is highly conserved and shown to have an important impact on lifespan in different organism. Pioneering studies in *C. elegans* revealed that mutation of *age-1* and of the insulin receptor homolog *daf-2* increases lifespan (Kenyon et al., 1993). Further studies of IIS downstream components reducing insulin signalling enhanced an anti-ageing effect in different organisms (chapter 4) (Kenyon, 2010; Tatar et al., 2003).

Drosophila has seven insulin-like peptides (dILPs) (Brogiolo et al., 2001), and transduction of the insulin signalling is mediated through conserved homologous components, including the *Drosophila* insulin receptor, the insulin substrate homolog CHICO, *Drosophila* PDK1 and AKT (Giannakou and Partridge, 2007; Kenyon, 2010). A key downstream target of AKT is the transcription factor dFOXO, the homolog of mammalian FOXO1, FOXO3a, and FOXO4. Insulin signalling inhibits dFOXO transcriptional regulation by cytoplasmic translocation via 14-3-3 (Tzivion et al., 2011b).

Genetic manipulation of IIS components directed to reduce IIS signalling extends lifespan, reduces fecundity, increases stress response, and affects metabolism. Reduced IIS results in activation of dFOXO by phosphorylation and nuclear translocation (Kenyon, 2010). Indeed, over-expression of dFOXO in the muscle or fat body (equivalent to the mammalian liver) is sufficient to extend lifespan (Demontis and Perrimon, 2010; Giannakou et al., 2004; Hwangbo et al., 2004). Interestingly, increased xenobiotic response and longevity of IIS reduced flies are dependent upon dFOXO, whereas the other IIS reduced phenotypes are dFOXO independent effects (Slack et al., 2011). Thus, the question arose whether dFOXO enhancement of longevity is a result of a higher ability to clear toxins. Hence, downstream components regulating detoxification are of major interest to test this hypothesis in epistatic analysis.

The identification of the hormone nuclear receptor DHR96 as a direct target gene of dFOXO (Alic et al., 2011) led to the initiation of my thesis project. The interesting findings that DHR96 is a key regulator in xenobiotic response (King-Jones et al., 2006), and moreover that the DHR96 homolog DAF-12 is a key regulator in *C. elegans* longevity (Mooijaart et al., 2005) give this protein an important position according to IIS reduced signalling.

1.3 Hormone nuclear receptors

Hormone nuclear receptors (HNRs) act as transcription factors that directly regulate the expression of target genes in response to hormones. They are involved in diverse biological processes including reproduction, lifespan, diapause, detoxification and sex determination (Aranda and Pascual, 2001). The typical nuclear receptor structure, as shown in figure 1.3, is characterised by an N-terminal DNA-binding domain, a hinge region comprising the nuclear localisation signal, and a C-terminal ligand-binding domain (LBD) (Mangelsdorf and Evans, 1995). In addition to its function in ligand binding, the LBD induces dimerization, and for some it facilitates the recruitment of a chaperon-mediated complex whose dissociation is required for nuclear translocation.

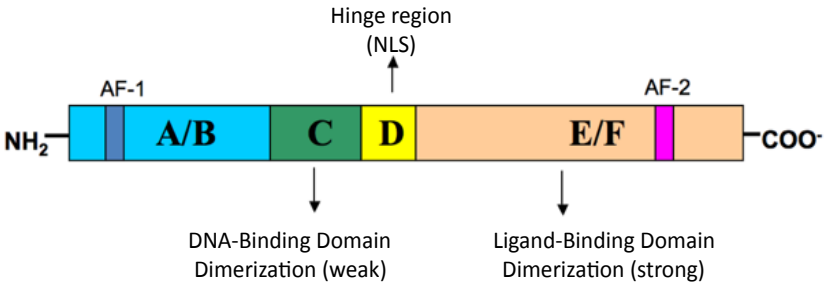


Figure 1.3 Nuclear Receptor (NR) Organisation. The structure of NRs are defined by a highly variable N-terminal A/B domain, a DNA-binding-domain containing two zinc fingers for specificity and weak dimerization, a hinge region harbouring the NLS region and the ligand-binding domain, which is required for ligand binding, dimerization and interaction to chaperones. Transactivation region AF-1 contributes ligand-independent regulation of the NR.

Ligands are mostly lipophilic hormones (e.g. sterols, bile acids and fatty acids) and can be generated via three different ways: (1) the active hormone is synthesized in a classical endocrine organ and enters the target cells, (2) the ligand is generated from a precursor or prohormone and is activated in the target cell, (3) the ligand may be a metabolite synthesized within the target cells (Aranda and Pascual, 2001). Localisation differs between nuclear receptors. Some are located in the cytoplasm, bound to the machinery of a chaperone complex. Ligand binding to the transcription factor induces conformational changes of the NR, leading to the dissociation from the complex, nuclear translocation, and the induction of target gene expression (Yamashita, 1998). Others are solely nuclear. Upon initiation of transcription, nuclear receptors

form multi-protein complexes, which target a specific DNA-sequences in target genes, known as the hormone response elements (Härd et al., 1990). Furthermore, many NRs are known to bind as homo- or heterodimers to the response elements (Forman and Samuels, 1990; King-Jones et al., 2006) (figure 1.4).

Ligand binding to HNRs is not always inductive for target gene expression; ligand binding to nuclear receptors can also repress transcript expression (Bodenner et al., 1991; Carr and Wong, 1994). However, a ligand-independent pathway is also possible for nuclear receptors. Activity may be regulated by other means, for example by phosphorylation (Kato et al., 1995; White et al., 1997).

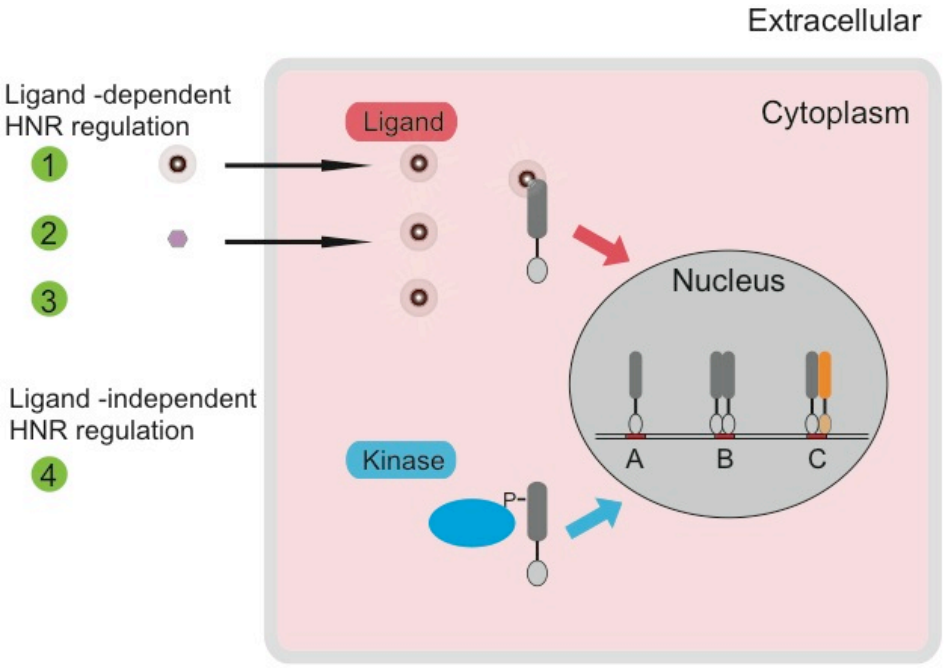


Figure 1.4 Regulation of Nuclear Receptor (NR) activity. NRs can be regulated in a (1-3) ligand-dependent or a (4) ligand-independent manner. The ligand can be synthesized in different ways: (1) it is synthesized in an endocrine organ and enters the target cell. (2) It enters the target cell as a pre-hormone, and has to be activated within the target cell. (3) It is generated within the target cell. (4) In a ligand-independent regulation, the Nuclear Receptor is regulated by posttranslational modification, for example by phosphorylation. Activated NRs bind to hormone response elements (HRE), and dependent on HRE they modulate expression of target genes in form of (A) monomers (B) homodimers(C) or heterodimers.

1.4 DHR96 in *Drosophila melanogaster*

Studies concerning the nuclear receptor DHR96 have mostly focused on developmental research, where DHR96 functions in Triacylglycerol homeostasis (TAG) and in cholesterol metabolism (Horner et al., 2009; Sieber and Thummel, 2009). TAG homeostasis plays a key role in fat metabolism, and TAG represents a fat intermediate that gets stored in adipose tissues in time of abundant nutrients (Lusis and Pajukanta, 2008). In contrast, starvation leads to the release of TAG in the form of fatty acids as an energy supply. In this context it has been shown that TAG levels in adipose tissues are reduced in *dhr96* null mutants, resulting in sensitivity to starvation. *Dhr96* over-expression has the contrary phenotype of increased TAG levels and starvation resistance (Sieber and Thummel, 2009).

Drosophila and *C. elegans* are not able to synthesize cholesterol, and their homeostasis is therefore completely dependent on food uptake (CLARK and BLOCK, 1959; Crowder et al., 2001). As cholesterol is a precursor of many ligands for hormone nuclear receptors, it is especially relevant in this context. A further important aspect is its critical role in health and disease, as defects in cholesterol homeostasis play a role in various diseases, for example in Alzheimer's and cardiovascular diseases (Tabas, 2002). During larval development it has been found that cholesterol uptake is affected in *dhr96* null mutants due to misregulation of *npc1b*, an ortholog of mammalian NPC1L1 (Niemann-Pick C1-like 1). As a result, *dhr96* null mutants show an accumulation of cholesterol when exposed to a high cholesterol diet. A low cholesterol level in the medium leads to decreased survival rate of the mutants (Horner et al., 2009). Furthermore, it has been suggested that a critical low cholesterol level induces activation of DHR96, as a protective mechanism to cholesterol deficiency (Bujold et al., 2010).

In the adult stage, DHR96 functions in cholesterol and TAG homeostasis via the *Drosophila* LipA homolog *magro* and *npc1b* (Sieber and Thummel, 2009; 2012). *Magro* is highly expressed in the midgut (intestine), where it is secreted into the intestinal lumen to digest dietary triacylglycerol. In the *dhr96* null mutant *magro* is down-regulated, thus dietary TAG and cholesterol esters cannot be hydrolysed, and accumulate in the lumen (Sieber and Thummel, 2012).

In addition, in the adult stage DHR96 is known to regulate the xenobiotic response (King-Jones et al., 2006). This topic will be introduced in chapter 3.

1.5 Project aims

That IIS pathway is involved in lifespan regulation has been show in different organisms, but how this effect occurs is not clear. In *C. elegans*, cytoprotective mechanisms represent important factors for the lifespan extension in IIS-reduced worms. Indeed, many other long-lived animals also exhibit an increased cytoprotective response.
In *Drosophila*, effects of reduced-IIS on xenobiotic response and lifespan are dependent upon dFOXO activity, a downstream key regulator of the IIS pathway. The question arose whether improved detoxification might confer longevity in IIS-reduced flies. Interestingly, *dhr96* is a target gene of dFOXO and might represent an interesting candidate for xenobiotic response and longevity.

As DHR96 is involved in xenobiotic metabolism, and studies from the *dhr96* homolog *daf-12* in *C. elegans* revealed an important role in ageing, the first approach was directed to investigate the role of this hormone nuclear receptor in lifespan and in life history traits. The second approach included epistatic analysis between the IIS network and DHR96 to address the question of whether DHR96 might mediate IIS phenotypes. A further aim was to identify possible downstream targets that have relevance for DHR96 phenotypes.
In addition, the known DAF-12 ligand, dafachronic acid, has facilitated different DAF-12 studies, whereas the ligand of DHR96 and its regulation is not known. Thus, we attempted to identify the DHR96 ligand (s).

These studies are summarized in different chapters with the following structure:
(1) Chapter 3 covers studies on *dhr96* transgenic flies and their effects upon life history traits.
(2) Epistatic analysis between DHR96 and the IIS network are reported in Chapter 4.
(3) Chapter 5 includes studies on DHR96 downstream components.

(4) Results from chapter 5 initiated immunological studies on *dhr96* transgenic flies.

(5) DHR96 ligand identification is the topic of the last chapter 7.

Chapter 2 Materials and methods

2.1 Nucleic acid-based methods

2.1.1 Cloning

2.1.1.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used as a technique to amplify target DNA using specific primer pairs. All primers were synthesised by the company Biomers, purified by HPLC, and dissolved in nuclease-free water to a final concentration of 100 pmol/µl. A complete oligonucleotide primer list used in this thesis is attached in supplement S.2.1.

Pfu Polymerase (Promega) was used for PCR reactions requiring high fidelity. The standard protocol for *Pfu* DNA Polymerase-mediated PCR is summarized in table 2.1.1. The Recommended thermal cycling condition is described in table 2.1.2. The annealing temperature is dependent on primer pair properties and was adapted to 5°C below the lowest melting temperature of each used primer pair.

Component	**Final concentration**	**Volume**
Pfu Buffer with MgSO4 (10x)	1x	5 µl
dNTP (10mM)	200 µM	5µl
5’ primer (5 pmol/µl)	0,5 µM	5 µl
3’ primer (5 pmol/µl)	0,5 µM	5 µl
Template DNA	150 ng	2-5 µl
Pfu Polymerase (2–3u/µl)	2–3u	1 µl
H_2O_{dd}		to 50 µl

Table 2.1.1 Standard protocol for PCR reaction using *Pfu* DNA polymerase (Promega).

Step	Temperature	Time	Cycle number
1. Initial Denaturation	95 °C	1 minute	1
2. Denaturation	95 °C	1 minute	35
Annealing	42-65 °C *	45 seconds	
Extension	72 °C	1 minute per kb	
3. Final extension	72 °C	10 minutes	1
4. Final hold	4 °C		

Table 2.1.2 Thermo cycler settings for PCR reaction. * Annealing temperature was adapted 5°C below melting temperature of primer pairs.

For genotyping purposes, PCR reactions were performed using HotStarTaq *Plus* DNA Polymerase (Qiagen) (table 2.1.3) and carried out with the following PCR program, shown in table 2.1.4.

Component	**Final concentration**	**Volume**
HotStarTaq Plus Master Mix (2x)	1x	10 µl
5' primer (5 pmol/µl)	0,5 µM	2 µl
3' primer (5 pmol/µl)	0,5 µM	2 µl
Template DNA	150 ng	2-5 µl
H_2O_{dd}		to 20 µl

Table 2.1.3 PCR reactions using HotStarTaq Plus DNA Polymerase (Qiagen).

Step	Temperature	Time	Cycle number
1. Initial Denaturation	95 °C	5 minute	1
2. Denaturation	95 °C	30 seconds	35
Annealing	42-65 °C *	30 seconds	
Extension	72 °C	1 minute per kb	
3. Final extension	72 °C	10 minutes	1
4. Final hold	4 °C	-	-

Table 2.1.4 Thermo cycler settings for HotStarTaq Plus DNA Polymerase mediated PCR reaction. * Annealing temperature was adapted 5°C below melting temperature of primer pairs.

2.1.1.2 In-Fusion® HD cloning

The In-Fusion HD Cloning Kit allows fast, directional insertion of DNA fragments. Primers were designed for the gene of interest by using the GENtle program, but with an extension of 10-15 base pairs, which were complementary to the ends of the linearized vector. Insert was amplified by PCR, the vector linearized by digestion (see chapter 2.1.1.3), and both products were isolated by gel extraction (see chapter 2.1.1.4). To obtain the concentration of the purified DNA fragments, 2 µl of each sample was tested on an agarose gel next to an appropriate DNA ladder with known concentration. The In-Fusion reaction was set up as shown in table 2.1.5.

Component	Volume
In-Fusion HD Enzyme Premix	2 µl
Linearized vector	x µl (50-100 ng)
Insert	x µl (10-200ng)*
H_2O_{dd}	To 10 µl

Table 2.1.5 InFusion reaction. * Volume was dependent on the size of the insert: <0.5 kb: 10-50ng, 0.5 to 10kb: 50-100ng.

The reaction was induced for 15 minutes at 50°C, and subsequently cooled on ice. DNA were transformed into bacteria as described below, and resulting colonies were tested by restriction digestion on an agarose gel. Where the fragmentation pattern was positive, samples were sequenced with 100% coverage to ensure fidelity.

2.1.1.3 Restriction digestion

To prepare DNA for cloning or analysis, DNA was digested by restriction enzymes (New England BioLabs, NEB), special endonucleases isolated from bacteria, which cleave DNA at specific sites. Digestions of vectors or control restriction digestion were conducted as shown in table 2.1.6.

Component	Volume
DNA (200ng-1µg)	2-10 µl
10x NEB Buffer	5 µl
Enzyme(s)	1 µl (each)
H_2O_{dd}	50 µl

Table 2.1.6 Digestion of DNA by restriction enzymes (NEB).

Corresponding NEB Buffers to each restriction enzyme, and if required BSA were used according to the manufacturers' instructions (NEB). Reactions were incubated at 37 °C for 1 hour.

2.1.1.4 Agarose gel electrophoresis and plasmid purification

DNA fragments were separated by length by running linear DNA (PCR products, digested plasmids) on a 1% TAE agarose gel (supplement S.1). To visualize DNA, the gel was stained with 0.5 µg/ml Ethidium Bromide (Sigma-Aldrich) or SYBR®-Safe (1:10.000, Life Technologies). Samples were mixed with 6x DNA loading dye buffer (thermo scientific), which contained the marker bromophenole blue. Dependent on the size range of the DNA fragments, different ladders were used to determine DNA size. The electrophoretic separation of DNA was performed at 80-120 V in 1x TAE buffer. DNA was visualized with UV-light and results were imaged by a gel documentation system (G-BOX, Syngene).

For cloning or sequencing, DNA fragments of interest were excised from the gel with a sterile scalpel or razor blade. The DNA was subsequently purified from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.1.1.5 Transformation

In-Fusion ligation samples were transformed into XL2-Blue MRF' Ultracompetent (Stratagene) strains. Alternatively E. coli XL1-Blue competent cells (Stratagene) were used. To amplify plasmids, transformation in chemically competent One Shot TOP 10 (Invitrogen) was performed. A 100µl aliquot of or XL2-Blue or XL1-blue bacteria was thawed on ice for 10 min. 5, 10 and 15 µl of

the InFusion ligation was added to 100µl aliquots of cells and gently mixed. For the transformation into One Shot TOP 10 bacteria, 100ng plasmid was used in a 50µl aliquot of cells. After an incubation time of 30 minutes, cells were heat shocked for 30 seconds in a 42 °C water bath, and immediately placed back on ice for 2 minutes. 500 µl pre-warmed S.O.C medium was added to the cells and samples were incubated at 37°C for 60 minutes at 225 rpm in a thermomixer (Eppendorf). 50µl of the culture was spread on pre-warmed LB-plates (Lysogeny broth, supplement S.1) containing the appropriate antibiotic (ampicillin, kanamycin, 50-100 µg/ml). The plates were incubated at 37°C over night and single colonies were used for mini- or maxipreparation next day.

2.1.1.6 Mini- and maxipreparation of plasmid DNA

The minipreparation is a method to purify small amounts of DNA from *E.coli* cultures. Single colonies were inoculated in 2 ml selective LB-media, which contained the appropriate antibiotic, and incubated at 37 °C and 225 rpm in a thermomixer overnight. Bacteria were transferred to microcentrifuge tubes and pelleted at 8000g for 1 minute at 4 °C. To obtain DNA of high purity, the QIAprep Spin Miniprep Kit (Quiagen) was used according to the manufactures' protocol, and DNA was eluted in 50µl nuclease-free water.

Maxipreparation ensures isolation of higher DNA amounts. 2 ml starter cultures, as prepared for minipreparation, were amplified in 200-400ml selective LB-media at 37°C and 225 rpm in an incubator shaker (New Brunswick Scientific) overnight. Bacterial cultures were harvested at 4°C and 6000g for 15 minutes (JA10.5000 Rotor, Beckmann centrifuge). DNA was purified by using the QIAGEN Plasmid Maxi Kit and following the manufacturer's instruction. Plasmid DNA was eluted in 1 ml nuclease-free water.

2.1.2 RNA analysis

2.1.2.1 RNA extraction

RNA was extracted from *Drosophila* whole bodies and specific tissues to quantify gene expression. Tissues and bodies, which were stored at -80 or in liquid nitrogen until RNA extraction, were transferred to FastPrep™ Lysing Matrix tubes (MP Biomedical) and homogenized in 700 µl Trizol Lysis Reagent (Invitrogen) using the FastPrep®-24 instrument (MP Biomedical) at maximum speed for 20 seconds. Each sample was mixed with 200µl chloroform and incubated at room temperature for 3 minutes. After centrifuging for 15 minutes at 13500g at 4 °C, the upper clear phase was transferred into nuclease free tubes. RNA was precipitated by adding 1 volume of isopropanol and 1/10 volume of 3M NaOAC, incubated at -80 °C for 40 minutes and pelleted by 10.000 g at 4°C for 10 minutes. The RNA pellet was washed three times with ice-cold nuclease free 70% ethanol. To remove ethanol residue after the last washing step, pellets were air-dried. RNA was suspended in 20µl nuclease-free water.

2.1.2.2 DNase treatment

TURBO DNA-free™ Kit (Ambion) was used to remove genomic DNA contamination following manufacturer's instruction. RNA concentrations and RNA quality were measured by using the NanoDrop 2000c Spectrophotometer (PEQLAB Biotechnologie GmbH) or the Eppendorf BioPhotometer plus instrument. Samples were diluted to an end concentration of 500 ng/µl

2.1.2.3 cDNA synthesis

The synthesis of cDNA from RNA was performed using the SuperScript® VILO™ cDNA Synthesis Kit, according to manufacturer's instructions. Reactions were set up as shown in table 2.1.7, gently mixed and incubated first at 25 °C for 10 minutes, then at 42 °C for 60 minutes. The reactions were terminated by incubation at 85 °C for 5 minutes.

Components	Volume (20µl)	Master mix (for 15 rxn)
5X VILO™ Reaction Mix	4 µl	60 µl
10X SuperScript® Enzyme Mix	2 µl	30 µl
RNA (up to 2.5 µg)	4 µl	-
DEPC-treated water	10 µl	150 µl

Table 2.1.7 VILO reaction to synthesise cDNA from extracted RNA samples.

2.1.2.4 Quantitative real-time PCR (qRT-PCR)

To quantify mRNA level of a gene of interest in a specific tissue or in the whole body, qRT-PCR analysis were performed by using the TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems). TaqMan primers were provided by Applied Biosystems. A detailed list of used real-time primers is attached in supplement S.2.2. PCR reactions for each cDNA sample were conducted according to the protocol of the company and are summarized in table 2.1.8. Components were pipetted by a JANUS pipetting robot on a 384 well plate. Depending on the number of candidates and cDNA samples, master mixes were prepared. Each cDNA-Primer sample was tested in quadruplets.

Component	Volume
Primer	0,5 µl
cDNA	1 µl
TaqMan® Master Mix	5 µl
Nuclease-free water	3,5 µl

Table 2.1.8 Reaction for qRT-PCR using TaqMan® Universal PCR Master Mix and TaqMan probes.

The PCR program as shown in table 2.1.9 was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Step	Temperature	Time	Cycle number
1. Initial Denaturation	95 °C	10 minutes	1
2. Combined annealing and extension step	95 °C	15 seconds	40
	60 °C	1 minute	

Table 2.1.9 7900HT Fast Real-Time PCR setting for qRT-PCR reaction

2.1.2.5 Data analysis and statistics of qRT-PCR data

Data was evaluated by using sequence detection system's software 2.3 (Applied Biosystems). Data were validated using the ΔΔCt method and normalized to different internal controls (Rpl32, Actin, α-Tubulin). Student's *t*-test was used to evaluate significance of data.

2.2 Protein-based methods

2.2.1 Protein extraction

For proteomic analysis, 10 day old flies were collected in microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C. Protein from at least 15 whole flies of each genotype was extracted by using RIPA buffer ingredients with 0.1% SDS. Flies from each genotype were homogenised in 350µl lysis buffer with EDTA free protease inhibitor mix (7x concentration, Roche) by using a pestle and motor mixer (VWR international). Samples were incubated for 30 minutes on ice and centrifuged for 10 minutes at 10.000 g at 4 °C. Supernatant was transferred to new microcentrifuge tubes and protein concentration was measured subsequently by using bradford assay.

2.2.2 Bradford assay

A BSA standard curve, in a range of 0.25 to 10 µg/µl was used to calibrate extracted samples. 2µl of standard, a blank and sample was mixed each with 200 µl bradford dye solution (BioRad). Measurement was performed with the Infinite M200 photometer plate reader (Tecan) by an absorption at 595 nm. The concentration of extracts was calculated in excel by adjusting to the calibration

curve. Samples were mixed with SDS-sample lamelli buffer (Biomol) to a concentration of 3 µg/µl and heated at 95 °C for 5 minutes.

2.2.3 SDS Gel electrophoresis

Proteins were size-separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), using 12% Mini-PROTEAN® TGX™ Gels with 15 wells and a capacity of 15 µl per well (BioRad). Separation was conducted with 30µg each sample at 100 V for about 1 hours in a Tris/Glycin/SDS buffer (BioRad). To determine the size of proteins the Precision Plus Protein™ Dual Colour Standard ladder (BioRad) was used.

2.2.4 Western Blot

Samples were transferred from the SDS gel to a PVDF membrane (Amersham Hybond™–P, GE Healthcare) with a Trans Blot instrument (BioRad) in a TRIS/Glycine/Methanol Buffer (BioRad) following the manufacturer's instructions. Protein transfer was controlled by staining the membrane with Ponceau S (Serva). Blots were blocked with a 5% non-fat milk (Roth)/TBST buffer for one hour at room temperature. Membranes were incubated with primary antibodies over night at 4 °C on a shaker. Used antibodies and sera were diluted in 5% milk/TBST buffer and are listed in table 2.2.1.

Antibody	Dilution	Company	Host
Monoclonal Anti-α-Tubulin	1: 50.000	Sigma-Aldrich	Mouse
Anti-actin	1: 50.000	Sigma-Aldrich	Rabbit
Monoclonal anti-FLAG M2	1: 10.000	Sigma-Aldrich	Mouse
Anti-DHR96 serum	1:500	Janne Toivonen / Eurogentec	Rabbit

Table 2.2.1 Antibody list

Membranes were rinsed three times with TBST for 10 minutes, before incubating them with an appropriate second antibody (Invitrogen) for an hour at

room temperature. Secondary antibodies were diluted 1: 50.000 in a 5% non-fat milk buffer. After washing blots again three times with TBST for 10 minutes, membranes were developed with a standard ECL WB detection kit or a ECL plus WB detection kit (GE Healthcare) if increased sensitivity was required. Both reagents were used according to the protocol of the company and visualised with either a Luminescent Image Analyzer (Fujifilm) or a developer (AGFA, Curix60).

2.3 Standard methods for *Drosophila melanogaster*

2.3.1 *Drosophila melanogaster* strains

The white Dahomey *wolbachia* plus (w^{Dah} w^{+}) fly strain was used as a wild type stock. These flies contain the intracellular bacterium *Wolbachia.* All *Drosophila* lines were backcrossed for 8-10 generations into w^{Dah} w^{+} background. Fly strains are listed in table 2.2.2.

Wild type, balancer and mutant flies		
Fly strain	**Reference**	**Details**
White Dahomey *wolbachia* plus (w^{Dah} w^{+})	(Grönke et al., 2010)	Wild type *Drosophila* stock
w^{Dah} w^{+}, *CyO*	Bloomington *Drosophila* Stock Center	Balancer fly on the 2nd Chromosome, homozygous lethal, Curly wings
w^{Dah} w^{+}, *TM3Sb*	Bloomington *Drosophila* Stock Center	Balancer fly on the 3rd, Chromosome, homozygous lethal
w^{Dah} w^{+}, *CyO*, *TM3Sb*	Sonita Afschar	Double balancer on the 2nd and 3rd Chromosome
w^{Dah} w^{+}, $dfoxo^{\Delta 94}$	(Slack et al., 2011)	*dfoxo* null mutation on the 3rd Chromosome
w^{Dah} w^{+}, $dhr96^{\Delta}$	(King-Jones et al., 2006)	*dhr96* null mutation on the 3rd Chromosome

GAL4 driver lines		
Fly strain	**Reference**	**Details**
w^{Dah} w^{+}; *daughterless* Gene-Switch	(Tricoire et al., 2009)	Ubiquitous expression of GAL4, Chromosome 2
w^{Dah} w^{+}; *tubulin* Gene-Switch	(Fernandez-Ayala et al., 2009)	Ubiquitous expression of GAL4, Chromosome 2
w^{Dah} w^{+}; *mhc*-GAL4	Bloomington *Drosophila* Stock Center	Muscle-specific driver, Chromosome 3
w^{Dah} w^{+}; *daughterless* Gene-Switch; *dfoxo* $^{\Delta 94}$	Sonita Afschar	Ubiquitous driver in a *dfoxo* null background
w^{Dah} w^{+}; *mhc*-Gal4/ *dhr96* $^{\Delta}$	Sonita Afschar	Muscle-specific driver in a *dhr96* null background
w^{Dah} w^{+}; *dilp2*-GAL4	(Broughton et al., 2010)	MNC-specific driver (median neurosecretory cell)
UAS-responder lines		
Fly strain	**Reference**	**Details**
w^{Dah} w^{+}; UAS-*dhr96*-R539C-D2	J. Toivonen, Lab collaboration	LBD-mutated UAS-*hr96* line on Chromosome 3
w^{Dah} w^{+}; UAS-*dhr96*-WT-9M	J. Toivonen, Lab collaboration	Wild type UAS-*dhr96* line on Chromosome 2
w^{Dah} w^{+}; UAS-*dhr96*-R539C-D2; *dfoxo* $^{\Delta 94}$	M. Piper (UCL, London), Lab collaboration	LBD-mutated UAS-*dhr96* line in a *dfoxo* null background
w^{Dah} w^{+}; UAS-*dhr96*-WT-9M; *dfoxo* $^{\Delta 94}$	M. Piper (UCL, London), Lab collaboration	Wild type UAS-*hr96* line in a *dfoxo* null background
w^{Dah} w^{+}; UAS-*dfoxo*	(Giannakou, 2004)	*dfoxo* inserted through attp40 sites into the 2nd Chromosome
w^{Dah} w^{+}; UAS-*dfoxo*; *dhr96*$^{\Delta}$	Sonita Afschar	UAS-*dfoxo* in a *dhr96* null background
w^{Dah} w^{+}; UAS-*rpr*;	(Broughton et al., 2010)	UAS-*reaper*
w^{Dah} w^{+}; UAS-*reaper*; *dhr96*$^{\Delta}$	Sonita Afschar	UAS-reaper in a *dhr96* null background

Table 2.2.2 *Drosophila* strains.

2.3.2 *Drosophila melanogaster* food media

General fly stocks were kept on a standard sugar/yeast/agar medium (1x SYA medium) (Ashburner, 1989). Lifespan experiments were also performed on SYA medium with a higher yeast concentration (1.5x SYA medium). The ingredients are summarized in table 2.2.3. Agar was boiled in distilled water first, then sugar and yeast were added, and mixture was re-boiled while stirring it. The media was cooled to 55°C and two anti-fungal reagents (nipagin and propionic acid) were added.

Components	1 x SY-Medium	1.5 x SY-Medium
Distilled water (ml)	700	700
Agar (g)	15	15
Sugar (g)	50	50
Yeast (g)	100	150
Water to add at the end (ml)	170	144
Nipagin (ml)	30	30
Propionic acid (ml)	3	3

Table 2.2.3 Recipe for 1 liter *Drosophila* food media.

Fly experiments with wild type flies, mutants or transgene flies using a constitutive driver were performed on 1x SYA medium in glass vials. Fly experiments with the need of an inducible driver (chapter 2.3.5) were arranged on 1.5x SYA medium in plastic vials (9.5 cm x 2.5 cm diameter) containing the drug Mifepristone (RU-486, Sigma-Aldrich, solved in ethanol) or ethanol.

2.3.3 Fly stock maintenance

Flies were kept in glass bottles (13.5 cm x 6 cm diameter) on a standard 1x SYA medium in a controlled temperature (CT) room with a 12:12 light:dark cycle, 65% humidity and a temperature of 18°C for general stocks and 25 °C for experiments.

2.3.4 Crossings and egg collection

Drosophila melanogaster flies are virgins in the first 6 hours at 25 °C or first 16 hours at 18 °C after they enclose (Ashburner, 1989). Therefore, bottles were cleared of flies, and virgins were collected within this time window. Females and males were separated on a gas pad under carbon dioxide anaesthesia treatment using a Binocular (Leica). Genders were distinguished by size and sex-specific structures. Crossings were performed in glass bottles on 1x SYA food at room 25°C over night. To standard larval density and age of experimental flies, parental flies were transferred to plastic cages with grape plates smeared with fresh yeast paste (S.I.Lesaffre). After an egg-laying time of 12 hours, eggs were washed in PBS from grape plates into falcon tubes and were allowed to settle by gravity. Supernatant was removed and 20µl of compact egg/PBS solutions were dispensed in glass bottles, resulting in between 300-350 eggs per bottle (Clancy and Kennington 2001).

2.3.5 The GAL4-UAS system

The GAL4-UAS system is a powerful genetic tool, which allows the expression of a gene of interest in *Drosophila melanogaster* (Brand and Perrimon, 1993).
The binary system, shown in figure 2.1, requires two fly lines: A driver line which harbours the yeast transcription factor *gal4* under the control of an enhancer, and a UAS-responder line, which carries the gene of interest under the control of a UAS-promoter, a GAL4-binding site. Crossing these fly lines results in progeny containing both factors. The GAL4 protein binds to the UAS-promoter site, which induces the expression of the gene of interest. Dependent on the enhancer, expression can be directed in the whole body, or in specific tissues. Ubiquitous driver lines can differ in their expression pattern.

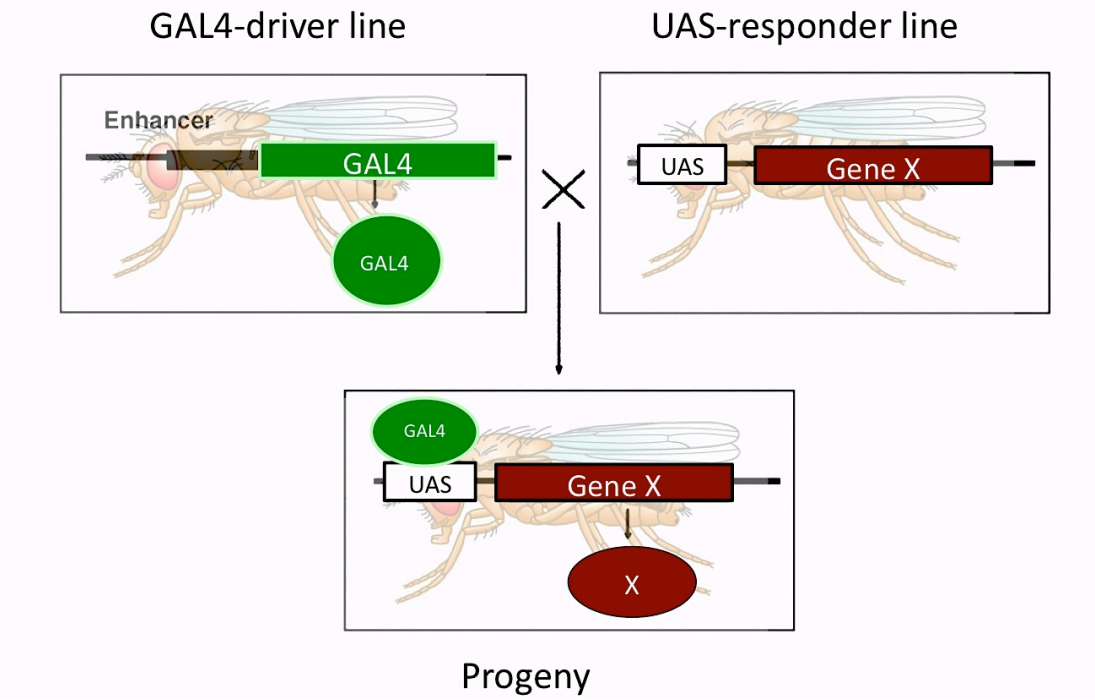


Figure 2.1 GAL4-UAS system. The GAL4-UAS system allows expression of a gene of interest in whole bodies or specific tissues. A GAL4-driver line that express *gal4* (a transcription factor in yeast) and a UAS-responder line that harbours the gene of interest under a UAS promoter are required for the GAL4-UAS system. Progeny contains both components, resulting in the expression of the gene of interest by the GAL4 transcription factor.

The Gene-Switch GAL4-UAS system is an inducible system and allows expression in a time dependent manner (Osterwalder et al., 2001) (figure 2.2). It consists of GAL4-progesterone receptor fusion protein, whose transcriptional activity is dependent on the steroid RU486 (mifepristone, Sigma-Aldrich). This drug was dissolved in ethanol and added to the food media to an end concentration of 200μM. As a control, the same volume of ethanol alone was added to the food media.

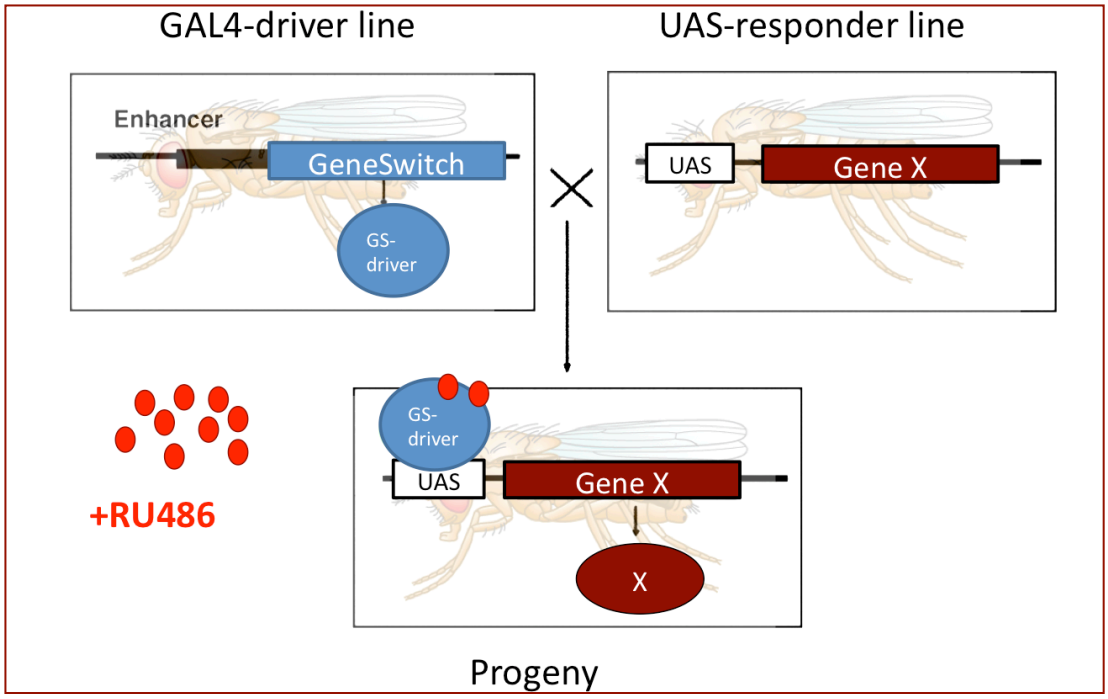


Figure 2.2 The Gene-Switch (GS) GAL4-UAS system. This system allows the expression of a target gene (Gene X) in a time and tissue dependent manner. The drug RU486 induces activity of the GAL4 GS fusion transcription factor.

Chapter 3 DHR96 regulates life history traits in *Drosophila melanogaster*

3.1 Introduction

The worm homologue of DHR96, DAF-12, is a key regulator of longevity. In *Drosophila*, *dhr96* null flies are characterized by a short lifespan (Janne Toivonen, unpublished data) and decreased xenobiotic resistance (King-Jones et al., 2006). Interestingly, *dhr96* has been identified as a target gene of the transcription factor dFOXO (Alic et al., 2011), which is a key regulator in the IIS pathway and modulates lifespan in *Drosophila.* Thus I hypothesised that DHR96 might play a role in regulation of lifespan, possibly downstream of the IIS pathway.

The goal of the work reported in this chapter was to investigate if over-expression of *dhr96* could increase lifespan and resistance to xenobiotics in *Drosophila*. A main aim was to discover if there is evolutionary conservation of the molecular and functional roles of DHR96 and the *C. elegans* homologue DAF-12. Ligand-insensitive *daf-12* mutants extended lifespan (Fisher and Lithgow, 2006), and I therefore investigated the effects of over-expression for both wild type (*dhr96*) and a putative ligand-insensitive mutation version of *dhr96* (*dhr96-lbd*).

3.1.1 DHR96 is a key regulator of xenobiotic response in *Drosophila*

The xenobiotic pathway is a protective system in almost all organisms, and essential for organismal survival (Jakoby and Ziegler, 1990). Xenobiotics are chemical compounds from the environment, such as drugs, poison or pollutants, and possibly endogenously generated lipophilic toxins (Niwa et al., 2009). Xenobiotic metabolism leads to the neutralisation and clearance of toxins by detoxification enzymes (Jakoby and Ziegler, 1990).

The detoxification process is divided into three phases: (I) In the first phase, enzymes modify xenobiotic substrates by the insertion of a polar or reactive group. The most well-known group catalysing these reactions is the cytochrome P450 family. (II) In the second step, modified substrates are conjugated with anionic molecules. The most prominent group here is the family of the glutathione S-transferase (GST) (Jakoby and Ziegler, 1990) (Liska, 1998). (III) Because of the negative charge, transporter proteins can bind xenobiotic conjugates and excrete them out of the cell (Commandeur et al., 1995).

In the adult stage, DHR96 mediates the xenobiotic response by regulating the expression of detoxification enzymes. Although *dhr96* null mutants are fertile and viable, they show an elevated sensitivity to the xenobiotics phenobarbital and DDT (King-Jones et al., 2006). Phenobarbital is a xenobiotic drug that induces the expression of numerous detoxification genes (Waxman, 1999; Zelko and Negishi, 2000). In *dhr96* null mutants many of these detoxification genes are misregulated (King-Jones et al., 2006). Accordingly, at the third larval stage, DHR96 protein is detected predominantly in tissues that function in metabolizing xenobiotic compounds including the Malphigian tubules, fat body, salivary glands and gastric caeca of the midgut (King-Jones et al., 2006). *Dhr96* mRNA levels in adult *Drosophila* flies are elevated in the gut, tubules and the ovary, whereas *dhr96* is expressed at lower levels in the adult fat body (Chintapalli et al., 2007).

Also in other model organisms, mutant animals were used to study xenobiotic function of *dhr96* homologues. In the mammalian system, the DHR96 homologs CAR and PXR are well known as xenobiotic receptors, regulating expression of cytochrome genes in response to xenobiotic drugs like phenobarbital (Wei et al., 2002; Xie et al., 2000b). CAR and PXR deficient animals were used to investigate their function in the xenobiotic response, and to identify cytochrome target genes (Wei et al., 2002) (Willson and Kliewer, 2002).
In *C. elegans, daf-12*, *nhr-8* and *nhr-48* are the *dhr96* orthologues. NHR-8 overlaps in its function with DHR96 to regulate the xenobiotic response. LBD-deficient *nhr-8* mutants were characterized as sensitive to chloroquine and colchicinen (Lindblom et al., 2001). Moreover, long-lived *daf-12* mutants reveal

also increased stress resistance (Fisher and Lithgow, 2006). In deed, many long-lived animals show improved xenobiotic resistance (chapter 4.1.3 Xenobiotic response and Ageing). Hence, xenobiotic metabolism displays an important issue, as DHR96 is a key regulator in detoxification metabolism and possibly involved in ageing.

3.1.2 Life history traits and adult longevity in *C. elegans*

DAF-12, the *C. elegans* DHR96 homologue, regulates various life history traits, including developmental timing, arrest at the larval diapause, and adult longevity (Antebi et al., 2000; Gerisch et al., 2007; Bethke et al., 2009). The identification of the DAF-12 ligand, the dafachronic acids (Motola et al., 2006), has made a decisive contribution to understanding how DAF-12 activity affects life history traits and adult longevity.

Biosynthesis of DAF-12 ligand in *C. elegans* is dependent on environmental conditions, and its binding to DAF-12 is responsible for the switch between dauer formation and reproductive growth during larval development (figure 3.1.1) (Antebi et al., 2000; Gerisch et al., 2007a). In unfavourable situations (e.g., stress, starvation, overcrowding), reduced IIS and TGF-ß signalling result in complete or partial inhibition of DAF-12 ligand production in the cell, and DAF-12 remains in a ligand-unbound state. Under these conditions the nuclear receptor is associated to its co-repressor DIN-1 and leads to dauer diapause, a long-lived and stress resistant stage (long life history). In contrast favourable conditions activate IIS and TGF-ß signalling, that in turn induce biosynthesis of the dafachronic acids (Gerisch et al., 2007a; Riddle et al., 1981). There are two key regulators involved in DAF-12 ligand production, the Rieske-like oxygenase/DAF-36, which acts in the first step of cholesterol modification into 7-dehydrocholesterol, and DAF-9, which catalyses the last step of the dafachronic acid synthesis (Jia et al., 2002; Motola et al., 2006; Wollam et al., 2011). Ligand binding activates DAF-12 via conformational changes that subsequently induce the expression of genes that promote reproductive development and increased ageing (short life history).

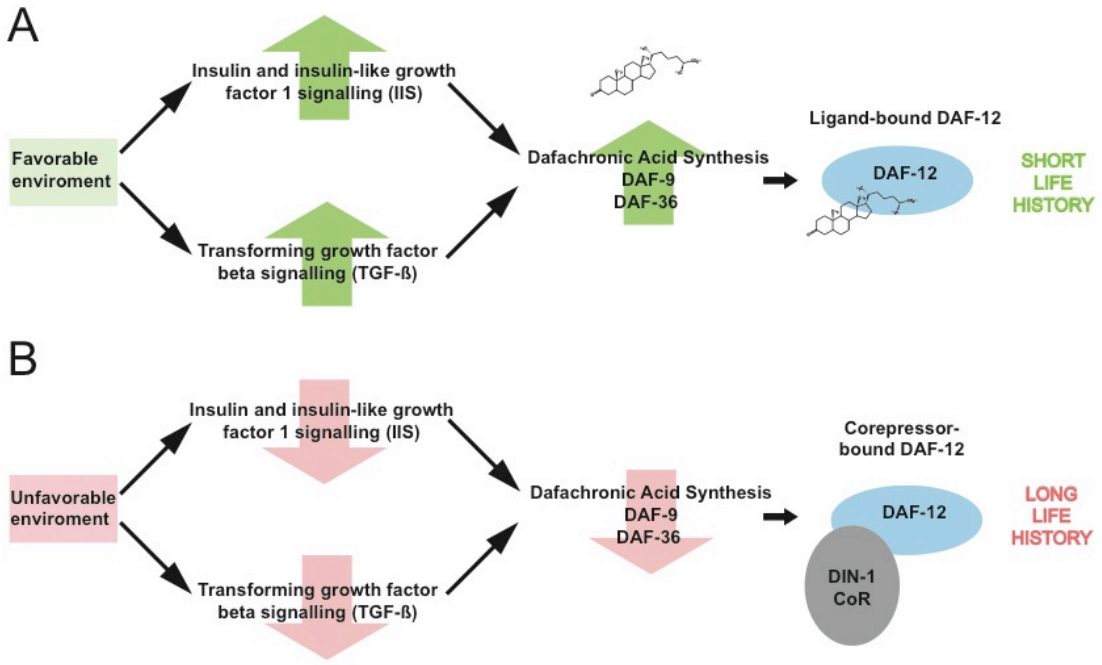


Figure 3.1.1 DAF-12 regulation of dauer longevity by (A) favourable or (B) unfavourable environment. Modified from Rottiers and Antebi (2006). (A) A favourable environment promotes dafachronic acid (DA) production via DAF-36 and DAF-9. DA binding to DAF-12 induces expression of genes that promote reproductive development and short life. (B) In the absence of the ligand, DAF-12 is bound to the co-repressor DIN-1, inducing stress resistance and longevity.

In the adult stage, signals from the germ line are involved in the regulation of adult longevity (figure 3.1.2). The *C. elegans* gonad consists of four precursor cells, Z1-Z4. The germ line emerges from Z2 and Z3, whereas somatic gonad arises from Z1 and Z4 cells. Removal of germ stem cells result in a 30-60% life span extension (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999). This effect is not caused by sterility of the worms, as ablation of all four precursor cells does not affect lifespan (Kenyon et al., 1993). Only the ablation of the germ line precursor (Z2 and Z3) enhances lifespan (Hsin and Kenyon, 1999). Transcriptional activity of DAF-12 and DAF-16 are required for the increase in lifespan of germ line ablated worms (Hsin and Kenyon, 1999). Here, DAF-12 mediated longevity requires ligand, as shown by the dependency upon DAF-36 and DAF-9 (Gerisch et al., 2007a; Rottiers et al., 2006).

In wild type worms, DAF-9 activity is inhibited in the somatic gonad by signals from the germ line. As a consequence, DAF-12 signalling is repressed due to diminished ligand synthesis in the somatic gonad. However, in long-lived animals with an ablated germ line, DAF-9 repression is abrogated, and synthesised dafachronic acid induces DAF-12 activity. In parallel, ablation of the germ line induces DAF-16 transcriptional activity by nuclear translocation in the intestine. Both transcription factors, DAF-12 and DAF-16, contribute to the lifespan extension in these animals.

Although DAF-16 and DAF-12 signalling are modulated in an independent manner, the pathways involved are suggested to be co-dependent for the lifespan extension of worms with ablated germ line (Berman and Kenyon, 2006).

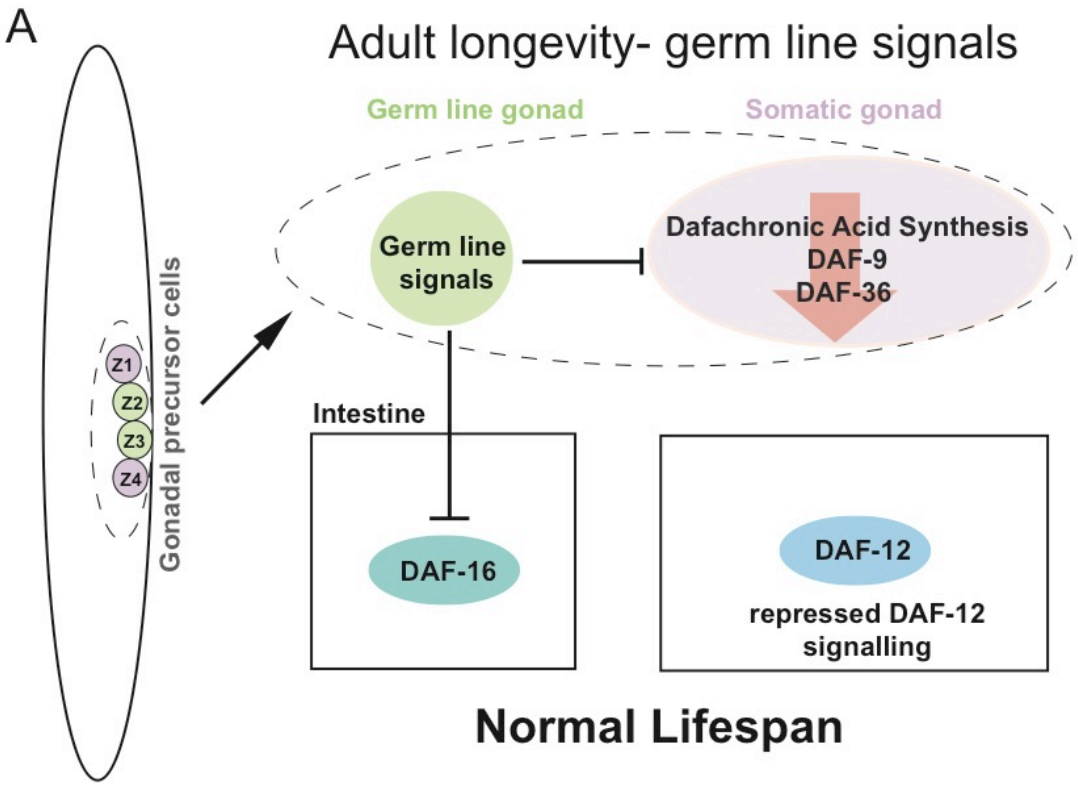


Figure 3.1.2 Germ line signals regulate adult longevity. Description see next page.

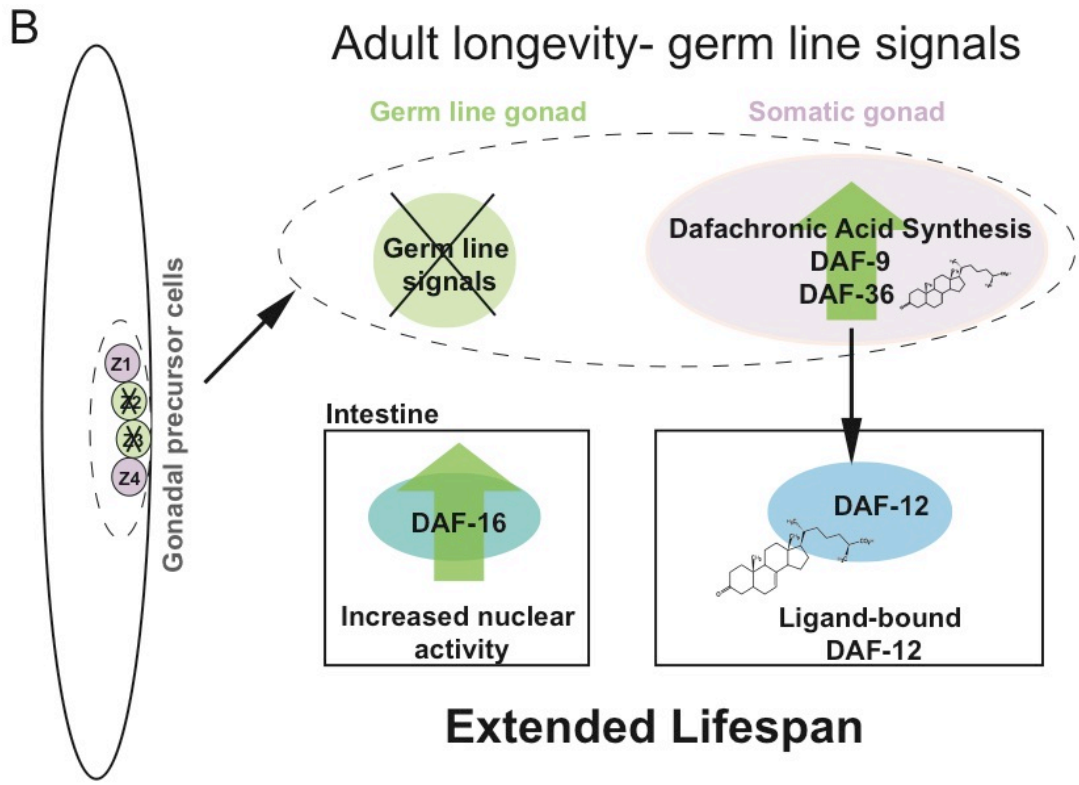


Figure 3.1.2 Germ line signals regulate adult longevity. (A) In wild type worms signals from the germ line inhibit the synthesis of DAF-12 ligand, the dafachronic acids (DA), and DAF-16 signalling. (B) Ablation of the germ line gonad extends adult longevity by induced DA synthesis and activation of DAF-12. Furthermore inhibitory effect on DAF-16 signalling is abrogated; thus DAF-16 induced activation promotes to an enhanced lifespan.

Although germ line longevity is enhanced by ligand-bound DAF-12, a mutation in the ligand-binding domain, which interrupts the ligand interaction, extends lifespan and increases resistance to oxidative stress (Fisher and Lithgow, 2006). *Daf-9* null mutants show a similar phenotype to the DAF-12 ligand-insensitive mutant, which is described as a weak constitutive partial dauer phenotype *daf-c* (Gerisch et al., 2001; Riddle et al., 1981). Larval *daf-9* null worms survive to give rise to adults that are long-lived (Gerisch et al., 2001; Jia et al., 2002). However, this effect is dependent upon DAF-12 and its co-repressor DIN-1 (Ludewig et al, 2004). Remarkably, hypomorphic mutations of *daf-9* and *daf-36*, in contrast, can decrease lifespan, suggesting that regulation is highly complex (Gerisch et al., 2007b).

Furthermore, adult longevity regulated by DAF-12 signalling is dependent on temperature. Although liganded DAF-12 enhances lifespan in the germ line ablated worms, unliganded DAF-12 activity together with the co-repressor DIN-

1S increases longevity at 15°C (Gerisch et al., 2007a). However, at 20°C unliganded DAF-12 worms are short-lived.

Drosophila DHR96 shares characteristics with DAF-12. Worms with predicted *daf-12* null-mutations are short-lived and show the *daf-d* phenotype, a stage in which worms are unable to enter the long-lived dauer larvae form (Riddle et al., 1981).

3.1.3 *Daf-12* mutants

In worms, *daf-12* mutants were characterized for lifespan traits. Interestingly, exchange of a single amino acid in the ligand-binding domain (R539C) of *daf-12*, which is predicted to interrupt binding (Fisher and Lithgow, 2006), results in a lifespan extension. As different *daf-12* alleles cause opposing phenotypes, it is essential to introduce DAF-12 and *daf-12* mutants.

For wild type *daf-12*, three different isoforms has been identified (12A1, 12A3 and 12B), modulating different nuclear receptor functions. Two of them contain DNA- and ligand-binding domains, whereas one only contains a LBD (isoform 12B) (Antebi et al., 2000). The expression pattern of a *daf-12* fusion construct with *gfp* (Chalfie et al., 1994), shows that it is present in tissues, including epidermis, vulva, somatic gonad, intestine, pharynx, sex myoblasts, as well as in tissues with no known DAF-12 role, including nervous system and body wall muscle. Its expression is detected throughout lifespan, but decreased transcript levels are reported in the larval dauer (Antebi et al., 2000). It is suggested that isoforms A1 and A2 are required for dauer formation in certain tissues or at specified time points, whereas the B isoform might have an autoregulatory function (Snow et al).

Mutants of *daf-12* generated by ethylmethanesulfonate (EMS) mutagenesis lead to various dauer phenotypes, thus a constellation of *daf-12* alleles in six classes, shown in supplement S.3.1, has been established (Antebi et al., 1998). Class 6, which includes the mutation in the ligand-binding domain (R539C), show a daf-constitutive phenotype and an inhibition of gonadal heterochrony

(Antebi et al., 2000). The affected amino acid of the R539C mutant is located in Helix 3 of the ligand-binding domain (12 helices in all). Comparison with related mammalian nuclear receptors with known LBD crystal structure revealed strong conservation of helices 3-5, 8 and 12 in the LBD (Antebi et al., 2000).

Remarkably, the double mutant allele *rh61rh411,* containing the R539C mutation, and in addition a mutation in the DNA-binding domain, results in mutants characterized as short-lived and show an opposing phenotype (Fisher and Lithgow, 2006). This suggests that lifespan extension of ligand-insensitive-mutants is dependent on subsequent regulation of target gene function, which is attenuated, when the DNA-binding function is affected.

Alignments of the *daf-12*-DBD to DBDs of other related nuclear receptor identified *nhr-8* (59%) and *nhr-48* (79%) as the closest *C. elegans* nuclear receptors (Sludder et al, 1999), and *dhr96* (63%), vertebrate *PXR* (57%) and *VitD* receptor (52%) as the closest orthologous DBD within the nuclear receptor family (Antebi et al., 2000). *Drosophila dhr96*, *C. elegans daf-12*, *nhr-8* and *nhr-48* share a sequence of 13 residues, which comprises the ESCKAFFR helix (Luisi et al, 1991; Schwabe et al, 1993). This feature functions to contact hormone response elements (HRE), which are short DNA binding sites in the genome. Due to the conserved sequence, HRE might be equal or similar between the NRs. The related mammalian NRs differ in the DNA recognition site (EGCKG), suggesting that the ESCKA family evolved after the metazoan radiation.

Alignments of the LBD sequences show that *dhr96* (40%), *nhr8* (36%), *Drosophila* ecdysone receptor (34%), and vertebrate thyroid hormone receptor (31%) are most closely related to *daf-12* LBD (Antebi et al, 2000). The residue responsible for life span extension, is located in helix 3. This helix together with helices 4-5,8 and 12 are regions with the highest conservation. Although it is known that nuclear receptors can act in a ligand-independent manner, the reported similarities in the LBD, and the fact that for many DHR96 orthologues ligands are already identified, enhance the likelihood that DHR96 activity is modulated by a ligand.

Based on the DAF-12 studies in *C. elegans*, two UAS-lines were used for the experimental approach in *Drosophila*, one which contains the wild type *dhr96*,

and one which contains the LBD-mutated *dhr96* with the *daf-12* equivalent mutation (R539C) (alignment see supplement S.3.2).

3.2 Materials and methods

3.2.1 *Drosophila* stocks

Two UAS-HR96 lines, generated by Janne Toivonen (UCL, London), were used for experiments. The first transgene line harbours the *dhr96* wild type construct on the second chromosome, integrated randomly by P-element insertion. The second transgene contains a UAS-HR96-R539C construct on the third chromosome, which is mutated in the ligand-binding domain. The mutation is equivalent to the *C. elegans* long-lived *daf-12 rh274* line, which is ligand-insensitive (Fisher and Lithgow, 2006). The amino acid that is exchanged in this transgene, is conserved between *dhr96*, *daf-12* and *nhr-8*. Thus, UAS-HR96-R539C might represent a ligand-insensitive construct. Also here, the P-element was integrated randomly. Both UAS-lines and driver lines (*daughterless* GS and *tubulin* GS) were backcrossed for 10 generation into a white Dahomey *Wolbachia* plus (w^{Dah} w^{+}) background. The cytoplasmic transmission of the *Wolbachia* bacteria occurs through female flies. Thus, the first crossing was done between w^{Dah} w^{+} virgin females and males of the fly lines of interest. All the following crossings were conducted between UAS- or driver virgins with w^{Dah} w^{+} males. For each backcross ≥30 virgins were mated with ≥30 male flies. To obtain homozygous fly lines, flies were crossed with the appropriate backcrossed balancer: CyO for lines containing an insert on the second chromosome and TM3Sb for transgenes on the third chromosome.

3.2.2 Lifespan assay

150 virgins were crossed with 80 male flies in glass bottles containing 1x SYA food, supplemented with live yeast (S.I.Lesaffre). The next day, flies were transferred to plastic cages to lay eggs on grape plates, supplemented with yeast paste, over night. Eggs were squirted into new bottles to ensure standard density of larvae (see chapter 2.3.4). After 8 days, bottles were emptied in the evening and 16 hours after clearing newly eclosed flies were transferred to fresh 1x SYA food and incubated at 25°C for 2 days to mate. 200 experimental flies per crossing were sorted under carbon dioxide anaesthesia treatment into

narrow plastic vials containing 1.5x SYA food with the drug RU486 or an equivalent amount of ethanol. Gas treatment was standardised, with a maximum treatment time of 4 minutes. Flies were separated by sex and 10 female or male flies were allocated per vial. The sorting day was classified as day 0 of the lifespan experiment.

All fly cultures were kept at 25 °C on a 12:12 light:dark cycle and 65 % humidity. Experimental flies were transferred to new vials three times per week and dead flies were counted.

3.2.3 Stress assays

Flies for stress assay analysis were prepared the same way as for lifespan experiments. At least 120 flies from each crossing were sorted according to their gender into wide plastic vials. 20 flies were placed per vial containing ethanol or the drug RU486 and flipped three times a week. Flies were assayed for stress resistance at the age of 10 days.

Drugs were supplemented to 1.5x SYA food after cooling it to 55°C. Fresh stress media was used for experiments, with a volume of 6ml per vial.

3.2.4 DDT and phenobarbital assay

DDT (Dichlordiphenyltrichlorethan; Greyhound) was dissolved in 100% ethanol at 275mg per litre 1.5x SYA food was used for experiments. Phenobarbital was dissolved in ethanol and prepared in the food to an end concentration of 5% (w/v).

Because of the toxicity of drugs, dispensing was performed under the hood. Flies exposed to drugs were not tipped into new vials as flies died within a few days and no progeny developed. Dead flies were counted every 4-8 hours during the day.

3.2.5 Hydrogen peroxide (H_2O_2) assay

H_2O_2 (Sigma Aldrich) was not prepared in SYA media due to indications that a component in the SYA food quenches the effect of this drug (unpublished data). 5% H_2O_2 was cooked in 1% agar.

3.2.6 Starvation assay

For starvation analysis, media without yeast and sugar was cooked. 1% agar (Sigma Aldrich) was boiled in water and dispensed into vials once it cooled below 60°C.

3.2.7 Fecundity assay

Fecundity from the same 5 vials of lifespan flies was measured at different time points throughout the lifespan. Female flies were exposed to new food to lay eggs for 16 hours. After removing them, the number of eggs was counted by using a hand counter under a microscope. Vials which were not analysed immediately were stored at -20°C and processed at a later date. Fecundity was measured by the number of eggs produced by female *Drosophila* flies in 16h and data display the cumulative eggs laid per female fly. Significance of data was tested by Wilcoxon rank test.

3.3 Results

3.3.1 Over-expression of *dhr96* and *dhr96-lbd* extended lifespan, increased stress resistance, and reduced fecundity in *Drosophila* females

3.3.1.1 Over-expression of *dhr96* in adult *Drosophila* flies using a *daughterless* GS driver

Constitutive over-expression of *dhr96* and *dhr96-lbd* in the whole body by using a *daughterless* driver resulted in lethality during development (Janne Toivonen, see figure 3.3.1). To avoid developmental lethality and facilitate investigation of ageing, studies on *dhr96* by the inducible Gene-Switch (GS) system were performed.

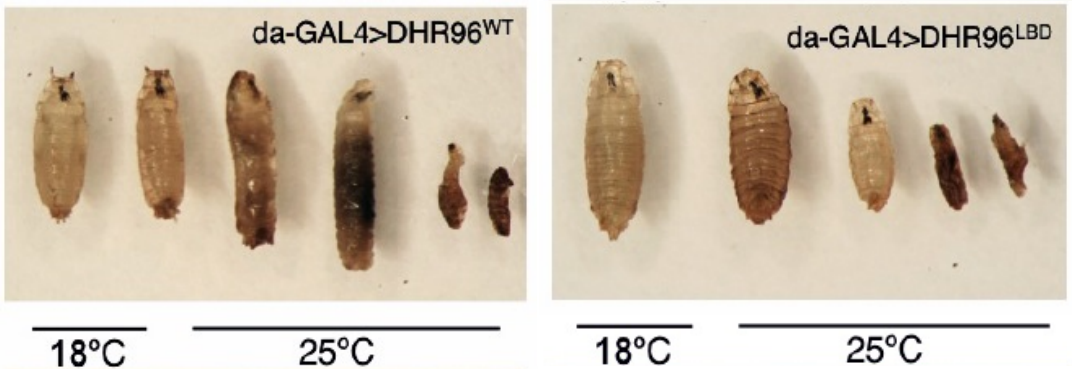


Figure 3.3.1 Constitutive over-expression of *dhr96* in the whole body caused developmental lethality. Experiment was performed by Janne Toivonen (UCL, London). Over-expression of (A) *dhr96* ($DHR96^{WT}$) or (B) *dhr96lbd* ($DHR96^{LBD}$) by using the *daughterless*-Gal4 driver revealed lethality in different stages of *Drosophila* development, and few survivors. Lower expression level at 18°C were increased for survival during the development.

For functional analysis in adult flies, *dhr96* and *dhr96-lbd* were over-expressed using the *daughterless* GS driver. Experimental flies used for qRT-PCR, lifespan, stress, and fecundity analysis (see below) stem from the same parental crossings.

Transcript levels of *dhr96* were controlled by qRT-PCR and revealed significant *dhr96* induction in both *dhr96* and *dhr96-lbd* over-expressing flies (figure 3.3.2,

p-value ***<0.001, Student's *t*-test). *Dhr96* transcript levels were induced by about 10-fold in *dhr96* over-expressor, and about 13-fold in *dhr96-lbd* over-expressing flies.

Wild type *dhr96* over-expressor showed a higher induction compared to ligand-mutated *dhr96* over-expressor. However, this difference was not significant (P-value: 0.07).

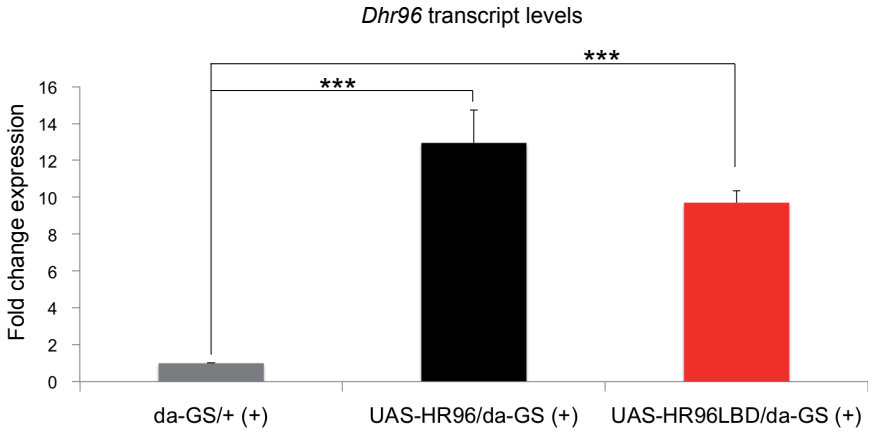


Figure 3.3.2 *Dhr96* transcript levels in *dhr96* and *dhr96-lbd* over-expressing females. Ubiquitous over-expression of wild type *dhr96* (UAS-HR96) or ligand-insensitive *dhr96* (UAS-HR96LBD) significantly induced *dhr96* expression (p-value ***<0.001, Student's *t*-test). *Dhr96* transcript levels were induced by 10-fold in wild type *dhr96* over-expressor, and by 12.5-fold in *dhr96-lbd* over-expressor.

3.3.1.2 Over-expression of *dhr96* and *dhr96-lbd* using a *daughterless* GS driver extended lifespan of *Drosophila* females

Over-expression of *dhr96* (UAS-HR96) or LBD-mutated *dhr96* (UAS-HR96LBD) by using the *daughterless* Gene-Switch driver (da-GS) extended longevity significantly for both transgene lines in female flies (figure 3.3.3 A, p-value ***<0.001, Log Rank Test). Median lifespan was increased by 15,97% and maximum lifespan by 9,4% in *dhr96* over-expressing flies, when compared to its non-induced ethanol control. *Dhr96-lbd* over-expression extended median lifespan by 20,3% and maximum lifespan by 13,3%. There was also no significant difference between *dhr96* and *dhr96-lbd* over-expressing flies.

There was no significant effect on lifespan between UAS- and driver controls on ethanol and on RU (figure 3.3.3 A and B).

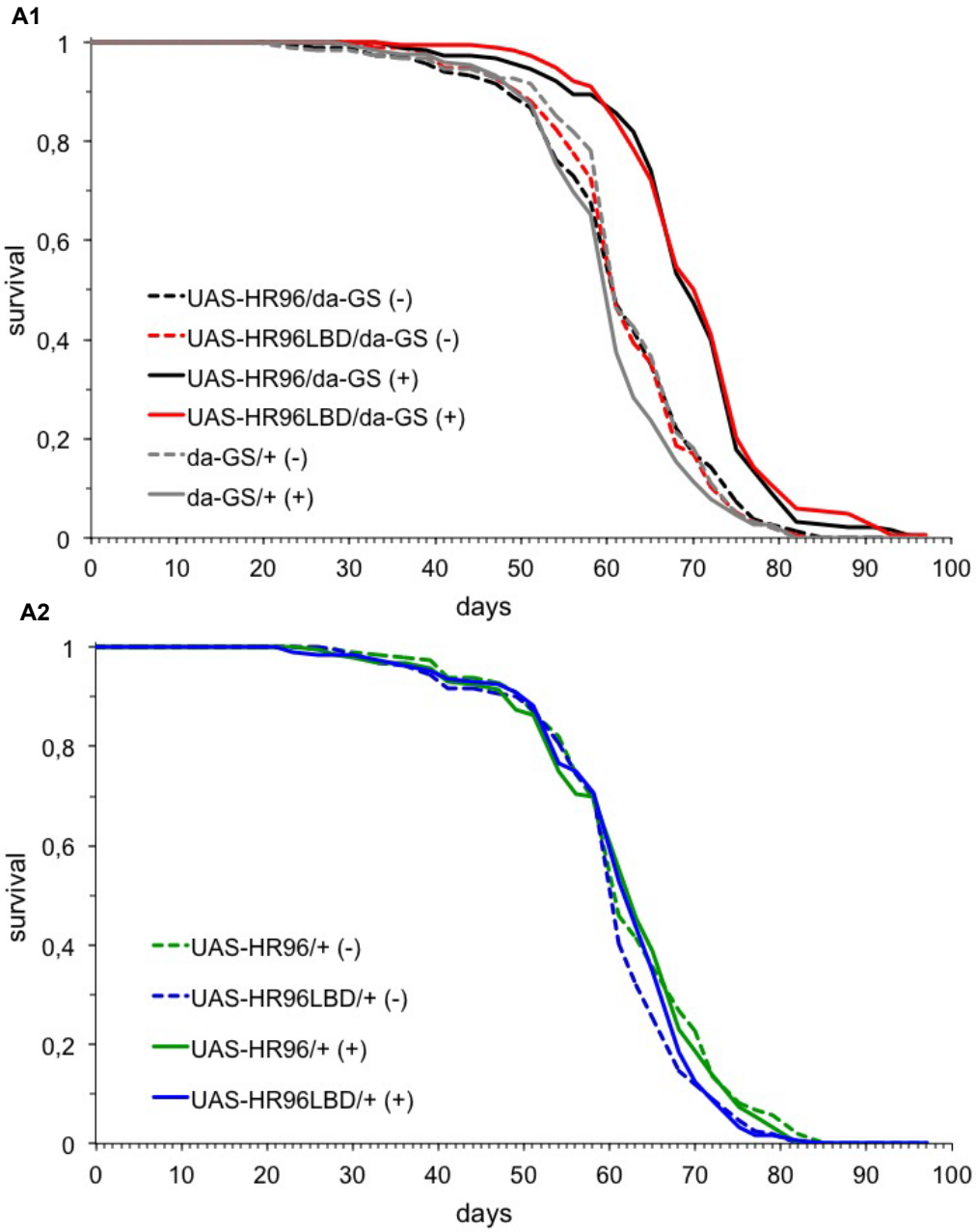


Figure 3.3.3 *Dhr96* over-expression extends *D. melanogaster* lifespan. (A1) Ubiquitous over-expression of wild type *dhr96* (UAS-HR96) or ligand-insensitive *dhr96* (UAS-HR96LBD) significantly extended lifespan (Log Rank Test, p-value ***<0.001). Expression was induced in the adult stage by using the *daughterless* Gene-Switch system (da-GS). RU feeding, marked as (+), induces gene expression. Control vials contained equivalent concentrations of ethanol (-). (A2) UAS-control flies on ethanol and RU did not show any effect on lifespan.

3.3.1.3 Over-expression of *dhr96* and *dhr96-lbd* using a *daughterless* GS driver reduced fecundity in adult *Drosophila* females

Fecundity was significantly reduced in *dhr96* and *dhr96-lbd* over-expressing flies compared to non-induced ethanol controls, or to driver controls (figure 3.3.4) (p-value ***<0.001, Kruskal-Wallis test, Dunn's test for multiple comparisons). The reduction in fecundity in the first and second time point (7

and 10 days) was significantly greater for *dhr96* over-expressing flies than for LBD-mutated *dhr96* over-expressing flies (p-value ***<0.001, Kruskal-Wallis test).

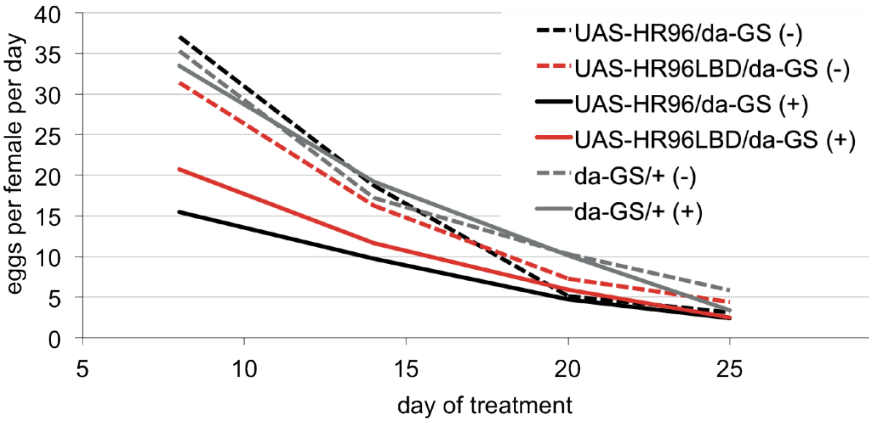


Figure 3.3.4 *Dhr96* or *dhr96-lbd* over-expression reduces fecundity. Driver control did not show any effect on fecundity when exposed to RU, whereas both over-expressor lines (UAS-HR96/da-GS (+) and UAS-HR96LBD/da-GS (+)) showed significantly reduced egg laying (p-value ***<0.001, Kruskal-Wallis test, Dunn's test for multiple comparisons).

3.3.1.4 Over-expression of *dhr96* and *dhr96-lbd* using a *daughterless* GS driver increased stress resistance of female flies

As DHR96 is involved in xenobiotic response (King-Jones et al., 2006), we investigated the effect of *dhr96* and *dhr96-lbd* in stress response.

RU-induced *dhr96* and *dhr96-lbd* over-expressing flies and controls were exposed at the age of 10 days to drugs and chemicals. Interestingly, *dhr96* and *dhr96-lbd* over-expression increased resistance to different xenobiotic drugs (figure 3.3.5 and 3.3.6).

Over-expression of both lines increased survival to the xenobiotics DDT (figure 3.3.5) and phenobarbital (figure 3.3.6 B) (p-value ***<0.001, Log Rank Test). Interestingly, the increase in resistance to phenobarbital was significantly higher in *dhr96-lbd* over-expressing flies than in the wild type *dhr96* over-expressing flies (p-value ***<0.001, Log Rank Test). Furthermore, only the *dhr96-lbd* over-expressor line showed a significant increase in resistance to H_2O_2 (figure 3.3.6 A). Survival in the *dhr96* over-expressor remained unchanged compared to non-induced and driver controls, when exposed to H_2O_2.

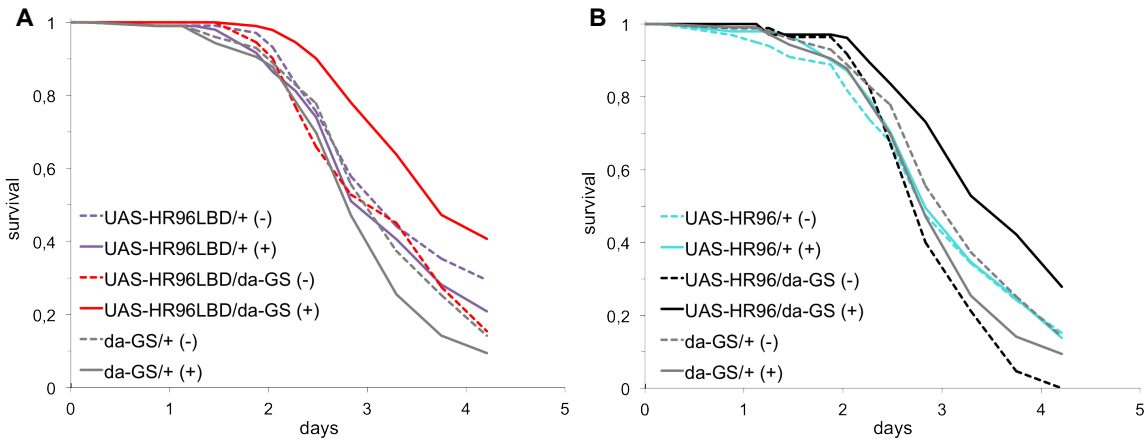


Figure 3.3.5 Over-expression of *dhr96* and *dhr96-lbd* by the *daughterless* GS driver increased resistance to DDT. (A) RU-induced expression of *dhr96-lbd* (UAS-HR96LBD/ da-GS) resulted in DDT resistance compared to driver- and UAS-controls on ethanol or RU. (B) The same effect was observed for *dhr96* over-expression (UAS-HR96/da-GS) (Log Rank Test, p-value ***<0.001).

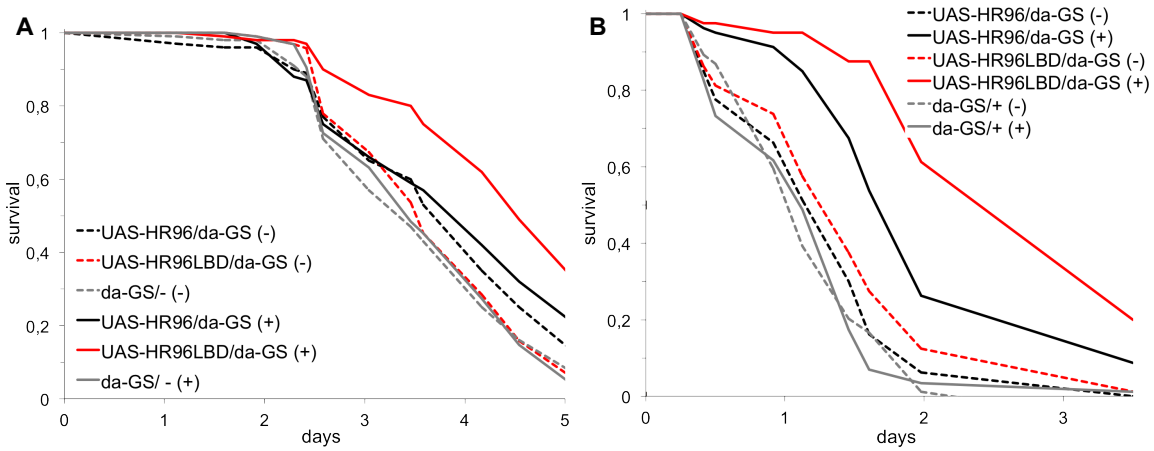


Figure 3.3.6 Responses of *dhr96* and *dhr96-lbd* over-expressors to phenobarbital and H_2O_2. (A) Only the RU-induced expression of *dhr96-lbd* resulted in significant H_2O_2 resistance (Log Rank Test, p-value ***<0.001) whereas *dhr96* over-expressor remained unchanged compared to its controls. **(**B) Both over-expressor lines showed significantly higher resistance to phenobarbital (PB) (Log Rank Test, p-value ***<0.001). *Dhr96-lbd* over-expressor showed significant higher survival compared to *dhr96* over-expressor.

Dhr96 over-expressing flies were sensitive to starvation (figure 3.3.7 B). Survival was significantly reduced in these flies compared to driver and UAS controls (p-value ***<0.001, Log Rank Test). In two repeats *dhr96-lbd* over-expressing flies showed significantly reduced survival to its ethanol control, but

the reduction did not reach significance when compared to the driver control on ethanol or RU (figure 3.3.7 A).

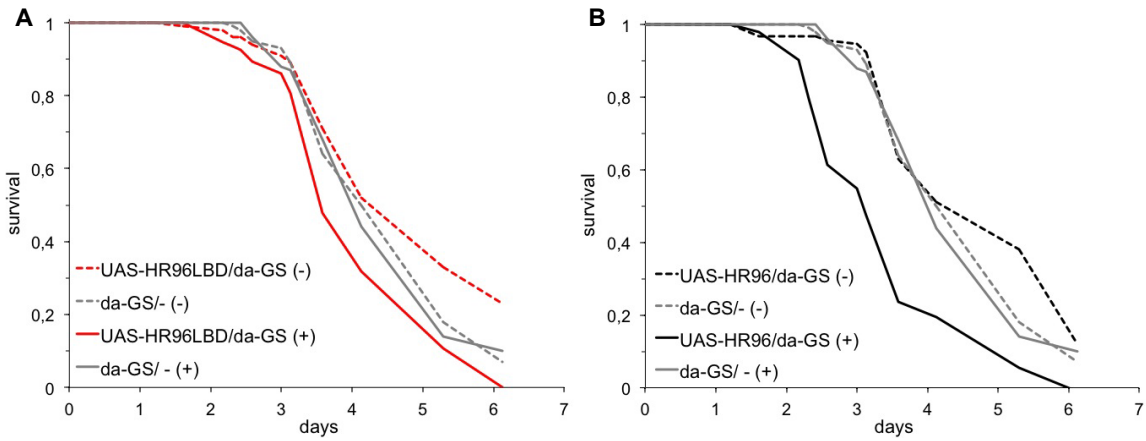


Figure 3.3.7 *Dhr96* over-expressor was sensitive to starvation. (A) *Dhr96-lbd* over-expressing flies were significantly sensitive to starvation when compared to its ethanol control (Log Rank Test, p-value ***<0.001), but not to the *daughterless* GS controls. (B) RU-induced expression of *dhr96* caused significant reduced survival in starvation conditions compared to driver control and its ethanol control (Log Rank Test, p-value ***<0.001).

3.3.2. Effects of *dhr96* and *dhr96-lbd* over-expressing flies was dependent upon *dhr96* induction levels in female flies

Over-expression of *dhr96* or *dhr96-lbd* using the ubiquitous *tubulin* Gene-Switch driver increased lifespan only for the LBD-mutated *dhr96* over-expressing females (Janne Toivonen, London, supplement S.3.3), whereas in my hands over-expression of both the *dhr96* and *dhr96-lbd* using the *daughterless* GS driver extended lifespan in female flies (figure 3.3.3). To investigate if these different results are caused by the use of different ubiquitous driver lines, lifespans of *daughterless* GS and *tubulin* GS driven *dhr96* and *dhr96-lbd* over-expression were conducted under same conditions.

3.3.2.1 Repeat lifespan and DDT assay of *dhr96* and *dhr96-lbd* over-expressing females using the *daughterless* GS driver

QRT-PCR analysis revealed that *dhr96* induction in both transgenic *dhr96* over-expressing flies was higher than in previous experiment. *Dhr96* over-expressing females showed an about 19-fold significant *dhr96* induction and d*hr96-lbd* over-expressing females an about 14-fold significant induction of *dhr96* transcript levels (p-value ***<0.001, Student's *t*-test) (figure 3.3.8). Interestingly,

dhr96 transcript levels were significantly higher in *dhr96* over-expressor than in d*hr96-lbd* over-expressor (Student's *t*-test, *p<0.05).

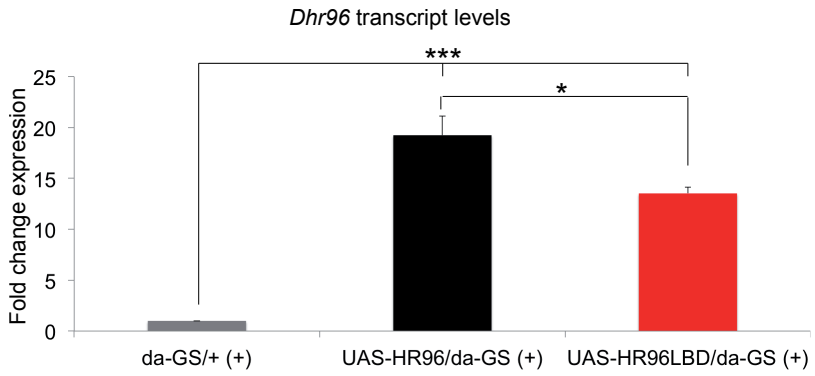


Figure 3.3.8 *Dhr96* transcript levels in *dhr96-lbd* over-expressing females using the *daughterless* GS driver. Ubiquitous over-expression of *dhr96* (UAS-HR96) and ligand-insensitive *dhr96* (UAS-HR96LBD) significantly induced *dhr96* expression (p-value ***<0.001, Student's *t*-test). *Dhr96* expression was significantly higher in wild type *dhr96* over-expressor (19-fold) than in LBD-mutated *dhr96* over-expressor (14-fold) (p-value *<0.05, Student's *t*-test).

Dhr96-lbd and especially *dhr96* over-expressing flies showed bacterial contamination during the lifespan experiment in form of an observed egg-jamming problem, which can occur when fecundity is strongly reduced. Thus, the survival was affected by bacterial contamination.

However, the repeat lifespan of *daughterless* GS-driven *dhr96* and *dhr96-lbd* over-expression confirmed significant increase in lifespan for both transgene lines (figure 3.3.9). The grade of lifespan extension was in this repeat smaller than in the previous experiment (figure 3.3.3). In *dhr96-lbd* over-expressing flies, median lifespan was increased by 12% compared to its ethanol control whereas *dhr96* over-expression extended median lifespan by only 4,5%. Wild type *dhr96* over-expressing flies indicated a smaller lifespan extension than LBD-mutated *dhr96* over-expressing flies, but this indication is not significant. There was no significant effect on lifespan between UAS-controls.

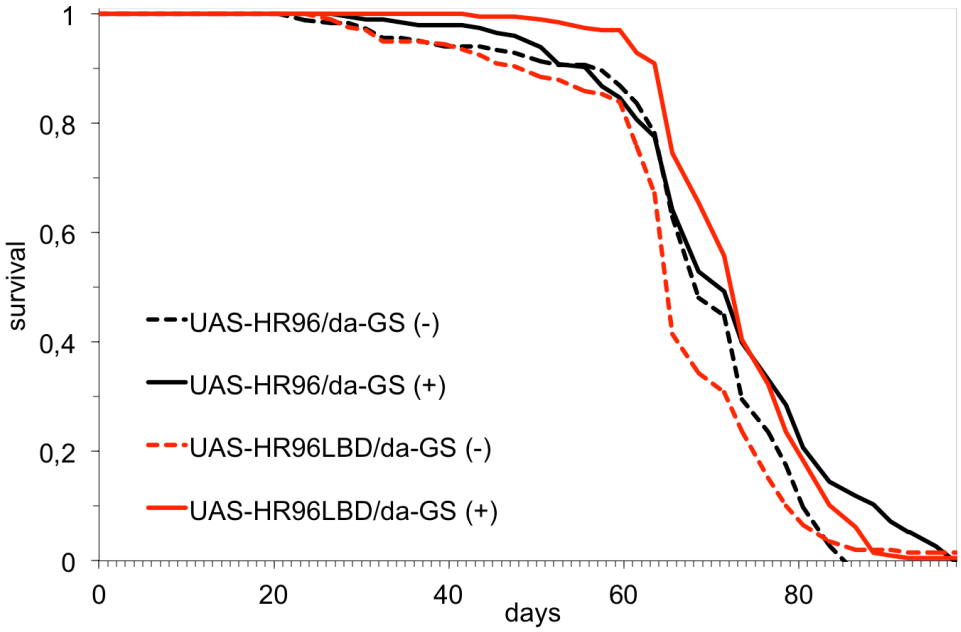


Figure 3.3.9 Repeat lifespan of *dhr96* over-expression in *D. melanogaster* females. Using the *daughterless* GS driver, over-expression of wild type *dhr96* (UAS-HR96) or ligand-insensitive *dhr96* (UAS-HR96LBD) significantly extended lifespan (Log Rank Test, p-value ***<0.001). There was no significant difference between the ethanol control flies. Extension is smaller than in previous experiment (figure 3.3.3).

Over-expression of both lines increased resistance to the xenobiotic DDT (Figure 3.3.10), and is therefore overlapping with previous result shown in chapter 3.3.1. Moreover, *dhr96-lbd* over-expressing females showed a higher increase in DDT resistance than wild type *dhr96* over-expressing females.

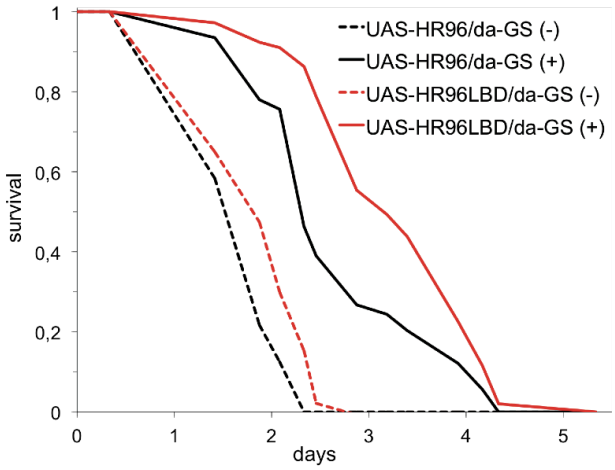


Figure 3.3.10 DDT repeat assay of *daughterless* GS *dhr96* and *dhr96-lbd* over-expressor. (A) Both over-expressor lines showed increased survival to DDT compared to controls, when using the *daughterless* GS driver (Log Rank Test, p-value ***<0.001). Increase was significantly higher in *dhr96-lbd* over-expressing flies compared to *dhr96* over-expressing flies (Log Rank Test, p-value **<0.01).

3.3.2.2 Over-expression of *tubulin* GS-driven *dhr96* and *dhr96-lbd* decreased lifespan and stress response

QRT-PCR analysis and western blot analysis revealed that *dhr96* expression was higher induced in *tubulin* GS-driven *dhr96-lbd* over-expressing females than in *daughterless* GS-driven *dhr96-lbd* over-expressing flies (figure 3.3.8 and 3.3.11, western blot see supplement S.3.4). *Dhr96-lbd* over-expressing females using the *tubulin* GS driver showed an about 32-fold significant induction of *dhr96* transcript levels. *Dhr96* expression of *dhr96* over-expressing flies was not tested.

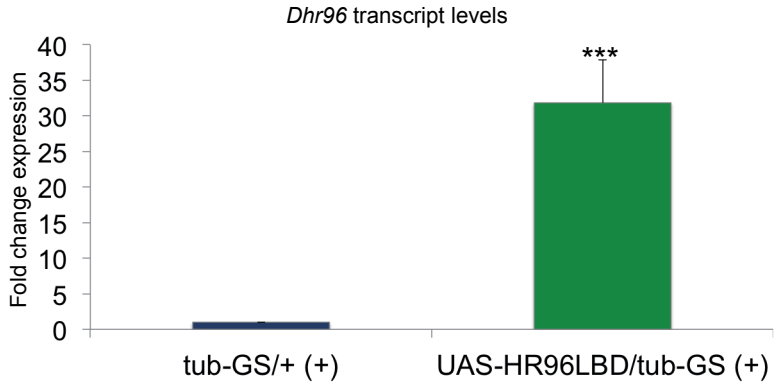


Figure 3.3.11 Induction of *dhr96-lbd* over-expressing flies by *tubulin* GS driver. Using the *tubulin* GS driver caused a 32-fold strong induction of *dhr96* in females over-expressing *dhr96-lbd* (p-value ***<0.001; Student's *t*-test).

Over-expression of both *tubulin*-driven *dhr96* and *dhr96-lbd* in female flies both caused a lifespan shortening effect compared to their ethanol controls (figure 3.3.12 A1) (p-value ***<0.001, Log Rank Test). The LBD-mutated *dhr96* expression (median lifespan: 56,5 days) decreased lifespan significantly more than the wild type *dhr96* expression (median lifespan: 62,5 days). There was also RU-induced toxicity in the driver line, as the *tubulin* GS-driver control on the drug RU showed a significant reduction by 8,5% in median lifespan compared to the driver control on ethanol. The UAS-control did not show any effects on lifespan (figure 3.3.12 A2).

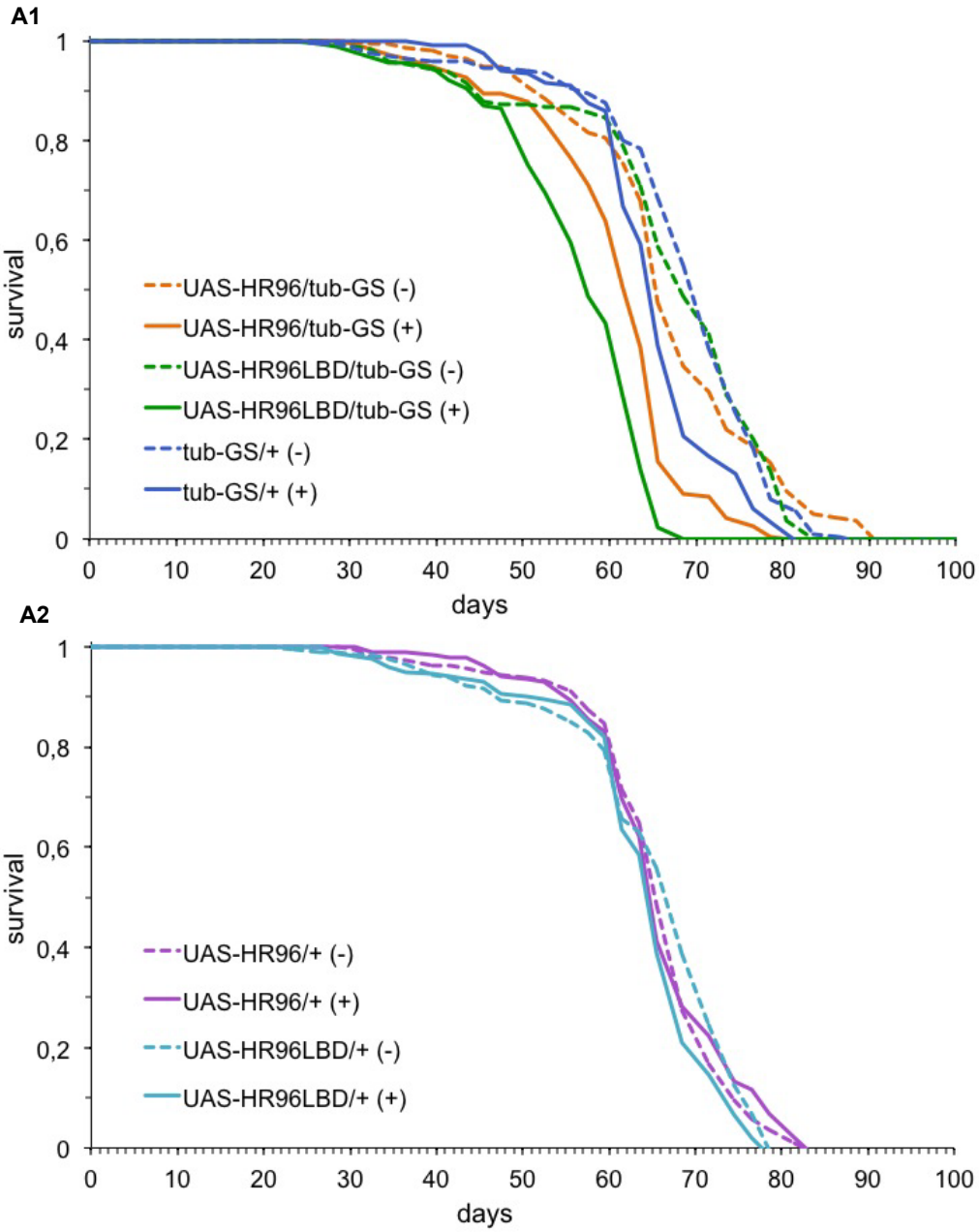


Figure 3.3.12 Tubulin GS-driven *dhr96* and *dhr96-lbd* over-expression shortens lifespan compared to control flies. (A1) Over-expression of both transgene lines by using the strong, ubiquitous *tubulin* GS diver significantly reduced longevity (Log Rank Test, p-value ***<0.001). The reduction in lifespan was higher for LBD-mutated *dhr96* (UAS-DHR96LBD) than for wild type *dhr96*. Driver control on RU showed a significant decrease in survival when compared to its ethanol control (Log Rank Test, p-value ***<0.001). (A2) There was no effect on lifespan between UAS-controls on ethanol and these lines on RU.

Fecundity was strongly reduced in the *tubulin* GS-driven *dhr96* flies (figure 3.3.13) (p-value ***<0.001, Kruskal-Wallis test, Dunn's test for multiple comparisons). These flies showed significantly higher reduced egg laying than *daughterless* GS-driven *dhr96* over-expressing flies. But also the *tubulin* Gene-

Switch driver control on RU showed significantly lower fecundity than its non-induced ethanol control. These flies were strongly affected by bacterial contamination as a consequence of reduced fecundity.

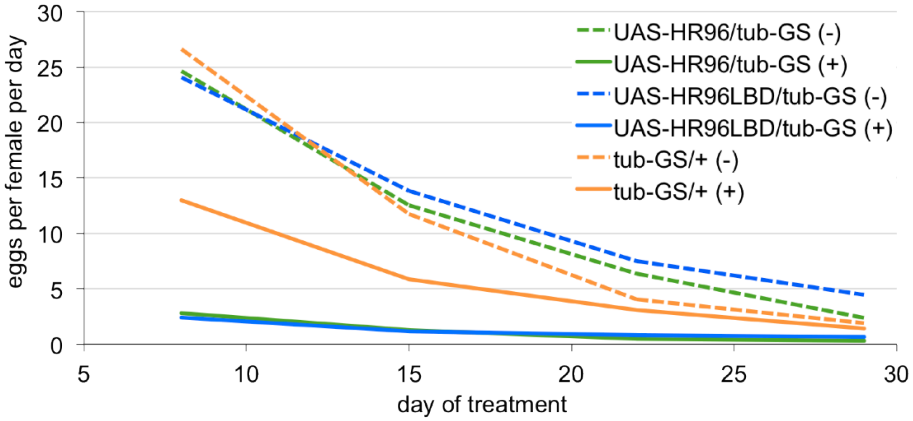


Figure 3.3.13 Using the *tubulin* GS-driver, *dhr96* and *dhr96-lbd* over-expression strongly reduced fecundity. *Dhr96* and *dhr96-lbd* over-expressing lines significantly decreased egg laying when compared to UAS-controls and the non-induced driver control. But also the RU-induced *tubulin* driver control significantly lowered fecundity compared to control flies (p-value ***<0.001, Kruskal-Wallis test, Dunn's test for multiple comparisons).

Furthermore, *dhr96* over-expressing flies using the *tubulin* Gene-Switch driver were significantly sensitive to the xenobiotic DDT compared to controls (p-value ***<0.001, Log Rank Test) (figure 3.3.14). There was no significant difference in survival between RU-induced and non-induced *tubulin* controls.

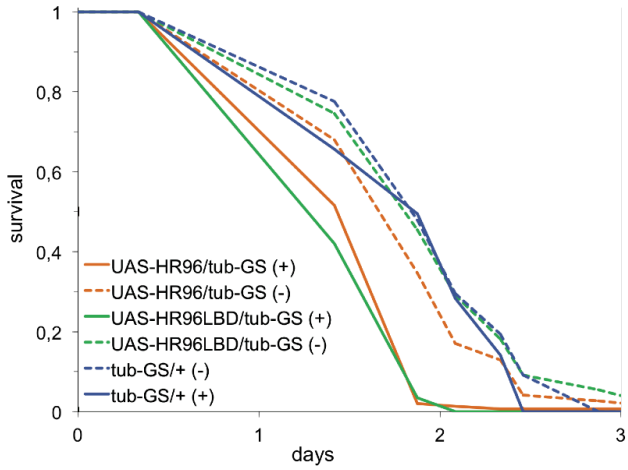


Figure 3.3.14 *Tubulin* GS *dhr96* and *dhr96-lbd* ove-rexpressor were sensitive to DDT. (A) Both over-expressor lines showed reduced survival to DDT compared to controls, when using the *tubulin* GS driver (Log Rank Test, p-value ***<0.001).

3.3.3 Quantitative RT-PCR analysis of genes involved in xenobiotic metabolism

Induction of *dhr96* expression in *dhr96-lbd* over-expressing flies was more than two times higher using the *tubulin* GS driver (32-fold induction) (figure 3.3.11) than with the *daughterless* GS driver (about 14-fold induction) (figure 3.3.8). Over-expression of *dhr96* transgenic lines using the *daughterless* driver increased lifespan and stress resistance whereas over-expression of *dhr96* transgenic lines using the *tubulin* driver decreased lifespan and DDT resistance. Transcript levels of several cytochrome genes were tested by quantitative real-time PCR (qRT-PCR) analysis in long-lived *dhr96-lbd daughterless* GS over-expressing flies and in short-lived *dhr96-lbd tubulin* GS over-expressing flies to identify enzymes which might confer increased or decreased resistance to xenobiotics.

The transcript level of cytochrome P450 *6g1* (*Cyp6g1*) was increased in the long-lived *dhr96-lbd* female over-expressor flies (figure 3.3.15) (*p<0.05, Student's *t*-test). The increase in *Cyp6g1* levels was not significant when whole bodies were used. However, transcript levels of *Cyp6g1* in dissected guts of *dhr96-lbd* female over-expressing females were significantly increased.

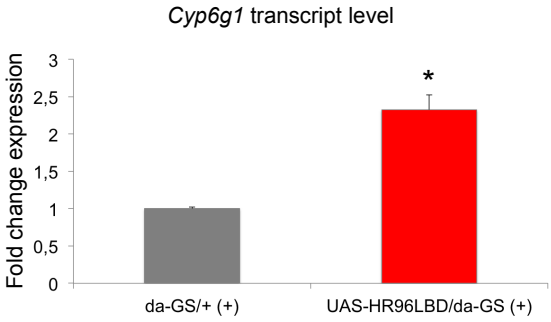


Figure 3.3.15 Cytochrome P450 *6g1* transcript levels in the gut of *dhr96-lbd* over-expressing flies. Over-expression of *dhr96-lbd* by the *daughterless* GS driver significantly up-regulated *Cyp6g1* expression in the gut (*p<0.05, Student's *t*-test).

QRT-PCR analysis on GS *tubulin*-driven *dhr96-lbd* over-expressors (whole body) showed a strong and significant down-regulation of *Cyp6g1* (p-value ***<0.001, Student's *t*-test) and glucose-6-phosphate-1-dehydrogenase or Zwischenferment (*Zw*) (p-value **<0.01; Student's *t*-test) (figure 3.3.16).

Transcript levels of *Cyp6g1* were reduced by 76%, and transcript levels of *Zw* were reduced by 46% in the *tubulin*-driven *dhr96-lbd* over-expressing flies compared to the driver control on RU.

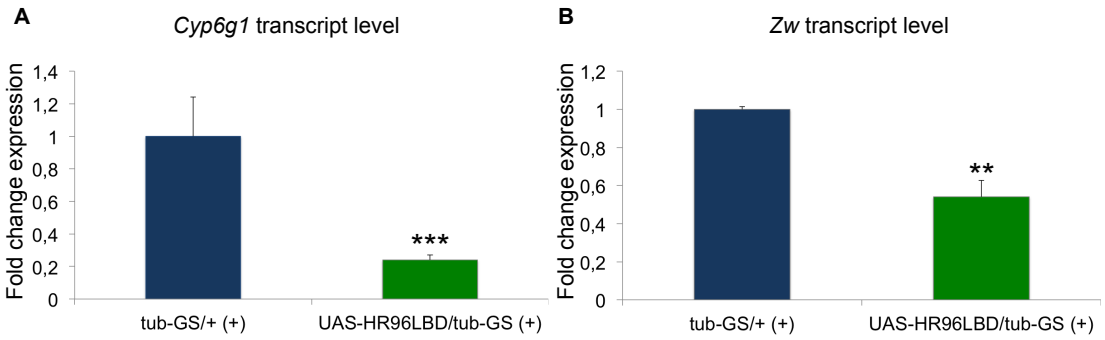


Figure 3.3.16 *Cyp6g1* and *Zw* transcript levels were altered in *dhr96-lbd* over-expressing flies. Using the tubulin GS driver, over-expression of *dhr96-lbd* caused a significant down-regulation of (A) *Cyp6g1* (p-value ***<0.001, Student's *t*-test) and (B) *Zw* (p-value **<0.01, Student's *t*-test).

3.3.4 Over-expression of *dhr96-lbd* increased DDT resistance in male flies, but not their lifespan

Experiments were also performed with male flies to investigate if phenotypes are gender-specific. Over-expression of *dhr96* with the ubiquitous *daughterless* or *tubulin* Gene-Switch driver lines did not affect lifespan in males (figure 3.3.17 and 3.3.18). There was no life shortening effect of the *tubulin* Gene-Switch driver on RU, as it was shown for the life span experiment in female flies.

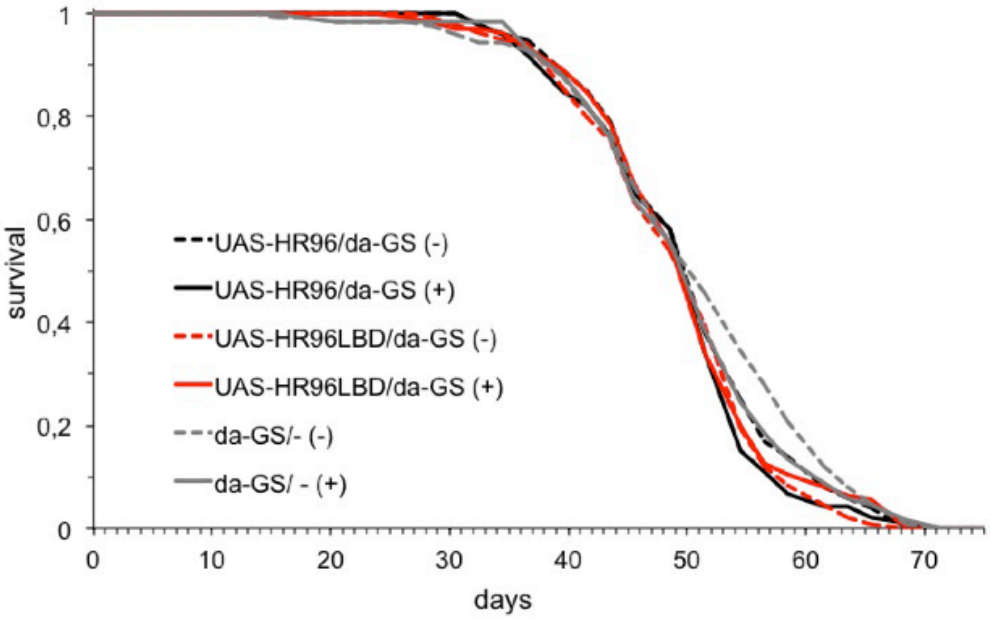


Figure 3.3.17 Using the *daughterless* GS driver, over-expression of *dhr96* did not affect lifespan in male flies. No significant difference was observed in male flies, when *dhr96* or *dhr96-lbd* was over-expressed by using the *daughterless* GS driver compared to control flies.

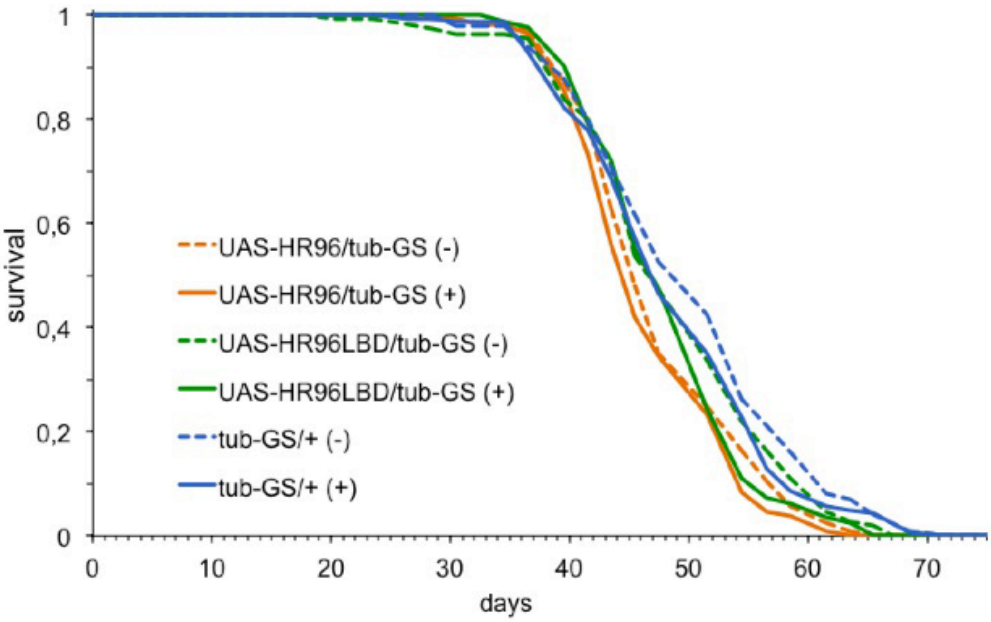


Figure 3.3.18 *Tubulin* GS-driven over-expression of *dhr96* and *dhr96-lbd* did not change lifespan in male flies. Also possibly stronger induction of *dhr96* did not affect lifespan in male *Drosophila* flies.

Treatment with the xenobiotic DDT using the *daughterless* GS driver revealed an increase in DDT resistance in *dhr96-lbd* over-expressing male flies (figure 3.3.19 A). DDT experiments with males were conducted on 150mg/L DDT, as male flies were more sensitive to DDT than female flies in pre-experiments (data not shown). Whereas in female flies *dhr96-lbd* over-expression enhanced

H_2O_2 resistance, over-expression of *dhr96-lbd* in male flies did not increase resistance to H_2O_2 (figure 3.3.19 B). Instead, a significant reduction in H_2O_2 resistance was observed.

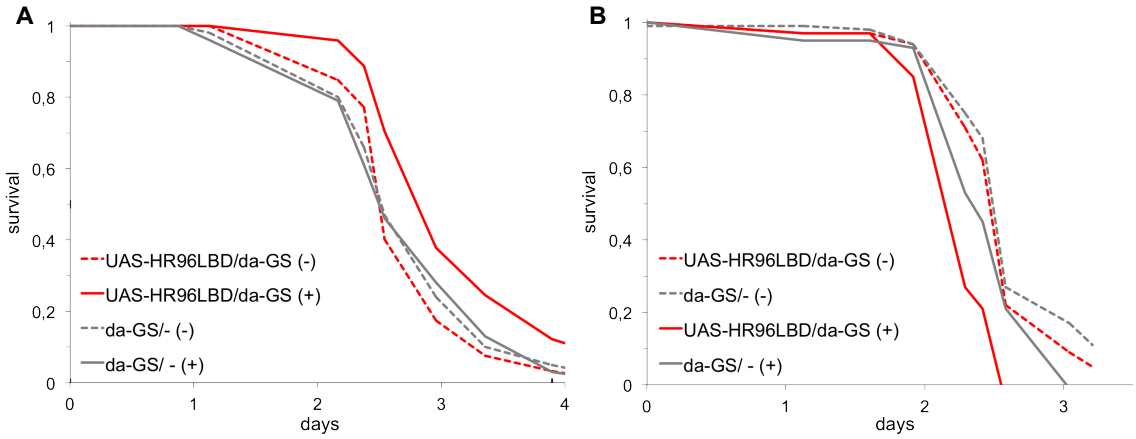


Figure 3.3.19 DDT and H_2O_2 stress assay with male *dhr96-lbd* over-expressing flies. Over-expression of *dhr96-lbd* significantly increased DDT resistance in male flies (Log Rank Test, p-value ***<0.001) (B), but also significantly reduced resistance to H_2O_2 (Log Rank Test, p-value ***<0.001).

Dhr96 and *dhr96-lbd* over-expressing male flies were significantly sensitive to starvation conditions when compared to ethanol controls (figure 3.3.20), as was the case for females.

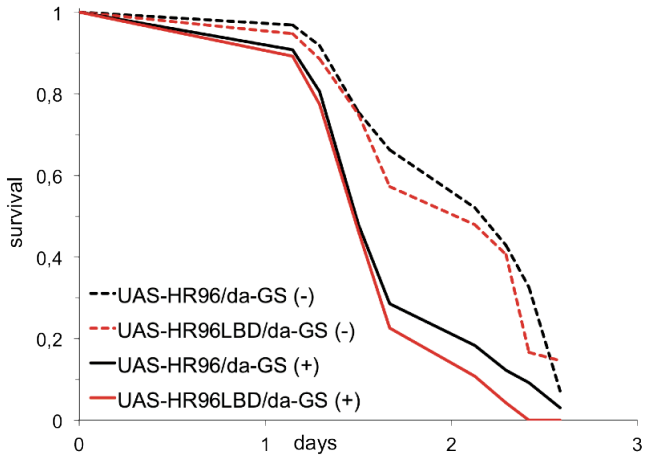


Figure 3.3.20 *Dhr96* and *dhr96-lbd* over-expressing males were sensitive to starvation. Using the *daughterless* GS driver, survival was significantly reduced in *dhr96-lbd* and *dhr96* over-expressor lines compared to ethanol controls, when flies were starved (Log Rank Test, p-value ***<0.001).

3.4 Discussion

3.4.1 Xenobiotic response of *dhr96* and LBD-mutated *dhr96* over-expressing flies

The xenobiotic response is an essential defence system to clear toxins from the body by detoxification enzymes. An involvement in detoxification for DHR96 was already shown in *dhr96* null mutants, which are sensitive to DDT and phenobarbital treatment (King-Jones et al., 2006). Thus, DHR96 was suggested to regulate the expression of detoxification enzymes (King-Jones et al., 2006).

I used genetic tools of *Drosophila melanogaster* to investigate the effects of *dhr96* over-expression. That *dhr96* over-expressing flies showed increased resistance to the xenobiotics DDT, phenobarbital and H_2O_2 (in addition to chloroquine, nicotine and paraquat by Janne Toivonen, UCL London) in females is novel.

In *Drosophila*, one study was focused on *Cyp6g1* and its correlation with DHR96 (Shah et al., 2011). Knockdown of this cytochrome gene decreased stress resistance, which would agree with these thesis results, in terms that *tubulin* GS-driven *dhr96* over-expressing flies showed reduced *Cyp6g1* transcript levels and were stress sensitive. However, ubiquitous expression of transgene *dhr96* and *dhr96* RNAi lines induces lethality, thus transgenic lines with weak *dhr96* expression were used in this study. Knockdown of *dhr96* increased resistance to imidaclopric (an insecticide and neurotoxin). This seems contrary to my data, as *dhr96* null flies showed a stress sensitive phenotype (chapter 4). It is not clear if developmental effect of transgene lines used in this paper might contribute to pleiotropic effects. In addition, *dhr96* over-expression caused changes in expression of a broad range of detoxification enzymes in a sex-specific manner, which is in agreement with my results. However, *dhr96* transgene lines were functionally not tested (e.g. survival assay) and induction of *dhr96* was also not measured in this study. This might be a critical point, as *dhr96* induces or represses *Cyp6g1* expression dependent on its transcript level.

In addition, transgenic mice of the mammalian DHR96 homolog PXR were investigated for xenobiotic studies. PXR knock-out mice, and transgenic mice

with an activated form of human PXR revealed that *CYP3A* is one of the specific targets (Xie et al., 2000a). Null mutants were incapable of *CYP3A* expression in response to xenobiotics, whereas the transgenic mice induced *CYP3A* expression and showed increased protection to the toxicants tribromoethanol and zoxazolamine. Interestingly, *Cyp6g1* is the homologous gene to mammalian *CYP3A*. Our results are in agreement with their findings, in that in both model organisms enhanced protection against toxins was observed with over-expression. Overlapping with this study, *Cyp6g1* was induced in *dhr96-lbd* over-expressing flies. Furthermore, it was shown that both human *CYP3A* and *Drosophila Cyp6g1* confer resistance to the same toxin methylmercury, indicating that role of this Cytochrome gene is conserved across species (Rand et al., 2012).

3.4.2 *Dhr96* over-expressing flies and *daf-12* ligand-insensitive mutants are long-lived

In *C. elegans*, the DHR96 homologue DAF-12 affects lifespan. Ligand-insensitive *daf-12* mutants are long-lived (Fisher and Lithgow, 2006), whereas *daf-12* null mutants are short-lived (Riddle et al., 1981). Also DHR96 affects lifespan, as *dhr96* null mutants were short-lived (Janne Toivonen, unpublished data), and over-expression of *dhr96* and *dhr96-lbd* (dependent on transcript levels) increased lifespan in females. Although phenotypes are similar in that *Drosophila dhr96-lbd* over-expression using the *daughterless* GS driver and *C. elegans* ligand-insensitive DAF-12 extend lifespan, conditions were not the same. In *C. elegans*, long-lived *daf-12* mutant were generated by EMS, which means that mutation is present in the genomic, endogenous *daf-12* gene (Antebi et al., 1998). However, in the *dhr96* over-expressor lines used in this thesis, *dhr96* was integrated in the *Drosophila* genome by P-element insertion. *Dhr96* gene expression was under the control of a UAS-promoter, and expression was induced by GAL4 binding in a normal wild type background. That means that *dhr96* or LBD-mutated *dhr96* was over-expressed in flies that harboured also the endogenous wild type *dhr96* and thus the lifespan extension may be independent of the ligand. Taking this information into account, the life span extension of ligand-insensitive *daf-12* is caused by the mutation in the

ligand-binding domain, whereas the lifespan extension in *dhr96* over-expressing flies might be due to the enhanced *dhr96* transcript expression and protein level.

It would be interesting to investigate, if ligand-insensitive DAF-12 might induce its own expression, as hormone nuclear receptors are regulated by feedback loops (Hammell et al., 2009; Sasaki et al., 1999), and confer enhanced longevity by a similar mechanism as for *dhr96* over-expressing flies. If not, it would be significant to investigate whether the two different manipulations (ligand-insensitive *daf-12* and over-expression of *dhr96*) would cause the same changes, for example the same changes in the transcriptional regulation of target genes leading to an altered lifespan. At least for DAF-12, it lifespan extension is dependant on the DNA-binding domain, as *rh61rh411* double mutants, which contain an additional mutation in the DBD-domain, are short-lived (Fisher and Lithgow, 2006).

3.4.3 *Dhr96* expression level was a critical criterion for xenobiotic response and lifespan

The expression level of *dhr96* and/or expression pattern was shown to play a major role as these can cause contrary effects, a beneficial or a damaging phenotype. Whereas *daughterless* GS-driven *dhr96* over-expressing flies (12-16 fold induction) were long-lived and showed increased DDT resistance, *tubulin* GS-driven *dhr96* over-expressors (32-fold induction) were sensitive to DDT and showed decreased lifespan. These non-beneficial effects were accompanied by sensitivity to bacterial infection during the lifespan. Thus, high level of induction causes non-beneficial effects and is toxic.

The expression pattern might also be critical, as the *daughterless* GS driver and the *tubulin* GS driver induce *dhr96* expression in different tissues, with different induction levels. So far, the tissue(s) that might confer a lifespan extension have not been identified. Different tissue-specific drivers were tested and none of them resulted in increased longevity (Matt Piper and Janne Toivonen, UCL London, unpublished data). That may be due to expression levels, or that over-expression in more than one tissue may be required to extend lifespan. In case

it might be level dependent, a tissue-specific, inducible system would be useful to modulate induction levels. However, a beneficial effect was seen in the Malphigian tubules. Over-expression of *dhr96* and *dhr96-lbd* in the Malphigian tubule increased resistance to DDT (Matt Piper, see figure 3.4.1, unpublished data). Taking into account that *dhr96-lbd* over-expression induced *Cyp6g1* expression, these results are in agreement with studies showing that tissue-specific over-expression of *Cyp6g1* in the Malphigian tubule confers DDT resistance, whereas knockdown of *Cyp6g1* in this tissue causes sensitivity to DDT (Yang et al., 2007).

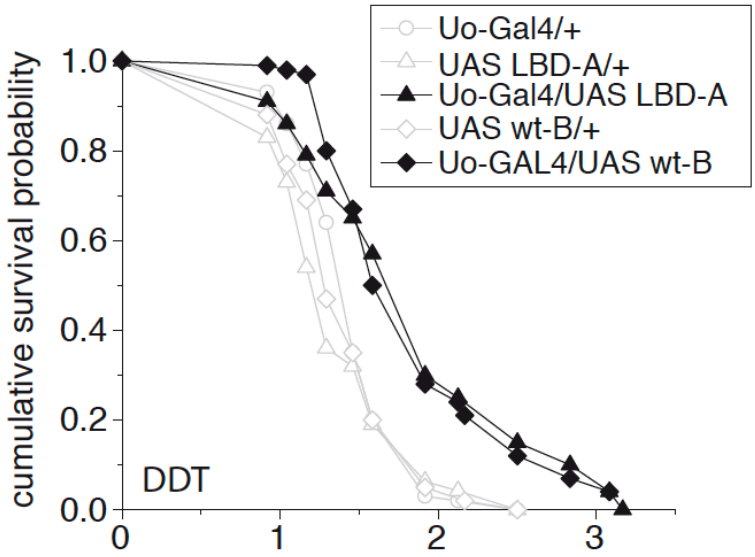


Figure 3.4.1. Increased DDT resistance by over-expressing *dhr96* and *dhr96-lbd* in the Malphigian tubule. Experiment was performed by Matt Piper, (UCL London). Induced expression of *dhr96* (UAS wt-B) and *dhr96-lbd* (UAS LBD-A) in the Malphigian tubule (Uo-GAL4) confer significantly DDT resistance compared to driver (Uo-Gal4/+) and their UAS-controls (UAS-LBD-A/+,; UAS-wt-B/+) (DDT concentration 275 mg/L).

3.4.4 *Dhr96* over-expressing flies versus *dhr96-lbd* over-expressing flies

Dhr96 and ligand-mutated *dhr96* over-expression gave differing phenotypes in lifespan using the *tubulin* GS driver (Janne Toivonen, supplement S.3.3), and in the xenobiotic response using either the *tubulin* GS or the *daughterless* GS driver (Janne Toivonen, chapter 3.3.2).

The *dhr96-lbd* over-expressor possessed stronger resistance to different xenobiotic drugs than the *dhr96* over-expressor. Moreover, *dhr96-lbd* over-

expressing females were less affected by bacterial contamination, and indicated a better lifespan extension than *dhr96* over-expressing females in the repeat experiment using the *daughterless* GS driver (chapter 3.3.2). It is suggested, that the stronger reduced fecundity of *dhr96* over-expressing flies might be a possible reason that *dhr96* over-expressing flies were more affected by bacterial infection. Thus, *dhr96-lbd* over-expressing females might display more robust phenotypes.

These differences between *dhr96* and *dhr96-lbd* over-expressing flies might occur due to different induction levels. *Dhr96* over-expressing flies showed a stronger induction of *dhr96* transcript levels than *dhr96-lbd* over-expressing flies. In the first lifespan experiment, induction was not significantly different and lifespan curves looked very similar between the two transgenic over-expressors. In the repeat experiment, levels of *dhr96* were in general higher for both over-expressors. As *dhr96* over-expressing flies showed higher induction levels, that might have caused non-beneficial effects, possibly in fecundity and subsequently in sensitivity to bacterial infection. In experiments with *dhr96* and *dhr96-lbd* over-expressing flies using the *tubulin* GS driver these effects were even stronger. In *tubulin* GS-driven *dhr96-lbd* over-expressing females, induction of *dhr96* transcript levels was much higher than in experiments using the *daughterless* driver (32-fold versus 12-16-fold). Both (*tubulin* GS-driven) over-expressor flies showed strong reduction in fecundity and were strongly sensitive to bacterial infection. The strong induction affected survival due to bacterial infection.

Two possible reasons for the different *dhr96* transcript levels are suggested. As the induction of *dhr96* and *dhr96-lbd* is dependent on RU, one possible explanation is that *dhr96* over-expressing flies might differ in their feeding behaviour and eat more RU-containing SYA media. That would also give different results in stress assays between *dhr96* and *dhr96-lbd* over-expressing flies. Another reason might be that the two UAS-lines are integrated in different insertion sites in the *Drosophila* genome. The chromosomal position effects could cause different induction levels between the UAS-HR96 and the UAS-HR96LBD lines (Tubon and Yin, 2008). Nowadays genes of interest are

inserted in so-called attP insertion-sites, which are defined insertion sites in the *Drosophila* genome to provide similar induction level and to prevent site specific effects (Groth et al., 2004). For future experiments *dhr96* and *dhr96-lbd* has been cloned in attP-flanked vectors and injected in flies (supplement S.3.5). These flies will be used in stress assays that exclude the route of drug feeding.

3.4.5 Gender-specific effects of *dhr96* and *dhr96-lbd* over-expression

Although *dhr96* over-expression in male flies increased resistance to DDT, lifespan was not affected. Two different driver lines with different induction levels, *daughterless* GS and *tubulin* GS, were used. In female flies, over-expression of *dhr96* transgenes showed opposing effects between the two driver lines. Using the *daughterless* GS driver, *dhr96* and *dhr96-lbd* over-expression extended lifespan, whereas using the *tubulin* GS driver to over-express *dhr96* and *dhr96-lbd* shortened lifespan. In male flies neither drivers had any beneficial or non-beneficial effect on lifespan, but beneficial effects in DDT resistance. Thus, DHR96 modulates xenobiotic resistance in a way that does not translate to increased longevity in males, at least in these conditions. As induction levels turned to be a significant criteria for lifespan, it might be that other RU concentration are required to extend lifespan of *dhr96* or *dhr96-lbd* over-expressing male flies. Thus, to define whether the lifespan effect is restricted to females, future studies will include lifespan analysis of *dhr96* or *dhr96-lbd* male flies on different RU concentration combined with qRT-PCR analysis of *dhr96* induction levels.

So far, *dhr96* and *dhr96-lbd* female transgenes enhanced DDT, phenobarbital and H_2O_2 resistance whereas male *dhr96-lbd* over-expressing flies showed increased resistance only to DDT. Interestingly, chemical H_2O_2 treatment decreased survival in male flies, suggesting that oxidative stress response to H_2O_2 is regulated in a gender specific manner. QRT-PCR analysis of key anti-oxidant enzymes, including Superoxide Dismutase (SOD) and catalase, between transgene males and transgene females, might contribute to support this conclusion. In addition, oxidative damage by ROS (reactive oxygen species) represents a feature in many ageing studies (HARMAN, 1956). Many

long-lived animals show increased resistance to oxidative stress mediated by an enhanced activity of anti-oxidant enzymes, which metabolize toxic ROS (reactive oxygen species). Furthermore, over-expression of *sod* or *catalase* extends lifespan in *Drosophila melanogaster* (Orr and Sohal, 1994). Oxidative stress response might be therefore a gender-specific pro-ageing possible mechanism in long-lived *dhr96* and *dhr96-lbd* transgene females.
Differences between male and female *dhr96-lbd* over-expressing flies might be caused by gender-specific responses. Studies in *Drosophila* have already shown that transcriptional expression of cytochrome genes by xenobiotic or chemical drugs differ between gender (Le Goff et al., 2006). Moreover, the expression profile of *dhr96* transgene indicated gender-specific regulation. Thus, it is necessary to test male *dhr96* over-expressing flies against a wide range of xenobiotic drugs to support this conclusion.

In females, the RU fed *tubulin* GS control was short-lived compared to its ethanol control. This was not observed in male flies, indicating that the site effect of the driver line is also gender specific. Furthermore, the driver line showed an effect on fecundity in female flies, which intensifies this argument.

Many studies correlate effects on the reproductive system with the lifespan of organism (Hansen et al., 2013). Reproduction is a cost-intense process, thus reduced fecundity or abolished reproduction affects the energy storage of an organism, in terms that fat reservoir is not consumed and is available for other biological processes. This ‘energetic trade-off’ as a consequence of reduced reproductive rate might represent a possible mechanism to extend lifespan. In *C. elegans*, ablation of the germ line leads to an extension in lifespan (Hsin and Kenyon, 1999). Interestingly, the *C. elegans* dFOXO homologue DAF-16, as well as the DHR96 homologue DAF-12 are required to extend lifespan in germ line ablated nematodes (Hsin and Kenyon, 1999).
In *Drosophila*, lifespan modulation by the ablation of germ line cells (GC) is not as clear as in worms and can show pleiotropic effects. GC ablation by using *grandchildless*-mutants does not affect the lifespan of the organism (Barnes et al., 2006) whereas GC ablation in *Drosophila subobscura grandlchildless*-mutants extends lifespan (SMITH, 1958). Although both mutants lack the

primordial germ line, they cause different phenotypes. *Grandchildless*-mutants have their impact during development. It is suggested that developmental effects on the somatic gonad might supress the lifespan extension in *grandchildless*-mutants (Flatt et al., 2008b). Another study avoided possible developmental effects by inducing the ablation of the germ line in later stages. They could show an extended lifespan and altered insulin signalling, implying that ageing regulation by gonadal activity is conserved between worms and flies (Flatt et al., 2008b). Moreover, other methods to down-regulate reproduction, including the ovo^{D} sterile mutation (Clancy et al., 2001; Sgro and Partridge, 1999), reduced egg production (Partridge et al., 1987) or mating (Fowler and Partridge, 1989), revealed enhanced lifespan in *Drosophila*.

Some IIS long-lived mutants in flies show reduced fecundity. MNC ablation in the brain of flies or *dfoxo* over-expression in the fat body reduces egg laying (Broughton et al., 2005; Giannakou, 2004). In *Drosophila*, different studies have uncoupled lifespan and fecundity in IIS-reduced signalling. $Chico^{1}$ heterozygous mutants show normal fecundity and have extended lifespan (Clancy et al., 2001). Furthermore, it was shown that effects on fecundity is a dFOXO independent effect whereas lifespan extension of IIS-reduced flies requires dFOXO (Slack et al., 2011). Thus, in *Drosophila*, longevity and reproduction can be uncoupled. However, that does not subsequently mean that germ line signalling is not required for DHR96 lifespan extension. Signals from the reproductive system might be necessary to extend lifespan in *dhr96-lbd* over-expressing flies, as shown for DAF-12.

Another interesting fact, which might give the reproductive tissue an importance for DHR96 regulation is, that *dhr96* expression is highest during adult stage in the ovary (http://flybase.org/reports/FBgn0015240.html). Furthermore, it has been suggested that DHR96 might modulate epithelial follicle stem cell (FSC) proliferation via ribosomal protein S6 kinase (S6K) activity in the fly ovary in response to dietary conditions (Hartman et al., 2013). Interestingly, S6K acts as a key component of the TOR (target of rapamycin) pathway, affecting processes including protein translation and growth in response to insulin signalling and nutrition (Marygold and Leevers, 2002). Reduced S6K-activity by over-expression of a dominant negative *S6-kinase* were reported to extend lifespan in male flies (Kapahi et al., 2004). In our hands and laboratory

conditions these results were not reproducible. However, heterozygous *S6-kinase* knockout enhanced longevity in female flies but not in males (Andrew Finlayson, unpublished data). If S6K activity might be regulated by DHR96, it would represent an important candidate, in terms that the role of DHR96 in lifespan might be linked to S6K regulation.

One possibility to investigate is, whether lifespan extension is due to signals from or in the female reproductive system in *Drosophila*, is the use of a mutant stock, called ovo^{D}. Female germ line development is blocked in these mutants, which are as a consequence sterile and long-lived (Mevel-Ninio et al., 1991; Oliver et al., 1987). If lifespan extension of *dhr96* and *dhr96-lbd* over-expressing flies were lost in these mutants, the lifespan effect would be dependant on the female germ line.

The conclusion of this chapter is that over-expression of *dhr96* and *dhr96-lbd* using the *daughterless* GS driver extended lifespan in female flies and increased stress resistance in a possibly gender-specific manner. Cytochrome P450 *6g1* (*Cyp6g1*) was up-regulated in the gut of *dhr96-lbd* over-expressing female flies and might confer DDT resistance.

Chapter 4 Does DHR96 act downstream of the IIS network?

4.1 Introduction

4.1.1 Long-lived IIS mutants

The Insulin/insulin like signalling (IIS) network is evolutionally conserved in multicellular organisms, and is involved in diverse biological process, including growth, reproduction, and stress resistance. Lowered activity of components in the IIS signalling network can increase lifespan and retard many age-related phenotypes in diverse species including mammals (Bonkowski et al., 2006; Cohen et al., 2006; Fontana et al., 2010; Giannakou and Partridge, 2007; Kenyon, 2005; 2010; Selman et al., 2008; Tatar et al., 2003; Wessells et al., 2009).

Pioneering studies began in *C. elegans* by genetic screens of long-lived mutants (Klass, 1983), and uncovered *age-1* mutants, the mammalian phosphatidiylinositol 3-kinase ortholog, to extend lifespan (Friedman and Johnson, 1988). Moreover, worms enter a dauer formation (daf) stage in unfavourable conditions (chapter 3.1). Mutation genes regulating dauer formation can show a constitutive dauer phenotype, a stage characterized by a long life and increased stress resistance (Klass, 1983). The worm insulin receptor *daf-2* was discovered by Johnson to regulate dauer formation, and Kenyon studies revealed that down-regulation of *daf-2* leads to dauer formation at 25°C, but circumvents dauer at lower temperatures (20°C) and double adult lifespan (Kenyon et al., 1993). Furthermore, *daf-2* longevity is dependent upon the transcription factor DAF-16 (Kenyon et al., 1993) and the heat-shock transcription factor HSF-1 (Hsu et al., 2003; Morley and Morimoto, 2004). DAF-16 is the *C. elegans* orthologue of the FOXO transcription factor family (Forkhead bOX-containing protein, subfamily O) (Lin et al., 1997). Insulin signalling reduces FOXO activity by phosphorylation, and consequent nuclear

exclusion. In contrast, reduced IIS caused by *daf-2* mutation enhances nuclear localisation of DAF-16 and its transcriptional activity (Kenyon, 2010).

In flies, studies with different IIS mutants again revealed a link between the IIS network and ageing (figure 4.1.1). *Drosophila* produces seven insulin-like peptides (dILPs), which modulate growth control dependently on the insulin receptor (Brogiolo et al., 2001). *Dilp-2,3,5* mutants are long-lived (Grönke et al., 2010) and ablation of the median neurosecretory cells (MNC), which produces these three ligands, also results in longer lifespan (Broughton et al., 2010). Downstream IIS components also modulate lifespan. For instance, over-expression of a dominant negative version of the insulin receptor (*dnIR*) (Slack et al., 2011), and mutation in *chico* (Clancy et al., 2001), which encodes for the insulin receptor substrate in *Drosophila*, increase lifespan. Reduced IIS also decreases growth and fecundity, and increases xenobiotic and oxidative stress response. Growth and fecundity are affected in *chico*^{1} homozygous mutants, but these phenotypes are not coupled with the lifespan effect, as heterozygous *chico*^{1} mutants have a long lifespan with no altered growth or fecundity effects (Bohni et al., 1999; Clancy et al., 2001).

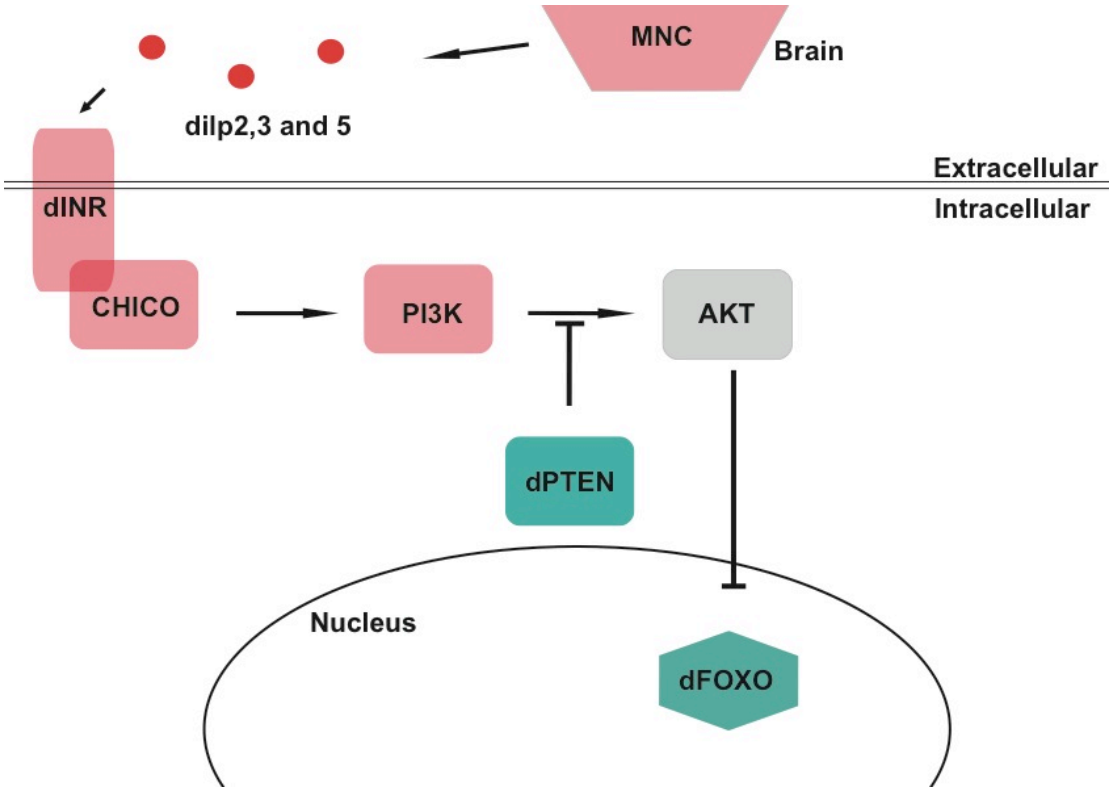


Figure 4.1.1 Reduced IIS extends lifespan. Altered activity of components in this pathway affect lifespan. Up-regulation of *dfoxo* and *dpten* activity (green) as well as down-regulation of *dilp2,3,5* production by MNC ablation, mutation in *chico* and the insulin receptor (red) extends lifespan in *Drosophila*.

Many recent studies have focussed on the IIS downstream target dFOXO (figure 4.1.2). Over-expression of this transcription factor in the gut/fat body (Giannakou et al., 2004; Hwangbo et al., 2004) or in the muscle (Demontis and Perrimon, 2010) extends lifespan. Muscle-specific *dfoxo* over-expression induces 4E-BP signalling, which is a key regulator of protein translation. Endocrine signals, regulated by 4E-BP and activated in these transgenic flies, causes reduced insulin signalling in the whole body (Bai et al., 2013; Demontis and Perrimon, 2010; Hwangbo et al., 2004). As a consequence, *dfoxo* is induced in the whole body, contributing to the enhanced lifespan. However, tissue-specific over-expression of *dfoxo* in the gut and fat body in a *dfoxo* null background extends lifespan to a similar extend as over-expression in a wild type background (Alic et al., 2014). That indicates that the lifespan extension caused by *dfoxo* over-expression in the gut/fat body does not require *dfoxo* induction in another tissue to increase lifespan (Alic et al., 2014). Moreover, induced transcriptional expression of *dfoxo* in the median neurosecretory cells

(MNC) by using a *dilp2*-GAL4 driver also extends lifespan. Interestingly, *Dilp2*, *3* and *5* levels are not reduced in these flies, indicating that the lifespan effect is independent of *dfoxo* induction in other tissues (Alic et al., 2014).

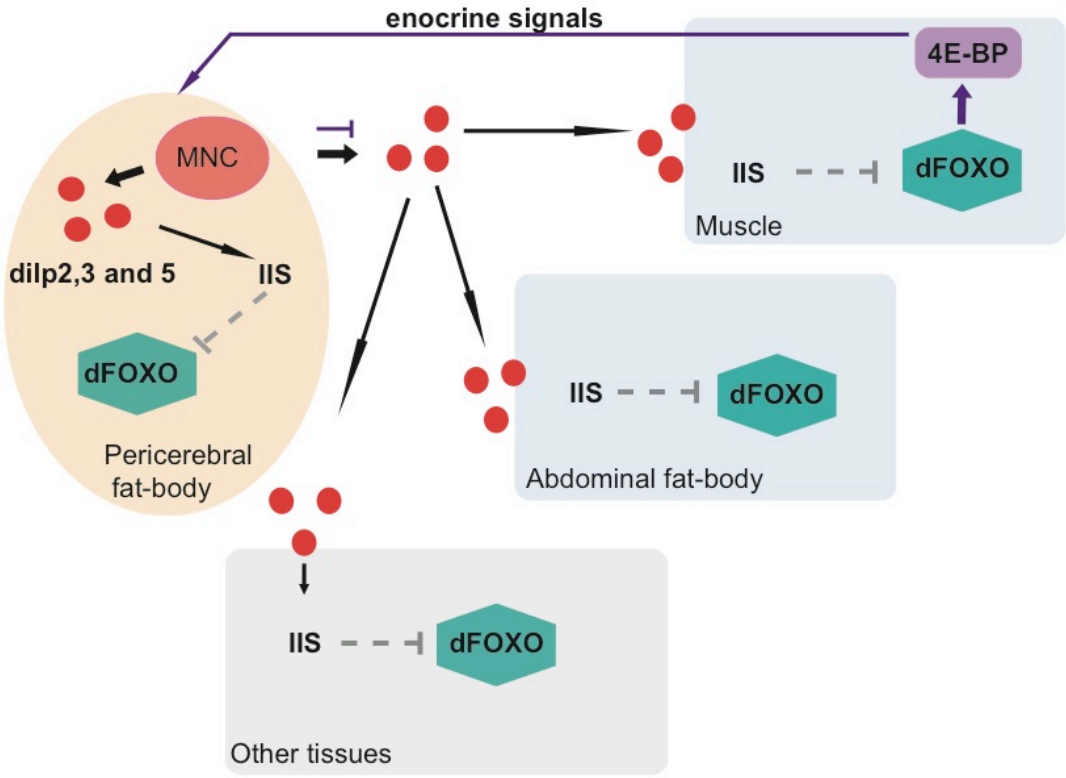


Figure 4.1.2 dFOXO regulation of longevity. Over-expression of *dfoxo* in the brain, gut/fat body or muscle extends lifespan in *Drosophila*. The lifespan effect of *dfoxo* over-expression in the muscle is non-autonomous and mediated via endocrine signals induced by 4E-BP (purple arrow). As a consequence *dilp2, 3, and 5* expression level is decreased, leading to reduced IIS in the whole body. Over-expression of *dfoxo* in the gut/fat body extends longevity in an autonomous manner. Moreover, *dfoxo* over-expression in the brain enhances longevity without decreasing insulin signalling in other tissues.

4.1.2 Target genes of DAF-16/dFOXO

DAF-16 and dFOXO represent the key worm/fly forkhead transcription factor downstream of IIS, regulating the expression of target genes. Its transcriptional activity is induced in response to reduced IIS, which is beneficial for longevity. Thus, it is of major interest to uncover FOXO target genes, and identify those that contribute to an extended lifespan in IIS-reduced animals. Genetic approaches in *C. elegans* (Oh et al., 2006) (Schuster et al., 2010) and in *Drosophila* adult female flies (Alic et al., 2011) have identified direct targets transcriptionally regulated by DAF-16 and dFOXO.

DAF-16 affects development, metabolism, fecundity, stress resistance and ageing. In agreement with its diverse biological functions, ChIP (Chromatin Immunoprecipitation) sequencing using wild type worms, *daf-2* mutants (as DAF-16 is enhanced located in the nucleus), and a *daf-16* null worms revealed 103 direct targets, which regulate various biological processes including detoxification, development, transcription, apoptosis and more (Oh et al., 2006). DAF-16 can function hereby either as an activator or a repressor and agrees with previous studies (Lee et al., 2003; Murphy et al., 2003; Oh et al., 2006). Another study using the DamID technique (DNA adenine methyltransferase identification) identified about 900 DAF-16 targets using DAM-tagged *daf-16* transgenic lines, and worms containing the DAM-tag without the gene, both fed with *daf-2* RNAi. (Schuster et al., 2010). Many genes identified in the previous study by ChIP-seq (Oh et al., 2006) were enriched among the ~900 genes identified by DamID. Moreover, data were cross-referenced to up-regulated genes in *daf-2* versus *daf-16;daf-2* worms (McElwee et al., 2007) and revealed 65 regulatory targets (Schuster et al., 2010). Here, results indicate that DAF-16 as an activator regulates only a small subset of targets. For example, no detoxification genes were identified as a direct target, suggesting that this process is not directly regulated by DAF-16, and moreover might be mediated by other regulators, which are induced by DAF-16 (Schuster et al., 2010).

In *Drosophila*, dFOXO effects are restricted to the xenobiotic response and longevity in reduced IIS flies. In contrast, fecundity, growth and oxidative stress are dFOXO-independent traits (Slack et al., 2011) (figure 4.1.3). Thus, the suggestion arose that dFOXO might retard the ageing process through increased detoxification. Indeed, many IIS long-lived flies show an enhanced resistance to xenobiotics.

In a ChIP-chip (microarray hybridization technique) study with adult female flies, more than 700 putative target genes of dFOXO were identified (Alic et al., 2011). Interestingly, the genomic location bound by dFOXO does not change in response to stress induced by paraquat or starvation, or in response to reduced insulin signalling by ubiquitous expression of the dominant-negative form of the *Drosophila* insulin receptor. However, dFOXO binding was enhanced at sites that were already dFOXO-bound.

In adult female wild type flies under normal conditions, dFOXO directly modulates the expression of 356 genes. Functional evaluation discovered dFOXO to act either as an activator or a repressor. Furthermore, target genes were identified that encode for transcription factors, indicating that a second layer of regulators might contribute to effects downstream of dFOXO. One of these identified regulators was the hormone nuclear receptor DHR96.
Furthermore, comparison studies between *Drosophila* and *C. elegans* revealed that dFOXO transcriptional regulation of the IIS response is evolutionally conserved. 121 conserved genes, including *dhr96* and *daf-12*, were uncovered that are direct targets of both DAF-16 and dFOXO (Alic et al., 2011).

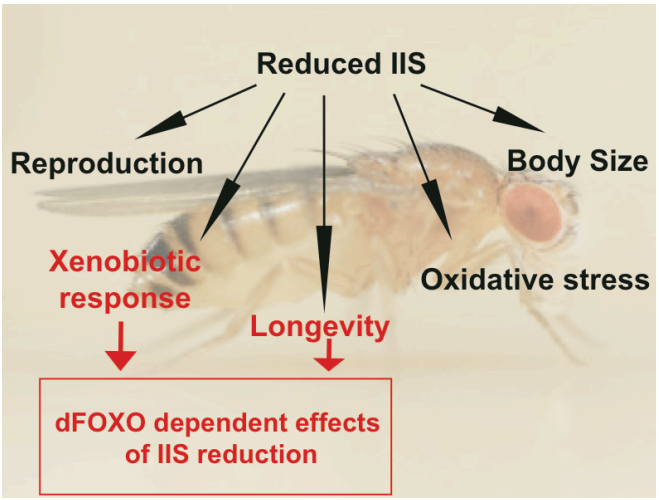


Figure 4.1.3. dFOXO dependent and independent effects in IIS-reduced flies. Reducing insulin signalling affects reproduction, body size, lifespan, xenobiotic response and oxidative stress. Lifespan and xenobiotic effects in IIS-reduced flies are dependent upon dFOXO, whereas effects in body size, reproduction and oxidative stress are independent upon dFOXO. Modified from Slack et al. (2011).

4.1.3 Xenobiotic response and Ageing

A variety of longevity studies in the last decade revealed genetic, pharmacological, and dietary interventions that extend lifespan in a range of model organisms. In many of these studies, long-lived animals were hallmarked by an increased cytoprotective response. Effectors of this response are involved in many different pathways, including the heat shock response, detoxification, inflammation, and anti-oxidation. A link between IIS signalling and detoxification was already proposed by Gems as the “Green Theory”, suggesting that ageing results from an accumulation of xenobiotic and endobiotic toxicity as a consequence of a declined detoxification response (Gems and McElwee, 2005). Thus, IIS-reduced mutants were predicted to

extend lifespan by an improved cytoprotective response as many detoxification genes are up-regulated.
The question arose if these protective mechanisms are required for the increased longevity or if they constitute a non-related consequence.

In *C. elegans*, analysis of long-lived *daf-2* mutants revealed that longevity requires the transcription factor Heat shock factor 1 (HSF-1) and DAF-16 (Hsu et al., 2003; Morley and Morimoto, 2004). HSF-1 is a key regulator of the heat-shock response, as it induces the expression of chaperones in response to stress to reduce proteotoxicity of protein aggregates. Furthermore, it is not only essential for *daf-2* longevity; over-expression of this effector itself also extends lifespan (Hsu et al., 2003). But not only chaperones are affected in *daf-2* mutant worms, microarray analysis uncovered genes that operate in the xenobiotic metabolism of toxic drugs, including *cytochrome p450 (cyp p450)* and *glutathione s-transferases (gsts)* (Gems and McElwee, 2005). A recent study discovered a set of cytoprotective genes (*hsp-6*, *hsp-4*, *gst-4* and *sod-3*) that is required for the lifespan extension in 3 long-lived mutants, including *daf-2* (reduced insulin signalling), *isp-1* (mitochondrial dysfunction), and *eat-2* (caloric restriction) mutants (Shore et al., 2012). This illustrates the importance of these defense systems in longevity.

In *Drosophila*, many long-lived IIS mutants are associated with a higher ability to detoxify xenobiotics or chemical drugs. Long-lived *dilp2,3,5*-mutants show increased resistance to DDT and H_2O_2 (Grönke et al., 2010). Ablation of the MNC, over-expression of a dominant negative version of the insulin receptor, or over-expression of dominant negative, catalytically inactive form of Type I *PI3-kinase*, which all reduce insulin signalling, extend lifespans and enhance resistance to paraquat and DDT (Slack et al., 2011). These effects are dependent on the transcription factor dFOXO.

Also mammalian longevity studies enhance the link between ageing and xenobiotic metabolism. Mutation of the *Snell dwarf* gene extends lifespan in mice by reducing growth hormone (GH) and IGF-1 signalling. Gene expression profiles from the liver of long-lived dwarf male mice revealed an up-regulation of

many detoxification genes (Amador-Noguez et al., 2004). Further analysis on fibroblast of long-lived Snell dwarf mutant mice showed increased level of *Nrf2* (NF-E2-Related Factor 2), a key transcription factor of detoxification genes (Leiser and Miller, 2010).

4.1.4 Project approach

Xenobiotic resistance is improved in many long-lived IIS-reduced animals, and in *Drosophila* increased lifespan and xenobiotic response of IIS-reduced flies require the activity of dFOXO (Slack et al., 2011).

Interestingly, dFOXO is required for basal *dhr96* expression levels (Alic et al., 2011). *Dhr96* over-expression extended lifespan and increased xenobiotic resistance in females, thus it might represent a crucial target of dFOXO, necessary for both increased xenobiotic resistance and longevity in IIS-reduced flies. However, expression of *dhr96* is not altered in IIS-reduced flies. Subsequently, dFOXO does not confer xenobiotic resistance and lifespan extension by inducing *dhr96* transcription, at least not in these circumstances. It could be that dFOXO induces *dhr96* expression in specific tissues or at later stages, or affect co-factors or the DHR96 ligand. It could also be that the increased xenobiotic resistance is mediated by other transcription factors and DHR96 activity is not required. Moreover, it is not clear whether xenobiotic response and longevity effects are dependent or not, but in *C. elegans* such a casual connection between IIS-reduced longevity and cytoprotective factors were shown.

Therefor, to investigate whether lifespan extension and the improved xenobiotic response of *dfoxo* over-expressing flies is mediated via *dhr96*, two different approaches were performed (1-2). A third approach was conducted to investigate whether DHR96 might modulate dFOXO activity.

1. *dfoxo* was over-expressed in a *dhr96* null background
2. MNC was ablated in a *dhr96* null background.
3. *Dhr96-lbd* as the robust allele has been over-expressed in *dfoxo* null flies.

(1-2) Over-expression of *dfoxo* in the muscle tissue or the ablation of the MNC extends lifespan and increases xenobiotic response. If the enhanced lifespan and stress resistance is conferred by DHR96, these effects should be lost in a *dhr96* null background.

(3) Over-expression of *dhr96* and *dhr96-lbd* resulted in an extended lifespan and increased xenobiotic resistance. If DHR96 acts downstream of dFOXO, phenotypes of *dhr96-lbd* over-expressing flies should not be affected in a *dfoxo* null background.

4.2 Materials and methods

4.2.1 Fly stocks

For epistatic analysis, transgenic flies were used to make flies with the relevant combination of mutants. The crossing scheme was dependent on the genomic location of the UAS- or driver-constructs, and of null mutation of interest. If genomic locations were on the same chromosome, flies had to be recombined by homologous recombination.

The UAS-*dhr96lbd* line in a *dfoxo*$^{\Delta 94}$ background was generated and provided by Matt Piper (UCL, London).

4.2.2 Generation of a *daughterless* GS driver in a *dfoxo*$^{\Delta 94}$ background

Daughterless GS flies are marked by red eyes, whereas *dfoxo*$^{\Delta 94}$ flies are white-eyed. The driver is inserted on the second chromosome and was therefore balanced over CyO. The deletion of *dfoxo* in *dfoxo*$^{\Delta 94}$ flies is located on the third chromosome, and thus was balanced with TM3Sb. Balanced flies were crossed together. To distinguish *daughterless* GS flies in a *dfoxo*$^{\Delta 94}$ background to *daughterless* GS flies in a normal wild type background, genomic DNA was extracted from the leg of single flies and PCR was performed with specific *dfoxo* primers (see below). Positive flies as shown in supplement S.4.1 were used for further crossings.

4.2.3 DNA extraction from *Drosophila melanogaster* leg and PCR

The middle leg was removed from live *Drosophila* flies using forceps, and these flies were transferred to single vials. Each leg was placed into a PCR tube filled with 50µl lysis buffer (squishing buffer with 0,2µg/µl protein kinase K (Roche), supplement S.1.1). For the extraction of genomic DNA, samples were set in the thermo cycler (Applied Biosystems) with the program shown in table 4.2.1.

Temperature	Time
65 °C	**60 minutes**
95 °C	**10 minutes**
4 °C	**Until removal**

Table 4.2.1 Thermo cycler program for genomic DNA extraction of *Drosophila* leg.

For genotyping purposes, PCR of extracted DNA was performed by using HotStarTaq *Plus* DNA Polymerase (Qiagen), following the protocol in chapter 2.1.1.1. Primers that were used to distinguish between *dfoxo*$^{\Delta 94}$ (Sol 236 and Sol 238) and wild type (Sol 236 and Sol 237) flies, are displayed in table 4.2.2.

Primer	Sequence
Sol 236	TTGCCGCTGACAATTATGATCAAG
Sol 237	AAGGTAGTGCCTATGATCCAG
Sol 238	GAGAACAACAAGAAGATAAGTCCGCC

Table 4.2.2 Genotyping primer for *dfoxo* wild type and *dfoxo* null flies. Sol 236 and Sol 237 are primers for wild type *dfoxo*, resulting in a fragment of a size of 600bp. Sol 236 and Sol 238 are primers to identify *dfoxo* null flies by giving a fragment at a size of 350bp.

PCR samples were analysed by agarose gel electrophoresis. Wild type *dfoxo* produces fragments at a size of 350bp using Sol 236 and Sol 237 primers. Flies carrying the *dfoxo*$^{\Delta 94}$ mutation show a DNA fragment at a size of 650bp using Sol 236 and Sol 237 primers. In a final crossing 25 positive *dfoxo*$^{\Delta 94}$ virgin females were crossed with 25 positive *dfoxo*$^{\Delta 94}$ male flies to produce homozygous offspring.

4.2.4 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 red colored eyes 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 (King-Jones et al., 2006) 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.

4.2.5 Generation of UAS-*reaper* and *dilp2*-GAL4 in a *dhr96* null background

The crossing for *dilp2*-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 balanced by an X-chromosome balancer (FM6).

4.2.6 Lifespan and fecundity

Lifespan and fecundity assays were set up as described in chapter 3.2.2 and 3.2.7. For the over-expression of *dfoxo* in a normal or in a *dhr96* null background, a constitutive muscle-specific driver was used. For the MNC ablation, a constitutive driver for the median neurosecretory cells, the *dilp2*-GAL4 driver, was used (Broughton et al., 2010). Due to the use of a constitute driver in these experiments, no induction by the drug RU486 was required and flies were sorted into 1x SYA glass vials. Lifespan experiment were conducted on 1x SYA food, as this type of SYA food was used in published longevity studies of MNC-ablated flies (Broughton et al., 2005) and *dfoxo* over-expression in the muscle (Demontis and Perrimon, 2010).

4.2.7 Stress assays

Stress assays were conducted as summarized in chapter 3.2.3. Due to the sensitivity of some fly lines, lower concentrations of DDT (Dichlordiphenyl-trichlorethan, Greyhound) were used for the DDT assay: 175mg/L or 100mg/L.

4.2.8 Quantitative real-time PCR analysis of dissected tissues (thorax and brain)

To ensure over-expression of *dfoxo* in muscle tissue, or ablation of the MNC in the brain, whole flies were transferred to microcentrifuge tubes and snap frozen in liquid nitrogen. Using a lab prepared sieve, frozen thorax (for muscle) and heads (for brain) were separated from the whole fly and stored at -80 °C. qRT-PCR was set up as described in chapter 2.1.2, and the RT-primers that were used are listed in supplement S.2.2.

4.3 Results

4.3.1 Functional analysis of *dfoxo* over-expressing flies in a *dhr96* null background

4.3.1.1 Lifespan extension of *dfoxo* over-expression in the muscle was not dependent upon DHR96

Muscle-specific over-expression of *dfoxo* in a wild type background significantly extended lifespan in females when compared to its driver and UAS-*dfoxo* controls (Log Rank Test, p-value ***<0.001) (figure 4.3.1 A). Median lifespan of *dfoxo* over-expressing flies (74,5 days) was increased by 7,1% compared to the UAS-*dfoxo* control (69,5 days) and by 3,5% compared to *mhc*-GAL4 control flies.

In a *dhr96* null background, over-expression of *dfoxo* in muscle revealed a significant lifespan extension compared to its driver- or the UAS-*dfoxo* control (Log Rank Test, p-value ***<0.001). Median lifespan of *dfoxo* over-expressing flies in a *dhr96* null background (74,5 days) was extended by 14,6% compared to its UAS-control (65 days) and by 11,2% compared to its driver control (67 days) (figure 4.3.1 B).
Control transgenic lines in a *dhr96* null background were short-lived compared to the same lines in a wild type background. Median lifespan of UAS-*dfoxo* was reduced by 6,9% and of *mhc*-GAL4 by 7,4%. Median lifespan of UAS-*dfoxo*/*mhc*-GAL4 was not changed by removal of genomic *dhr96*.

Interaction analysis using Cox proportional hazards (CPH) revealed that genomic *dhr96* (p-value ***<0.001) and over-expression of *dfoxo* (p-value ***<0.001) significantly affected lifespan, but these effects did not show a significant interaction (p-value=0.09) (supplement S.4.2). Thus, the degree on lifespan effect was not affected between the two *dhr96* backgrounds (*dhr96* versus *dhr96* null).

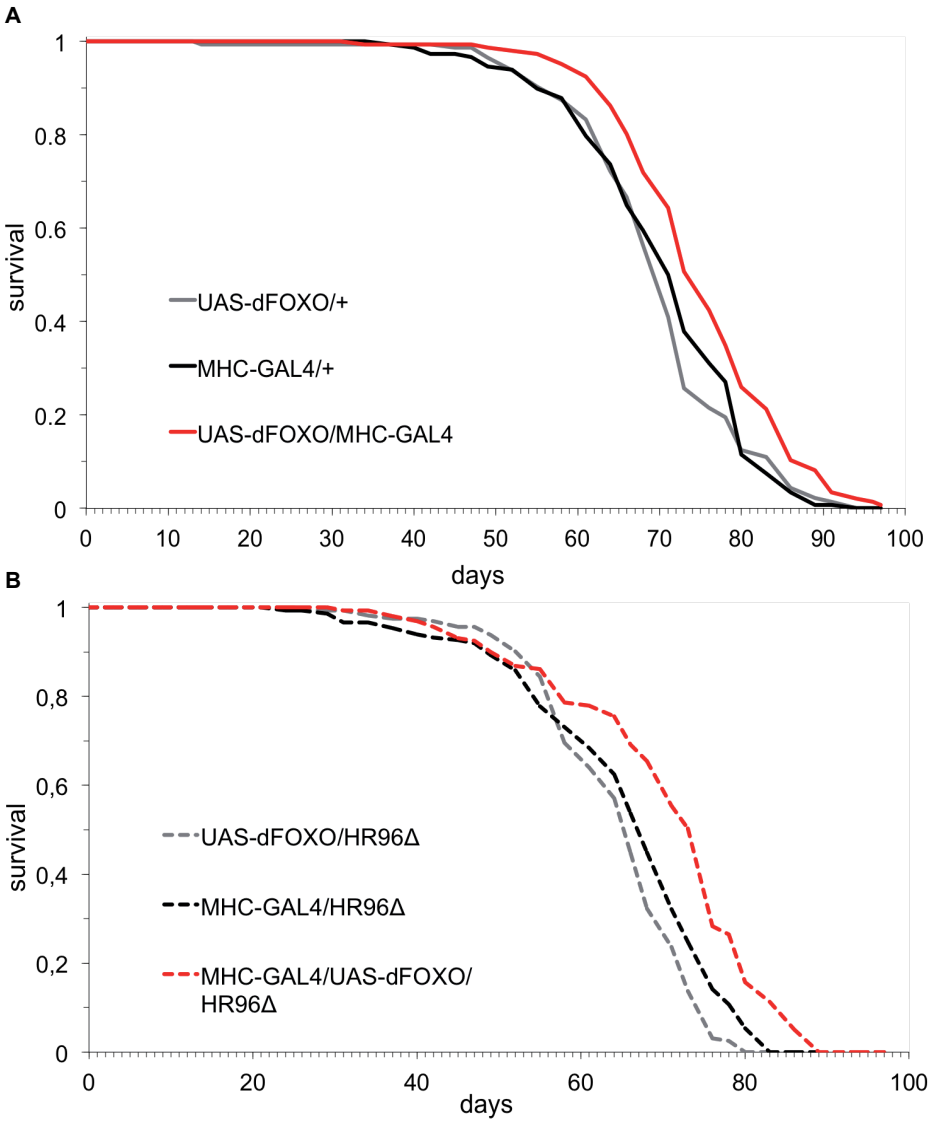


Figure 4.3.1 Lifespan of *dfoxo* over-expressing flies in a wild type and in a *dhr96* null mutant background. (A) Over-expression of *dfoxo* in the muscle significantly extended longevity in *Drosophila* females (Log Rank Test, p-value ***<0.001). (B) Lifespan was also increased when *dfoxo* was over-expressed in a *dhr96* null background (Log Rank Test, p-value ***<0.001).

4.3.1.2 DDT stress resistance of *dfoxo* over-expressing flies required *dhr96* expression

Flies in a *dhr96* null background were sensitive to the usual DDT concentration used, 275 mg/L, compared to flies in a wild type background (pre-experiments, data not shown). DDT assay was therefore performed on a lower concentration for transgenic lines in *dhr96* null background of 150mg/L. Over-expression of *dfoxo* in the muscle of female flies significantly increased resistance to the

xenobiotic DDT compared to the diver control or the UAS-*dfoxo* control (Log Rank Test, p-value ***<0.001) (figure 4.3.2 A). Increased DDT resistance was lost when *dfoxo* was over-expressed in a *dhr96* null background (figure 4.3.2 B). A direct interaction test by statistical analysis was not possible, as the two DDT assays were performed on two different DDT concentrations.

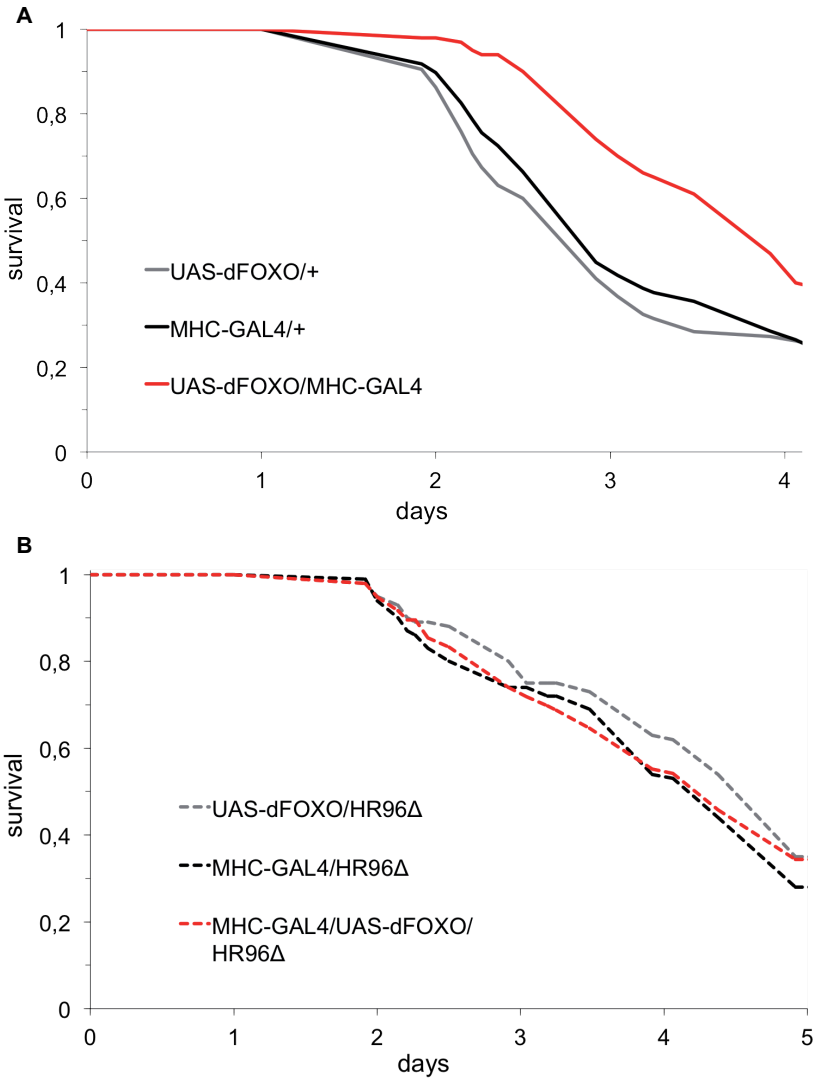


Figure 4.3.2 DDT stress assay of *dfoxo* over-expressing flies in a wild type and in a *dhr96* null mutant background. (A) Over-expression of *dfoxo* in the muscle increased resistance to DDT (275mg/L) (Log Rank Test, p-value ***<0.001). (B) Enhanced DDT resistance was lost, when *dfoxo* was over-expressed in a *dhr96* null background (150 mg/L).

4.3.2 Functional analysis of MNC ablation in a *dhr96* null background

4.3.2.1 Lifespan extension of MNC-ablated females was not dependent upon DHR96

Ablation of the MNC by over-expressing *reaper* with the *dilp2* specific GAL4-driver significantly extended lifespan in female flies (Log Rank Test, p-value ***<0.001) (figure 4.3.3 A). Median lifespan of MNC-ablated flies was enhanced by 11,1% compared to the UAS-*rpr*-control, and by 5,6% compared to the *dilp2*-GAL4 control.

Transgenic flies in a *dhr96* null background were significantly short-lived compared to the same lines in a wild type background: UAS-*rpr* by 8%, *dilp2*-GAL4 by 11% and UAS-*rpr*/*dilp2*-GAL4 by 7,1% (Log Rank Test, p-value ***<0.001).
Lifespan was also significantly increased when MNC were ablated in *dhr96* null female flies. In a *dhr96* null background, median lifespan of MNC-ablated flies was extended by 12% compared to the UAS-*rpr* control and by 7,6% compared to the *dilp2*-GAL4 driver control (Log Rank Test, p-value ***<0.001) (figure 4.3.3 B).

Statistical analysis by CPH revealed that genomic *dhr96* (p-value ***<0.001) and MNC ablation (p-value ***<0.001) had significant effects on lifespan, but these effects did not show any significant interaction (p-value=0.305) (supplement S.4.2).

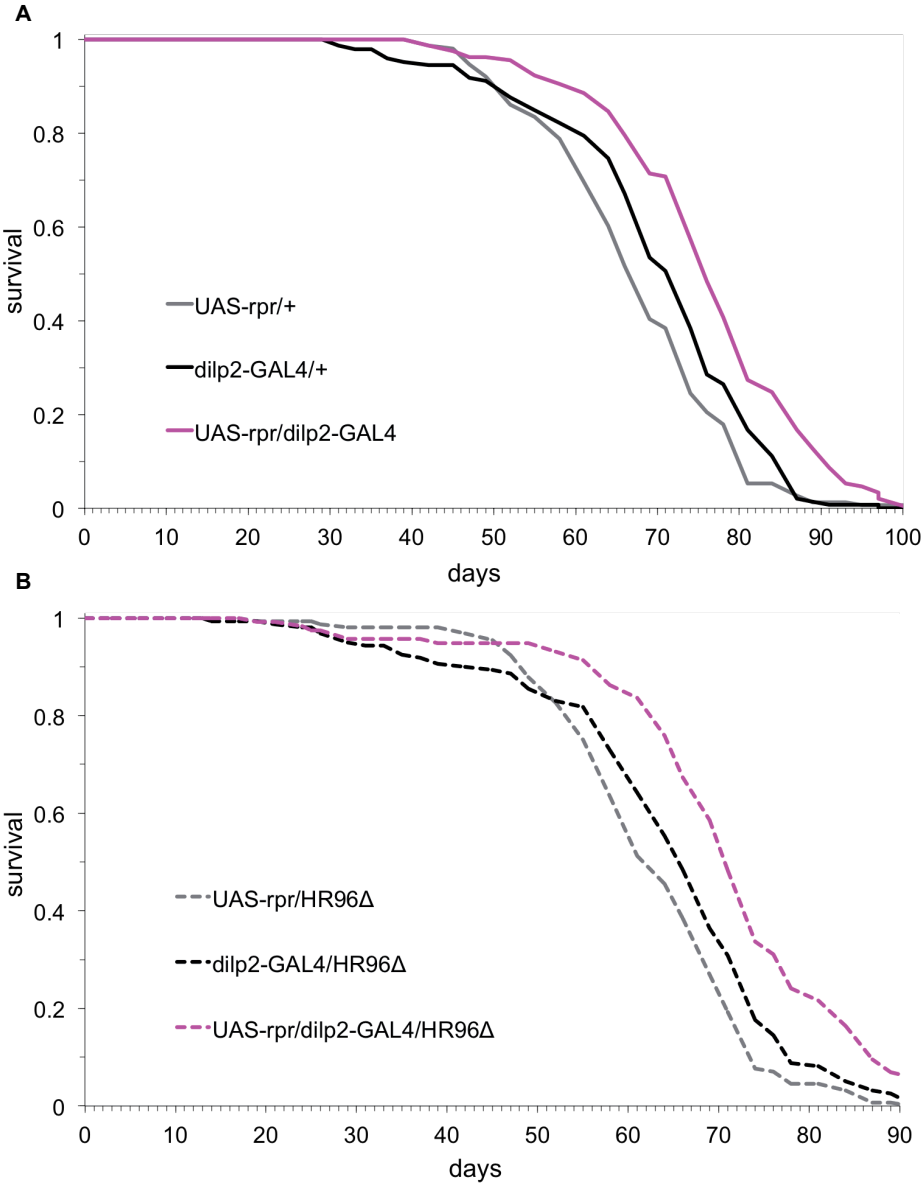


Figure 4.3.3 Lifespan of MNC-ablated flies in a wild type and in a *dhr96* null mutant background. MNC-ablated flies revealed a lifespan extension in both (A) a wild type and (B) a *dhr96* null background compared to their driver and UAS-controls (Log Rank Test, p-value ***<0.001).

4.3.2.2 Phenobarbital stress resistance of MNC-ablated flies required *dhr96* expression

Treatment of MNC-ablated flies in a wild type background with the drug phenobarbital resulted in a significant increase in survival compared to its UAS- and driver-control (Log Rank Test, p-value ***<0.001) (figure 4.3.4). This

increase in phenobarbital resistance disappeared when MNC was ablated in a *dhr96* null background.

Statistical analysis (CHP) revealed that the *dhr96* background (*dhr96* versus *dhr96* null) significantly affected lifespan, and that the two different responses between the two *dhr96* backgrounds were significant (p-value=0.0012).

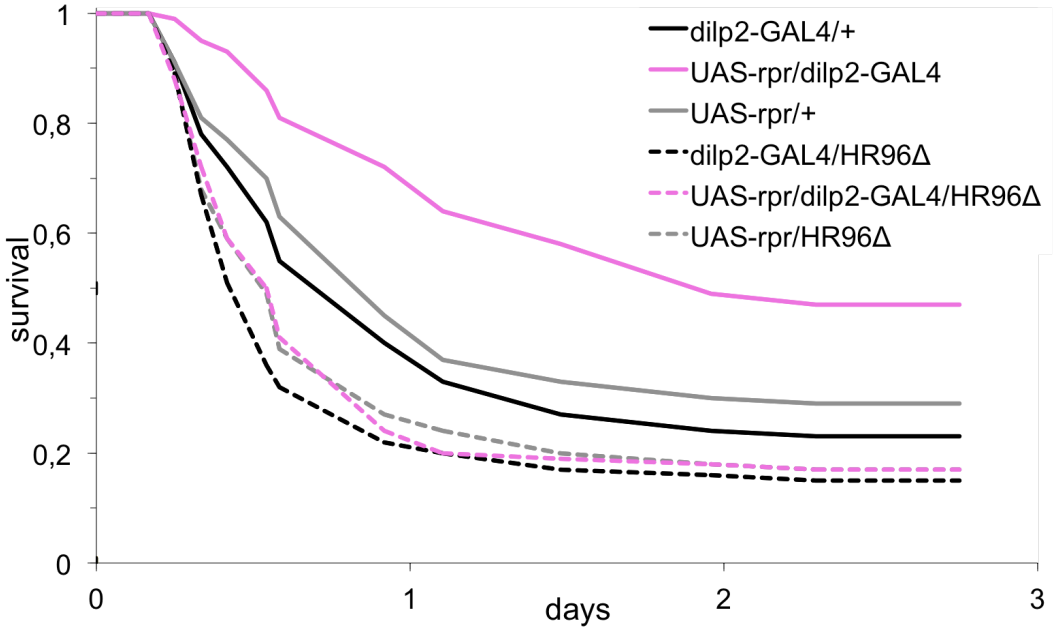


Figure 4.3.4 Phenobarbital stress assay of MNC-ablated flies in a wild type and in a *dhr96* null mutant background. Ablation of the MNC resulted in an increased resistance to the drug phenobarbital (Log Rank Test, p-value ***<0.001). This enhanced resistance was lost when MNC were ablated in a *dhr96* null background.

4.3.3 *Dhr96-lbd* over-expression in a *dfoxo* null background

4.3.3.1 Lifespan-extension of *dhr96-lbd* over-expressing females was not dependent upon *dfoxo*

Over-expression of *dhr96-lbd* in a wild type background (solid red line) resulted in a significant lifespan extension (Log Rank Test, p-value ***<0.001) (figure 4.3.5). Median lifespan in *dhr96-lbd* over-expressors (70,5 days) was extended by 12,8% compared to its ethanol control (62,5 days).

Over-expression of *dhr96-lbd* in a *dfoxo* null background significantly increased lifespan (Log Rank Test, p-value ***<0.001). Median lifespan (35 days) of this transgenic line was increased by 16,6% when compared to its ethanol control

(30 days). The experiment was repeated and confirmed that results were reproducible.

Cox proportional hazard (CPH) interaction test was used to determine whether the degree of lifespan extension between the two *dfoxo* backgrounds (*dfoxo* versus *dfoxo* null) was significantly affected. This statistical analysis revealed that the *dfoxo* background (p-value ***<0.001) and *dhr96-lbd* over-expression (p-value ***<0.001) significantly affected lifespan, but the two status did not show a significant interaction in their lifespan effects (p-value= 0.621) (supplement S.4.2).

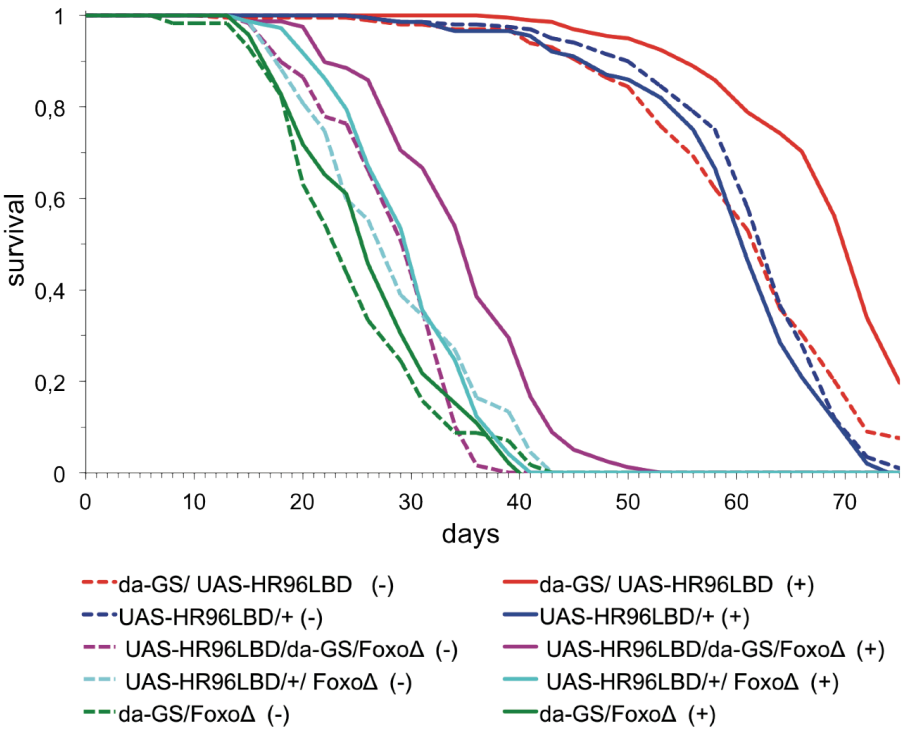


Figure 4.3.5 Lifespan of *dhr96-lbd* over-expressing flies in a wild type and in a *dfoxo* null mutant background. Over-expression of *dhr96lbd* resulted in an extension of longevity compared to controls (ethanol and UAS-controls). This effect remained when over-expression was conducted in a *dfoxo* null background (Log Rank Test, p-value ***<0.001).

Over-expression of *dhr96-lbd* in a wild type background reduced fecundity when compared to controls (figure 4.3.6, figure 3.3.4). The *dfoxo* null background decreased fecundity in all transgenic lines when compared to wild type flies. Over-expression of *dhr96-lbd* in a *dfoxo* null background did not reduce

fecundity to a greater extent than in *dfoxo* null control flies (Kruskal-Wallis test, Dunn's test for multiple comparisons).

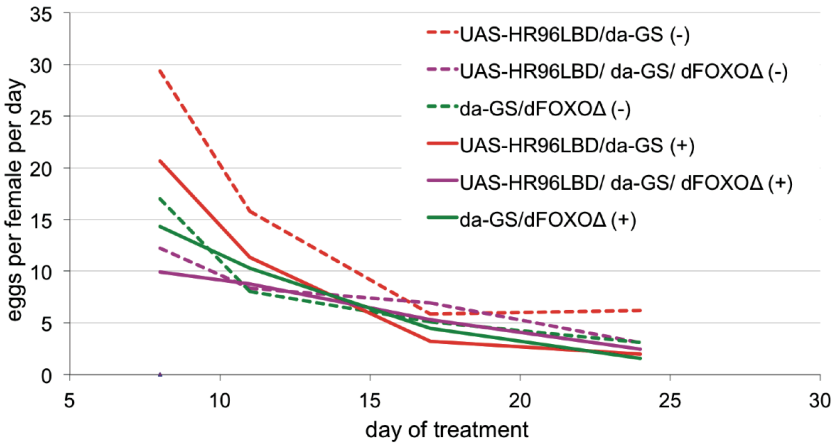


Figure 4.3.6 Fecundity of *dhr96-lbd* over-expressor in a wild type and in a *dfoxo* null mutant background. Over-expression of *dhr96-lbd* reduced fecundity compared to its ethanol control (Log Rank Test, p-value ***<0.001). Fecundity was also reduced in *dfoxo* null flies (Log Rank Test, p-value ***<0.001). Over-expression of *dhr96-lbd* in a *dfoxo* null background did not show significant changes compared to *dfoxo* null control flies.

4.3.3.2 DDT resistance of *dhr96-lbd* over-expressing flies was not wholly dependant on dfoxo

Treatment with DDT at a usual concentration of 275mg/L in 1.5x SYA food revealed an significant increase in DDT resistance for *dhr96-lbd* over-expressing flies in a wild type background, but not for *dhr96-lbd* over-expressing flies in a *dfoxo* null background (supplement S.4.3). As *dfoxo* null flies were strongly sensitive to DDT, the experiment was repeated with a lower DDT concentration (100mg/L DDT in 1.5x SYA food). In a *dfoxo* null background, flies with induced *dhr96-lbd* over-expression showed significantly increased DDT resistance compared to non-induced ethanol controls or controls on RU (Log Rank Test, p-value ***<0.001) (figure 4.3.7).

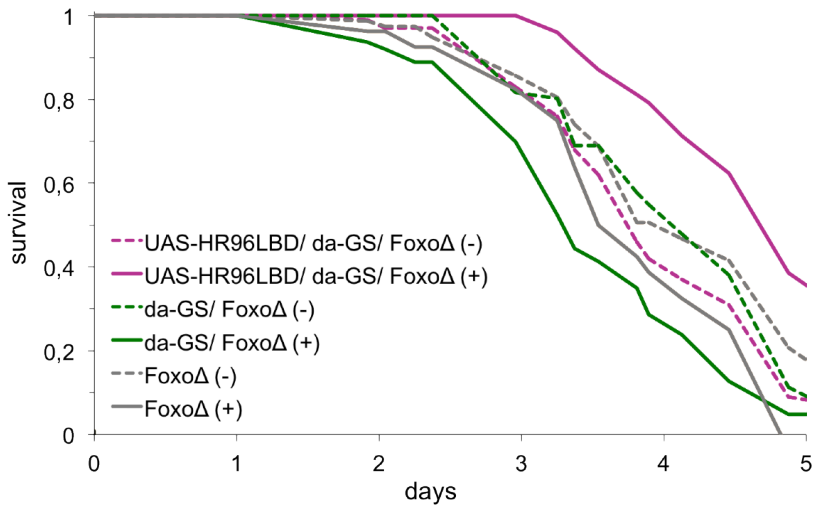


Figure 4.3.7 DDT stress assay (100mg/L) of *dhr96-lbd* over-expressing flies in a *dfoxo* null mutant background. *Dhr96-lbd* over-expressing flies in a *dfoxo* null background showed enhanced DDT resistance compared to RU and ethanol controls (Log Rank Test, p-value ***<0.001).

4.4 Discussion

4.4.1. *Dhr96-lbd* over-expression in *dfoxo* null flies

Over-expression of *dhr96-lbd* in a *dfoxo* null background significantly extended lifespan (figure 4.3.5) and increased DDT resistance (figure 4.3.7). Statistical analysis (CHP) on the lifespan effects between the two different backgrounds (with and without genomic *dfoxo*) revealed no significant interaction. Thus, longevity of *dhr96-lbd* over-expressing flies was not affected upon dFOXO activity.

Stress resistance was increased also in a *dfoxo* null background but direct comparison by statistical interaction were not tested, as experiment was performed on very low DDT concentration. However, results indicated that DDT resistance was not wholly dependent on genomic *dfoxo* expression.

Flies harbouring the *dfoxo* null mutation are strongly short-lived and sensitive to DDT. Although *dhr96-lbd* over-expression enhanced longevity and DDT resistance in a *dfoxo* null background, it did not restore these to levels seen in flies with a wild type background. That indicates that dFOXO affects a broad of additional genes or pathways, which show no dependency upon DHR96 signalling and are additionally important for modulating these lifespan and DDT resistance. This is in agreement with ChIP-chip of wild type and *dfoxo* null flies, showing that expression of more than 2000 genes was altered by removal of *dfoxo* (Alic et al., 2011). This modulation of target gene expression signifies the importance of dFOXO for ordinary maintenance.

4.4.2 Stress resistance of long-lived IIS-reduced flies in *dhr96* null flies

Ablation of the MNC, or over-expression of *dfoxo* in the muscle tissue both extended lifespan in agreement with published data (Broughton et al., 2010; Demontis and Perrimon, 2010). Increased DDT resistance in IIS-reduced flies was previously shown for *dilp2,3,5*-mutants (Grönke et al., 2010), MNC-ablated, flies over-expressing a dominant negative version of the insulin receptor, and

flies over-expressing a dominant negative, catalytically inactive form of *PI3-kinase* (Slack et al., 2011).

The increased DDT resistance for *dfoxo* over-expressing flies in the muscle tissue is novel. DDT detoxification in *Drosophila* occurs mainly in tissue with known xenobiotic function, including the fat body, gut, Malphigian tubules, and salivary glands. This supports the assumption of tissue-tissue communication. For longevity muscle-specific over-expression of *dfoxo* induces 4E-BP, which in turn reduces insulin signalling in the whole body by endocrine signalling (Demontis and Perrimon, 2010). Thus, it might be that in muscle-specific *dfoxo* over-expressing flies, endocrine signals from the muscle induce xenobiotic response in distal tissues.

Interestingly, the increased DDT resistance of *dfoxo* over-expressing flies was lost in a *dhr96* null background. This clarifies that dFOXO modulation of DDT resistance is dependent on DHR96 function. Results in chapter 3.3.3 indicated that *Cyp6g1* might mediate DDT resistance of *dhr96-lbd* over-expressing flies in the gut and Malphigian tubule. This is in agreement with a study showing that *Cyp6g1* expression is regulated by a tissue-specific enhancer in the gut and Malphigian tubule (Chung et al.). These findings indicate that muscle-specific *dfoxo* over-expression might promote stress resistance via direct or indirect tissue-tissue communication between the muscle and the gut/ Malphigian tubules.

An additional drug was tested in the MNC ablation epistasis experiment, namely the xenobiotic phenobarbital (PB). This drug was one of the first xenobiotics shown to induce expression of detoxification genes (Conney, 1967). Interestingly, the mammalian DHR96 homologues CAR and PXR are key regulators of the PB induced xenobiotic response (Honkakoski et al., 1998; Sueyoshi and Negishi, 2001). Treatment with PB causes nuclear translocation of CAR and PXR, and induces their transcriptional activity as heterodimers with the retinoid X receptor (CAR-RXR, PXR-RXR) (Sueyoshi and Negishi, 2001). The CAR and PXR response to PB is conserved in *Drosophila*, where DHR96 is also involved in the PB response. *Dhr96* null flies are sensitive to treatment with phenobarbital, implying that DHR96 function might be required to metabolize this xenobiotic (King-Jones et al., 2006). Furthermore, microarray analysis of

phenobarbital treated wild type and *dhr96* null flies revealed that there is a overlap of detoxification genes that are altered upon PB treatment and regulated by DHR96 (King-Jones et al., 2006). One of the PB-inducible genes is *Cyp6d1* (Scott et al., 1996), and a promoter assay of this gene revealed a putative binding site for the *Drosophila* Broad complex (BR-C) (Lin et al., 2010). *Br-c*, which encodes for an ecdysone responsive gene, and *dhr96* RNAi experiments using the *Cyp6d1* promoter assay, showed that these two transcription factors regulate *Cyp6d1* induction in response to PB; BR-C as a negative and DHR96 as a positive regulator (Lin et al., 2010).

As is the case for *dhr96-lbd* over-expressing flies, MNC-ablated flies showed enhanced resistance to phenobarbital. This resistance disappeared when MNCs were ablated in a *dhr96* null background, indicating that phenobarbital resistance in IIS-reduced flies is dependent upon DHR96. This conclusion enhances published findings that DHR96 is, like CAR and PXR, a key regulator in PB induced xenobiotic response. My results would also agree with the idea that DHR96 represents an activator in PB induced xenobiotic response.

4.4.3 Longevity of IIS-reduced flies in a *dhr96* null background

In contrast to DDT and phenobarbital stress resistance, lifespan extension of IIS-reduced flies did not require DHR96 activity. Lifespan of MNC-ablated and *dfoxo* over-expressing flies remained extended in a *dhr96* null background. These results separate DHR96-dependent and independent effects. Whereas DDT and phenobarbital resistance in IIS-reduced flies require DHR96 activity, the lifespan effect was mediated through other regulators. For dFOXO longevity, xenobiotic regulators are suggested to play an important role, as lifespan and xenobiotic response are dFOXO dependent effect in IIS reduced flies (Slack et al., 2011). Although DHR96 does not mediate this link, dFOXO regulates many other genes, which might modulate a cytopreotective mechanism to extend lifespan. One prominent key regulator in detoxification regulation is Nrf2 (NF-E2-Related Factor 2). In *Drosophila,* CncC (cap 'n' collar isoform-C) is the Nrf2 ortholog (Misra et al., 2011). Nrf2/CncC is a transcription factor, regulating the expression of detoxification enzymes and suggested to have anti-ageing function (Sykiotis and Bohmann, 2010). In a non-induced

condition, Nrf2 is located in the cytoplasm, where it interacts with the E3-ligase KEAP-1 (Kelch-like ECH-Associated Protein 1). This protein promotes the proteasomal degradation of Nrf2. Stress induces the release of Nrf2 from KEAP-1 and its translocation into the nucleus, where it induces the expression of target genes (Nguyen et al., 2009). Epistatic analysis between CncC and insulin signalling might help to understand if there is a correlation.
Interestingly, in *C. elegans* such an interaction between the Nrf2 analog SKN-1 and IIS signalling is shown (Tullet et al., 2008). Reduced insulin signalling promotes SKN-1 nuclear translocation and its transcriptional activity to enhance stress tolerance. However, although SKN-1 contributes to stress resistance in IIS-reduced worms, constitutive active SKN-1 increases longevity independently of DAF-16.

For DHR96 itself, it seems that the xenobiotic response and lifespan does not seem to be coupled. One indication is that *dhr96-lbd* over-expressing male flies, as shown in chapter 3.3.4, did not show enhanced lifespan although stress resistance was increased. Furthermore, over-expression of *Cyp6g1* did not extend lifespan, but increased DDT resistance, implying that this cytochrome gene is not essential for longevity (Matt Piper, unpublished data). In addition, N-terminally tagged *Flag-Strep-6xHis-dhr96* (*tagged-dhr96*) over-expressing flies (provided by Teresa Niccoli), which were planned for ChIP sequencing experiments, were used for functional analysis (supplement S.3.6). Over-expression of *tagged-dhr96* or *tagged-dhr96-lbd* using a *daughterless* GS driver did not increase lifespan, but enhanced DDT resistance. Thus, the tag (in frame to *dhr96* DNA binding domain) interfered in DHR96 function important for longevity but not for xenobiotic resistance.

In *C. elegans*, the relationship between DAF-12 and DAF-16 or DAF-2 is very complex, as DAF-12 longevity is already intricate. Different conditions can lead to opposing effects on longevity in *daf-12* mutants. Signals from the germ line and temperature are critical factors and were discussed in chapter 3.1.2. Furthermore, lifespan can be extended in a ligand-dependent and in a ligand-independent manner, which in turn is again dependent on environmental conditions. These different regulatory mechanisms in combination with tissue-

tissue signalling create a complex network, which poses a challenge to analyse and compare to *Drosophila* data.

The relationship between DAF-2 and DAF-12 was studied using different *daf-2* alleles. Several EMS generated *daf-2* alleles were investigated and dependent on their phenotype, alleles were sorted into two pleiotropic classes, which are summarized in supplement S.4.4. It was shown that *daf-12* affects longevity of *daf-2* mutants in an allelic-dependent manner. As an example, the non-null allele *daf-12 (m20)* (Loss of function, decreased lifespan) enhances longevity of *daf-2(e1370)* mutants (mutation in tyrosine kinase domain) but shortens lifespan of *daf-2(e1368)* mutants (mutation in ligand-binding domain) (Gems et al., 1998; Patel et al., 2008). Nevertheless, a recent study focused on epistatic analysis between DAF-2 and ligand-bound or ligand-unbounded DAF-12 (Dumas et al., 2013). The results indicated that the DAF-12 effect on *daf-2* longevity is also dependent upon whether DAF-12 is bound to the ligand or not. Thus, it is important to test also wild type *dhr96* in epistatic analysis.

Although the relationship between DAF-12 and DAF-16 is not fully understood, there is a co-dependency between the two regulators. DAF-12 signalling stimulates nuclear translocation of DAF-16, but constitutively nuclear DAF-16 is dependent on DAF-9, but not DAF-12 (Berman and Kenyon, 2006). This indicates that DAF-12 might have ligand-independent activities in worms (Antebi, 2013). In relation to possible ligand-dependent and ligand-independent DHR96 activities, it might be interesting, to test, whether over-expression of wild type or LBD-mutated *dhr96* induces dFOXO nuclear translocation, for example in S2 cells.

In induced IIS signalling, phosphorylated AKT kinase phosphorylates dFOXO leading to nuclear exclusion (Brunet et al., 1999). Reduced insulin signalling induces dFOXO nuclear translocation. Thus, the phosphorylation level of AKT and dFOXO in response to *dhr96* over-expression might be a further alternative to test DHR96 effects on dFOXO subcellular localization.

4.4.4 Conclusion

My results indicate that phenobarbital and DDT resistance in IIS-reduced flies is dependent upon DHR96 activity whereas longevity of *dhr96-lbd* over-expressor and IIS-reduced flies did not affect each other. Stress resistance results were obtained by feeding assays. As in chapter 3 discussed, feeding behavior might be affected in *dhr96-lbd* over-expressing flies. Thus, it is also here important to conduct stress assays via non-feeding assays to ensure results.

It is of major interest to uncover the different mechanisms that affect lifespan in DHR96 and in IIS signalling. ChIP sequencing analysis of *dhr96-lbd* over-expressing flies will help to attain a better understanding of DHR96 regulation and uncover target genes, which might mediate longevity. Moreover, epistatic analysis of IIS-reduced flies in a *dhr96* null background with a broader range of xenobiotics or oxidative stressors might help to separate detoxification processes that might not mediate longevity in IIS-reduced flies.

Chapter 5 Bioinformatic analysis of *dhr96* transcript profiles and qRT-PCR analysis of candidate genes

5.1 Introduction

Over-expression of *dhr96* and *dhr96-lbd* using the *daughterless* GS driver extended lifespan and increased xenobiotic resistance in females. However, the mechanisms leading to these phenotypes are not known. Thus, the next priority is to identify DHR96 downstream targets that might confer longevity and stress resistance.

In Chapter 4, *Cyp6g1* was indicated to confer DDT resistance in *dhr96* and *dhr96-lbd* over-expressing females. As *dhr96* and *dhr96-lbd* over-expression modulated other stress responses in addition, it is of interest to identify further DHR96 regulated detoxification genes (*cyps* and *gsts*).

Moreover, cytochrome enzymes play a major role in hormone metabolism. For the *dhr96* homologue gene *daf-12*, it has been shown that cytochrome P450 genes (DAF9 and Rieske) are involved in the ligand (dafachronic acid) biosynthesis pathway (Jia et al., 2002; Motola et al., 2006; Wollam et al., 2011). In *Drosophila*, cytochrome enzymes metabolize sterols from the precursor cholesterol. For example, Halloween cytochrome enzymes are identified to function in the biosynthesis pathway of 20-Hydroxyecdysone, which is the known ligand for the ecdysone receptor (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993). Thus, analysis of components of the ligand biosynthesis pathway is another possible way to detect the DHR96 ligand.

5.1.1 *Dhr96* ChIP sequencing and microarray experiments

Future aims are directed to identify genes important for longevity, detoxification and ligand biosynthesis, downstream of DHR96. ChIP experiments facilitate the studies of downstream processes.

So far, a suitable DHR96 antibody to purify DHR96 bound DNA has not been available, and tagged *dhr96-lbd* over-expressing flies failed to extend lifespan and so were not suitable, for use in ChIP sequencing experiments. New tagged transgenic *dhr96* lines were generated to facilitate IP of chromosomes bound DHR96, using the tag. Nevertheless, microarray data between long-lived or short-lived and control animals can give information on which genes are affected downstream of DHR96.

Microarray experiments were performed for *dhr96* null mutants versus wild type in a CantonS (CanS) background (King-Jones et al., 2006), and for long-lived w^{Dah+} ligand-insensitive *dhr96* versus not long-lived w^{Dah+} wild type *dhr96* over-expressing females using a ubiquitous *tubulin* GS driver (Janne Toivonen and Matt Piper, unpublished data). Using *daughterless* GS and *tubulin* GS driver led to different results in my hands, as wild type *dhr96* over-expressing flies extended lifespan in *daughterless* driven flies (Chapter 3), and over-expression of wild type or ligand-insensitive *dhr96* shortened lifespan when *tubulin* GS driver was used. The induction level was discussed as a possible reason, and food type represents an important factor. Although microarray data stem from over-expression of *dhr96* and *dhr96-lbd* using a *tubulin* GS driver, comparison of the two data sets is valid, as in their condition lifespan was only extended in *dhr96-lbd* over-expressing flies. Thus, micro array data includes changes from wild type *dhr96* over-expressing females which were not long-lived and changes from ligand-insensitive *dhr96* over-expressing females which were long-lived, and are therefore of major interest to identify longevity genes.

5.1.2 Approach for bioinformatics analysis of microarray data sets

Ligand-insensitive *dhr96* might induce compensatory changes in the expression of genes, which might regulate DHR96 or its ligand production in form of a feedback loop. Moreover, ligand-insensitive over-expressing flies were long-lived, but wild type *dhr96* over-expressors had no effect on lifespan using the *tubulin* GS driver (Janne Toivonen). Thus, the criterion was to search for cytochrome genes that show a differential expression between ligand-insensitive and wild type *dhr96* transcript profiles, or genes, which show a contrary effect between both. These genes are possibly relevant to lifespan, as

on the one hand they may be involved in life span extension, on the other hand they may be involved in ligand biosynthesis. For the second issue, genes that show changed expression in *dhr96* over-expressed wild type flies but not in ligand-insensitive *dhr96* over-expressed mutant flies have been also selected.

Flies used for microarray experiments were also tested for xenobiotic resistance (Janne Toivonen, unpublished data) and although lifespan was only extended for *dhr96-lbd* over-expressing flies using the *tubulin* GS driver, both lines were resistant to certain stresses and had decreased fecundity. The criterion to identify important candidates for detoxification or fecundity downstream of DHR96 was therefore to search for genes that were up- or down-regulated in both lines.

5.1.3 Candidates for qRT-PCR

High priority candidates selected from the microarray data sets were tested in qRT-PCR between *dhr96-lbd* over-expressing flies and driver or UAS-controls. Moreover, IIS components including *dfoxo* and *S6-kinase*, as well as longevity genes, anti-microbial peptide (AMP) genes and the gene *magro* (discussed below) were tested in qRT-PCR.

5.1.3.1 *Magro* – a downstream target of DHR96

Magro is reported as a target of DHR96 and represents therefore an interesting candidate (Sieber and Thummel, 2012).

Magro encodes for a lipase that is highly expressed in the midgut of *Drosophila* and is secreted into the intestine lumen (Sieber and Thummel, 2009). As with its mammalian homolog LipA, *magro* has esterase activity and digests dietary triacylglycerol (TAG) into free fatty acids and monoacylglycerols. Moreover it breaks down cholesterol esters into free sterols. Intestine cells absorb these digested forms, and resynthesized TAG and cholesterol esters circulate in the blood/haemolymph in form of lipoprotein particles to target tissues. TAG supply cells with energy, and are stored in the fat body. Excess conditions lead to the breakdown of TAG and cholesterol esters to promote energy metabolism and to

excrete cholesterol from the body. Misregulation in TAG homeostasis affects energy metabolism, and can cause physiological disorders like obesity or diabetes (Sieber and Thummel, 2009).

Drosophila is an cholesterol auxotrophic organism, unable to synthesize cholesterol and fully dependent on its uptake (Hoog, 1936). Cholesterol is significant for a broad array of biological functions, and altered regulation in cholesterol uptake affects normal physiology. Thus, regulators of these mechanisms are important for diverse biological function, from hormonal regulation to metabolism. Interestingly, DHR96 regulates the expression of genes, which encode proteins involved in TAG and cholesterol homeostasis like *magro* (Horner et al., 2009; Sieber and Thummel, 2009).

In *dhr96* null flies TAG levels are decreased, and cholesterol levels are increased. Altered expression of the genes *magro* and *npcb1* are suggested to partially cause these effects. Decreased transcription of *magro* reduces TAG levels in the body, as they cannot be absorbed by the gut. *Npcb1* mediates the absorbance of cholesterol and up-regulation of this gene in *dhr96* null flies increases cholesterol uptake. As *magro* is reduced, cholesterol efflux is unbalanced and consequently cholesterol levels are increased (Sieber and Thummel, 2012; Voght et al., 2007).

As expression levels of these genes are affected in *dhr96* null mutants, I investigated how the over-expression of *dhr96-lbd* modulates the transcript levels of these genes, as they display important links to energy and cholesterol homeostasis.

5.2 Materials and methods

5.2.1 Bioinformatic analysis

Microarray experiments and data of *dhr96* and *dhr96-lbd* over-expressing flies by using the *tubulin* GS driver were performed and provided by Janne Toivonen (UCL, London). Analyses were focused on transcript levels of cytochrome P450 genes with changed expression in only one of the two microarray sets, in both microarray sets, or with a contrary expression between both transcript profiles and were selected for further studies. The changes are shown as log2 fold changes (FC), indicating that positive log2 FC values are up-regulated genes and negative log2 FC describes down-regulated genes.

5.2.2 Fly strains and maintenance

Dhr96-lbd was over-expressed using the *daughterless* GS driver, and RNA of these and control flies (ethanol and driver control) were extracted at an age of 10 days for qRT-PCR analysis. Crossings and maintains were performed the same way as described in chapter 3.

5.2.3 RNA extraction and qRT-PCR

To test transcript levels of candidate genes in *dhr96-lbd* over-expressing flies, RNA was extracted from whole bodies or dissected tissues, including gut, and ovaries. Dissections were performed in Schneider's *Drosophila* medium (Gibco) using forceps. Quantitative RT-PCR was set up as described in chapter 2.1.2, and used Taqman primers are listed in supplement S.2.2. Data was analysed using sequence detection systems software 2.3 (Applied Biosystems). QRT-PCR data were validated using the $_{\Delta\Delta}$Ct method and normalized to three different internal controls (Rpl32, Actin, α-Tubulin).

Candidates which showed significant changes in transcript levels were repeated in two additional biological replicates.

5.3 Results

5.3.1 Analysis of transcript profiles

5.3.1.1 Candidates for longevity and for regulation of ligand synthesis

Bioinformatic analysis of transcript profiles from microarrays of wild type *dhr96* versus ligand-insensitive *dhr96* over-expressing flies revealed 18 cytochrome genes as candidates for ligand production and lifespan (table 5.3.1).

Three cytochrome genes (*Cyp18a1*, *Cyp12a4*, *Cyp6g1*) showed opposing expression levels between the wild type and ligand-insensitive *dhr96* over-expressing females. These genes were selected as high priority candidates for further analysis (chapter 6.3.3, *Cyp6g1* chapter 3).
Fifteen cytochrome genes showed a changed expression only in one situation but remained unchanged in the other. Strikingly, all eight cytochrome genes that are differentially expressed in ligand-insensitive *dhr96* over-expressor but unchanged in wild type *dhr96* over-expressing flies were down-regulated. Also the transcript levels of genes with opposing expression were all decreased in the ligand-binding mutants, even though *Cyp6g1* (involved in DTT resistance) and *Cyp12a4* are characterized as detoxification enzymes (Chung, 2008).
Cytochrome genes that were differentially expressed in *dhr96* over-expressing females but unchanged in *dhr96* ligand-insensitive over-expressing females showed low log2 fold changes in transcript level. *Cyp12a5* showed the highest fold change with an up-regulation of 0,35 log2 fold change. Two genes including *Cyp4p3* and *Cyp6u1* show a trend to be down-regulated, but did not reach significance. Excluding these two genes, all other cytochrome genes were up-regulated in *dhr96* over-expressing flies.

Cyp9h1 was selected from published transcript profiles between wild type CanS flies versus *dhr96* null flies and wilt type CanS flies versus *dhr96* heat-induced over-expressing flies (King-Jones et al., 2006). *Cyp9h1* is down-regulated in induced *dhr96* expressed flies, and up-regulated in *dhr96* null flies (table 5.3.1).

A Transcript profile of cytochrome p450 genes with contrary expression

		dhr96 over-expressing flies		*dhr96-lbd* over-expressing flies	
CG assigment	***Cyp* genes**	**q-value**	**fc (log2)**	**q-value**	**fc (log2)**
CG6042	*Cyp12a4*	0,011	0,292	0,085	-0,114
CG6816	*Cyp18a1*	0,037	0,229	0,047	-0,190
CG8453	*Cyp6g1*	0,080	0,094	0	-0,382

		dhr96 over-expressing flies		*dhr96* null mutants	
CG assigment	**Cyp genes**	w:hs*dhr96* Score	w:hs*dhr96* FC	CanS:*dhr96Δ* untreated Score	CanS:*dhr96Δ* untreated FC
CG17577	*Cyp9h1*	-7,44	-2,1	4,8	1,3

B Transcript profile of differentially expressed cytochrome p450 genes

		dhr96 over-expressing flies		*dhr96-lbd* over-expressing flies	
CG assigment	**Cyp genes**	**q-value**	**fc (log2)**	**q-value**	**fc (log2)**
CG4486	*Cyp9b2*	0,270	-0,070	0,007	-0,515
CG10242	*Cyp6a23*	0,260	-0,076	0,013	-0,472
CG8733	*Cyp305a1*	0,322	0,092	0,056	-0,427
CG2060	*Cyp4e2*	0,808	-0,009	0,000	-0,376
CG10842	*Cyp4p1*	0,724	-0,018	0,020	-0,323
CG3972	*Cyp4g1*	0,950	-0,002	0	-0,252
CG3466	*Cyp4d2*	0,352	0,086	0,095	-0,179
CG11567	*Cpr*	0,786	-0,010	0,005	-0,146

		dhr96 over-expressing flies		*dhr96-lbd* over-expressing flies	
CG assigment	**Cyp genes**	**q-value**	**fc (log2)**	**q-value**	**fc (log2)**
CG10843	*Cyp4p3*	0,098	-0,235	0,623	0,050
CG3567	*Cyp6u1*	0,077	-0,099	0,155	0,053
CG11821	*Cyp12a5*	0,030	0,350	0,856	-0,013
CG4120	*Cyp12c1*	0,047	0,340	0,196	0,119
CG4485	*Cyp9b1*	0,049	0,334	0,302	0,115
CG11466	*Cyp9f2*	0,002	0,290	0,985	0,001
CG10755	*Cyp4ae1*	0,062	0,240	0,879	0,012

Table 5.3.1 Transcript profiles from wild type *dhr96* versus ligand-insensitive *dhr96* over-expressing or *dhr96* null *Drosophila* flies. Cytochrome P450 genes were selected as candidate genes with the following framework conditions: (A) Genes which showed a opposing effect between both transgenic flies (*dhr96* and ligand-insensitive *dhr96*), or between *dhr96* null and wild type *dhr96* over-expressing flies. (B) Genes in which expression was changed in *dhr96* over-expressors, but not in ligand-insensitive *dhr96* over-expressors, or candidate cytochrome genes with changed expression in ligand-insensitive *dhr96* over-expressors but not in wild type *dhr96* over-expressors.

5.3.1.2 Candidates for increased stress response and reduced fecundity

Many cytochrome genes were either down-regulated or up-regulated in both the *dhr96* and the ligand-insensitive *dhr96* females. These genes were not included as putative candidates for DHR96 ligand production, but they might represent candidates for reduced fecundity and increased stress response as these phenotypes were observed in both *dhr96* and *dhr96-lbd* over-expressing females. Candidates were therefore listed in table 5.3.2 arrayed by their log2

fold change in *dhr96-lbd* over-expressing flies with the highest log2 fold change on top.

Other than in the list for candidates important for ligand production, log2 fold changes were higher for candidates important for fecundity or stress response. *Cyp313a* showed with a log2 fold change of 1,11 in *dhr96* over-expressing flies and a log2 fold change of 1,055 in *dhr96-lbd* over-expressing flies, the highest up-regulation compared to other *cyp* candidates. *Cyp6w1*, *Cyp6a8*, *Cyp6a2*, *Cyp6a21*, and *Cyp6a14* were down-regulated by a log2 fold change of about one or higher. Comparing up-regulated or down-regulated cytochrome gene levels between wild type *dhr96* and *dhr96-lbd* over-expressing flies revealed similar log2 fold changes for most cytochrome genes. Among up-regulated genes, *Cyp28d1* showed higher up-regulation in wild type *dhr96* over-expressing flies. Among down-regulated genes *Cyp6d5*, *Cyp4d1* and *Cyp4s3* showed higher log2 fold changes in ligand-insensitive *dhr96* over-expressing flies.

Up-regulated *cyp* genes in *dhr96* and *dhr96-lbd* over-expressing flies

		***dhr96* over-expressing flies**			***dhr96-lbd* over-expressing flies**		
CG assigment	**Cyp genes**	**q-value**	**fc (log2)**	**rank**	**q-value**	**fc (log2)**	**rank**
CG3360	*Cyp313a1*	0,036	1,111	1	0,049	1,055	1
CG4105	*Cyp4e3*	0,041	0,419	3	0,059	0,481	2
CG3616	*Cyp9c1*	0,048	0,334	5	0,088	0,240	3
CG12800	*Cyp6d4*	0,048	0,199	6	0,056	0,229	4
CG10833	*Cyp28d1*	0,015	0,828	2	0,073	0,212	5
CG8864	*Cyp28a5*	0,045	0,351	4	0,098	0,207	6
CG10246	*Cyp6a9*	0,073	0,173	7	0,098	0,131	7

Down-regulated *cyp* genes in *dhr96* and *dhr96-lbd* over-expressing flies

		***dhr96* over-expressing flies**			***dhr96-lbd* over-expressing flies**		
CG assigment	**Cyp genes**	**q-value**	**fc (log2)**	**rank**	**q-value**	**fc (log2)**	**rank**
CG8345	*Cyp6w1*	0,012	-1,722	1	0,017	-1,763	1
CG10248	*Cyp6a8*	0,034	-1,364	2	0,031	-1,553	2
CG9438	*Cyp6a2*	0,006	-0,909	4	0,002	-1,229	3
CG10247	*Cyp6a21*	0,002	-1,167	3	0,001	-1,112	4
CG8687	*Cyp6a14*	0,013	-0,902	5	0,040	-1,081	5
CG9081	*Cyp4s3*	0,039	-0,492	7	0,043	-0,833	6
CG3656	*Cyp4d1*	0,015	-0,382	8	0,037	-0,645	7
CG3050	*Cyp6d5*	0,001	-0,259	11	0,000	-0,641	8
CG3540	*Cyp4d14*	0,054	-0,550	6	0,085	-0,416	9
CG16761	*Cyp4d20*	0,057	-0,345	9	0,071	-0,378	10
CG2110	*Cyp4ad1*	0,080	-0,238	12	0,067	-0,342	11

Table 5.3.2 Transcript profiles of cytochrome genes from wild type *dhr96* versus ligand-insensitive *dhr96* over-expressing flies. Cytochrome P450 genes, which were up- or down-regulated in both transcript profiles (*dhr96* over-expressors and ligand-insensitive *dhr96* over-expressors) were selected as candidate genes for reduced fecundity and stress resistance.

5.3.2 qRT-PCR analysis of *dhr96-lbd* over-expression using the *daughterless* GS driver in females

5.3.2.1 qRT-PCR analysis of candidate genes in whole bodies

Candidates, including *Cyp12a4*, *Cyp18a1* and *Cyp6g1,* were selected from microarray analysis and tested by qRT-PCR for changed expression levels between *dhr96-lbd* over-expressing flies and control flies. Using whole bodies, *Cyp18a1* transcript levels were significantly reduced in *dhr96-lbd* over-expressing flies (p-value ***<0.001, Student's *t*-test) (figure 5.3.1 A). In contrast, *Cyp12a4* and *Cyp6g1* trended towards up-regulation, which did not reach significance (data not shown).

Remarkably, transcript levels of the anti-microbial peptide *metchnikowin* (*mtk*) were significantly down-regulated (p-value **<0.01, Student's *t*-test) (figure 5.3.1 B). The decrease amounted about to 70% in *dhr96-lbd* over-expressing flies when compared to driver control flies on RU. There was no significant change between ethanol and RU controls.

Hsp27 and *hsp26* were significantly up-regulated in the ligand-insensitive *dhr96-lbd* transcript profile, and represent possible longevity genes, as over-expression of *hsp26* or *hsp27* can extend lifespan in *Drosophila* (Wang et al., 2004). However, no change in expression levels was observed for either in *dhr96-lbd* over-expressing females (supplement S.5.1). *S6-kinase* and *dfoxo* transcript levels were investigated as well and revealed no significant changes in *dhr96-lbd* over-expressing females when whole bodies were used (supplement S.5.1).

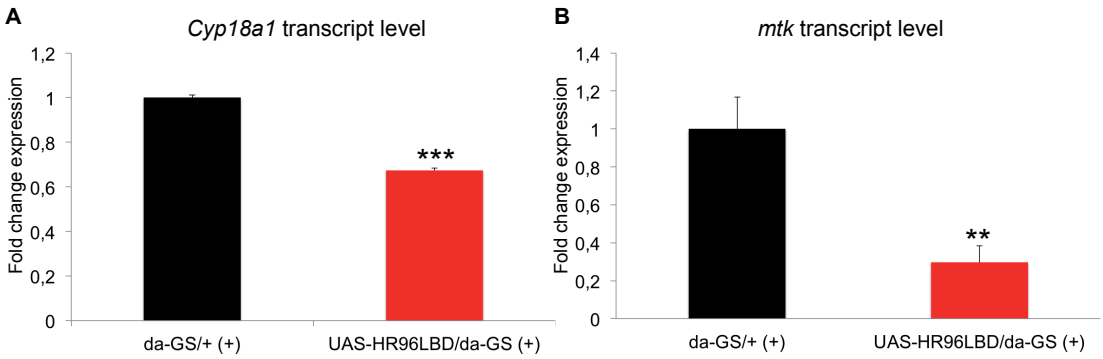


Figure 5.3.1 *Cytochrome P450 18a1* (*Cyp18a1*) and *metchnikowin* (*mtk*) transcript levels were reduced in *dhr96-lbd* over-expressing females. Over-expression of *dhr96-lbd* by the *daughterless* GS driver revealed to significantly down-regulate *Cyp18a1* and *mtk* expression in whole body of females (p-value **<0.01, p-value ***<0.001, Student's *t*-test).

5.3.2.2 qRT-PCR analysis of candidate genes in the gut of *dhr96-lbd* over-expressing flies

As expression changes in specific tissues might not be detectable when whole bodies are used, some candidates were tested in dissected tissues, including gut and ovaries.

In the gut of *dhr96-lbd* over-expressing females *dhr96* expression was induced by about 3.2 fold (p-value ***<0.001, Student's *t*-test) (figure 5.3.2 A). As shown in chapter 4, over-expression of *dhr96-lbd* in females significantly increased *Cyp6g1* levels in the gut (*p<0.05, Student's *t*-test) (figure 5.3.2 B). *Cyp12a4* expression levels were higher in *dhr96-lbd* over-expressing flies compared to driver control on RU, but the increase did not reach significance in the gut (figure 5.3.2 C). Interestingly, expression of *magro* was significantly reduced in these flies when compared to control flies (p-value **<0.01, Student's *t*-test) (figure 5.3.2 D).

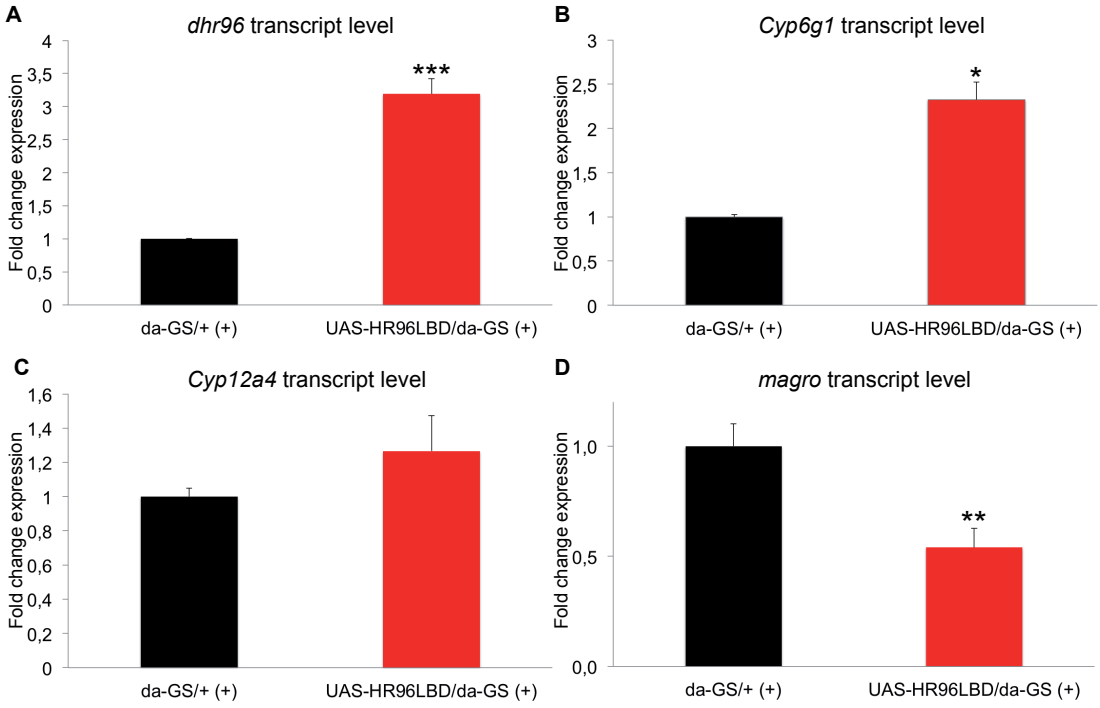


Figure 5.3.2 Quantitative RT-PCR of candidates in the gut of *dhr96-lbd* over-expressing females. (A) Over-expression of *dhr96-lbd* by the *daughterless* GS driver induced *dhr96* expression by 3.2 fold in the gut of females (p-value ***<0.001, Student's *t*-test). (B) *Cyp6g1* expression level was significantly increased (*p<0.05, Student's *t*-test), whereas *Cyp12a4* indicated increased expression levels, but increase did not reach significance. (D) *Magro* expression level was reduced in the gut of *dhr96-lbd* over-expressing flies (p-value **<0.01, Student's *t*-test).

5.3.2.3 qRT-PCR analysis of candidate genes in the ovary of *dhr96-lbd* over-expressing flies

Dhr96 expression was induced by a fold change of 7.4 in ovaries of *dhr96-lbd* over-expressing females (p-value ***<0.001, Student's *t*-test). However, there was no significant change observed in transcript levels of *S6-kinase*, *4E-BP*, *dfoxo*, *hsp26,* or *hsp27* (figure 5.3.3, supplement S.5.2). *Hsp26* and *hsp27* were tested in the ovaries as they show very high expression in this tissue (Chintapalli et al., 2007). Expression levels of *S6-kinase* showed a reduced trend in two biological replicates with p-values lower than 0.1 (Student's *t*-test).

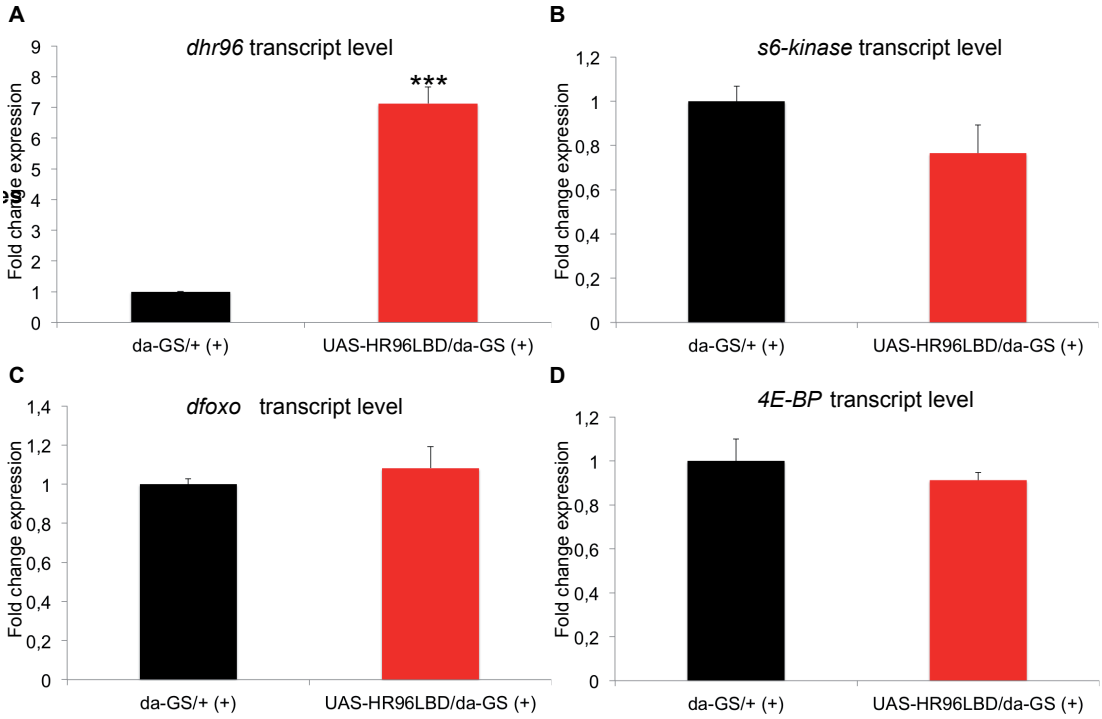


Figure 5.3.3 Quantitative RT-PCR of candidates in the ovaries of *dhr96-lbd* over-expressing females. Over-expression of *dhr96-lbd* by the *daughterless* GS driver induced *dhr96* by 7.4 fold in the ovaries when compared to driver control on RU (p-value ***<0.001, Student's *t*-test). No changes were observed for *S6-kinase*, *dfoxo* and *4E-BP* transcript levels. *S6-kinase* showed a trend, but did not reach significance.

5.4 Discussion

5.4.1 The majority of cytochrome genes were down-regulated in long-lived *dhr96-lbd* over-expressing females

Analysing microarray data of *dhr96* and *dhr96-lbd* over-expressing flies using the *tubulin* GS driver (provided by Janne Toivonen and Matt Piper) revealed that many cytochrome genes were down-regulated. Cytochrome genes that were differentially expressed between *dhr96* and *dhr96-lbd* over-expressing flies in the whole body, were all down-regulated in the *dhr96-lbd* over-expressing females. These findings overlap with the data from *C. elegans* trancript profiles of *daf-12* (Fisher and Lithgow, 2006). Cytochrome genes, which show differential expression levels between wild type and ligand-insensitive *daf-12*, were all down-regulated in the ligand-insensitive mutants.

These findings appear contrary, as *dhr96-lbd* over-expressing flies showed increased stress resistance. Thus, increased levels of these detoxification genes were expected. But in transcript profiles only a few cytochrome genes were up-regulated in both situations. One possible reason might be that increased expression is not visible in whole bodies and might occur only in certain tissues, as was shown for *Cyp6g1*. Furthermore it is known that over-expression of one detoxification gene can be sufficient to mediate resistance, so that the number of up-regulated genes might not be relevant (Daborn et al., 2002). In addition, transcript profiles showed increased expression levels for other detoxification enzymes, including some Glutathione S-transferases (*gsts*), which were not covered in this study.

To identify detoxification genes that are required for xenobiotic resistance in IIS-altered flies mediated by DHR96, transcript profiles between *dhr96* over-expressors and IIS mutants should be compared. If certain stress responses of IIS mutants were mediated by DHR96, the array profiles of the ligand-insensitive *dhr96* over-expressors or wild type *dhr96* over-expressor would be similar to the long-lived IIS profiles. This would restrict the number of possible candidates.

Besides their role in the xenobiotic metabolism, many cytochrome enzymes function in hormone metabolism, which in turn can affect a broad range of biological functions like reproduction, immunity and metabolism.
The resulting hormones can represent ligands for hormone nuclear receptors. Cytochrome genes modulate feed back loops, thus our expectation was to see compensatory effects in form of up-regulated genes in ligand-insensitive *dhr96* over-expressing flies. However, transcript profiles revealed that all cytochrome genes, which were differentially regulated to wild type *dhr96* over-expressing flies in the whole body, were down-regulated. Thus, the suggestion arose that DHR96 might be constitutively active without a bound ligand. However, it is also important to mention that certain tissues might be important for ligand biosynthesis and compensatory effects were not visible when whole bodies were used.
So far it is unclear, how DHR96 is regulated, and if it acts as a repressor, an activator, or both. Direct target genes would give a better understanding of DHR96 function. Thus, ChIP sequencing experiments are part of future experiments.

5.4.2 *Cyp18a1* transcript levels were down-regulated in *daughterless* GS-driven *dhr96-lbd* females

In *dhr96-lbd* over-expressing flies, *Cyp18a1* was significantly down-regulated. This is in agreement with microarray data, in which *Cyp18a1* levels are reduced in long-lived *tubulin* GS *dhr96-lbd* over-expressing flies.
CYP18a1 is important for sterol metabolism, and shares characteristics with DAF-9, which catalyses the last step in the biosynthesis of the dafachronic acids (Jia et al., 2002). CYP18a1 as well as DAF-9 belong to the 26-Hydroxylase-enzyme family and thus metabolize the same type of reaction. Conspicuously, CYP18a1 acts on 20-Hydroxyecdysone (Guittard et al., 2011), which is the known ligand for the ecdysone receptor (Koelle, 1992; Thomas et al., 1993; Yao et al., 1993), and convert that sterol into 20-hydroxyecdysonoic acid. An active function of that steroid is not known and it is only described as an inactive form of 20-Hydroxyecdysone, as it reduces ligand levels for the ecdysone receptor and thus its activity. Inactivation of the ecdysone receptor is

proven to be required for metamorphosis in *Drosophila,* and loss of function leads to lethality in early development (Guittard et al., 2011). Thus, modulation of *Cyp18a1* in constitutive *dhr96-lbd* over-expressing flies might be a reason for their developmental lethality.

Moreover, 20-hydroxyecdysonoic acid may be a possible ligand or precursor ligand for DHR96, or may act on its ligand biosynthesis pathway. In vertebrates cholestenoic acid acts as a natural ligand for the liver-X-receptor alpha (Song and Liao, 2000) that among others is also involved in cholesterol and triglyceride metabolism, which are both affected by DHR96. So far, several ligands are known for vertebrate nuclear receptors, but only a few are known in *C. elegans* and *Drosophila*.

5.4.3 *Magro* was down-regulated in *daughterless* GS-driven *dhr96-lbd* females

In *dhr96* null mutants, *magro* down-regulation affects TAG and cholesterol homeostasis. The uptake and efflux of these components is dependent on their form. TAG and cholesterol esters have to be digested for absorption, or excretion in or by the intestine cells. Knockdown of *magro* decreases the efflux of cholesterol in the intestine, resulting in increased cholesterol level in the whole body (Sieber and Thummel, 2012). *Dhr96* null mutants show reduced *magro* expression levels and similar phenotypes. Moreover, TAG levels are decreased in *dhr96* null flies, which is also partially caused by reduced *magro* expression and activity to digest TAG to an absorbable form. Induced expression of this lipase in *dhr96* null flies normalizes TAG levels (Sieber and Thummel, 2009).

Interestingly, reduced *magro* transcript levels were observed in the gut of *dhr96-lbd* over-expressing flies. This would lead to the conclusion that TAG and cholesterol uptake/efflux and metabolism might be affected in these long-lived flies. According to the *magro* RNAi studies, that could mean that cholesterol level might also be altered in *dhr96-lbd* over-expressing flies. But DHR96 affects other proteins involved in lipid metabolism, including the Niemann-pick genes *npc1b*, *npc1a*, *npc2a* and *npc2b*, which mediate cholesterol uptake or intracellular trafficking (Huang et al., 2005; 2007; Voght et al., 2007). The

decreased efflux might be a consequence of low cholesterol uptake in *dhr96-lbd* over-expressing flies. A next step would be to identify *Npc1b* transcript levels, since NPC1B mediates cholesterol absorbance, and moreover to identify TAG and cholesterol levels in *dhr96-lbd* over-expressing females. If these mechanisms were altered in *dhr96-lbd* over-expressing flies, a further step would be to investigate if *dhr96-lbd* phenotypes are dependent on these mechanisms by performing stress or lifespan experiments on food containing different cholesterol concentrations. For *dhr96* null flies at least it was shown that survival is decreased on low cholesterol food (Sieber and Thummel, 2009).

5.4.4 *Mtk* was down-regulated in *daughterless* GS driven *dhr96-lbd* females

The immune system represents a further important protective mechanism, and has been linked to ageing in many studies. Interestingly, transcript levels of the antimicrobial peptide *Metchnikowin* (*mtk*) were significantly decreased to about 70% in *dhr96-lbd* over-expressing females.

Further experimental analyses were focused on this topic, and will be therefore introduced and discussed in the following chapter 6.

5.4.5 Conclusion

This chapter focused on downstream targets of *dhr96* in *dhr96-lbd* over-expressing females and validated a list of possible candidates. Expression of *Cyp18a1*, *magro* and *mtk* were altered in long-lived *dhr96-lbd* over-expressing females. Further studies are needed to understand the effect of their altered transcription. *S6-kinase* represented an interesting candidate, as it might be regulated by DHR96 in the ovaries (Hartman et al., 2013). A trend was apparent, but did not reach significance. More biological replicates are needed for an accurate conclusion.

Candidates were extracted from microarray data from whole bodies of *dhr96* and *dhr96-lbd* over-expressing females. As genes might be differentially regulated in certain tissues a further step might be to perform arrays from specific tissues.

Chapter 6 Bacterial challenge of *dhr96-lbd* over-expressing females

6.1 Introduction

Although over-expression of *dhr96-lbd* using the *daughterless* GS driver extended lifespan and increased stress resistance, qRT-PCR analysis revealed a decreased basal expression level of the AMP gene *Mtk*. To investigate the effect on immune resistance, bacterial challenge of *dhr96-lbd* over-expressing females was tested by infection with the pathogen *Erwinia carotovora carotovora* (*Ecc*).

The experiments were performed in cooperation with Dr. Gerrit Loch (LIMES, laboratory of Prof. Hoch), who is an expert in the field of *Drosophila* immunology.

6.1.1 Immunity

Immunity has evolved as a host-defense mechanism in metazoans to protect the body from infectious, microbial organisms (Hoffmann, 2003). The innate immune system represents a first-line defense system and is conserved across phyla. Germ-line encoded pattern recognition receptors (PRR) detect microbial pathogens and induce immune signalling pathways. These activated cascades lead to the production of immune effectors to combat microbial infection (Janeway, 1989; Medzhitov and Janeway, 2000b; 2000a; 1998).

In *Drosophila*, two types of immune response are described: (1) the humoral or systemic response induces the production of antimicrobial peptides (AMP) in the fat body (equivalent to mammalian liver), and their release into the hemolymph (mammalian blood). (2) The cellular response or acute-phase response involves the activation of hemocytes (blood cells) by cytokines, leading to phagocytic activities (Gillespie et al., 1997; Hoffmann, 2003; Hoffmann and Reichhart, 2002; Rizki and Rizki, 1984).

Vertebrates have developed an additional, sophisticated immune system, the adaptive immunity (Alberts et al., 2007). Two classes of lymphocytes, the B-cells (humoral) and T-cells (cell-mediated) mediate the immune response. B-cells detect antigens of invaders and generate specific antibodies against them. Secreted and circulating antibodies in the blood bind to the foreign substrates and prevent their interaction with host cells. T-cells mediate the cell-based response, by eliminating the host cells directly or by inducing the activity of macrophages that eliminate microbial organism by phagocytosis. In contrast to innate immunity, the response in adaptive immunity is directed against specific pathogens, and can last for long periods. Clonal expansions in B-and T cells provide memorial recognition of these antigens. However, studies suggest that plants and invertebrates also have also trained or basal memorial immunity (Durrant and Dong, 2004; Kurtz, 2005; Netea et al., 2011; Quintin et al., 2014).

6.1.2 *Drosophila* humoral immunity

The humoral immune response is well characterized in *Drosophila* and includes two key immune signalling pathways, which are conserved across species (Hoffmann, 2003; Hoffmann and Reichhart, 2002). Both the Toll and Imd (immune deficiency) pathways activate NF-κB-like transcription factors (Dorsal/ Dif and Relish) respectively, which induce antimicrobial peptide (AMP) gene transcription. 20 AMP genes are categorized with seven classes: *Attacin* (4), *Cecropin* (4), *Drosocin* (1), *Diptericin* (2), *Defensin* (1), *Drosomycin* (7) and *Metchnikowin* (1). The resulting peptides are characterized by a small size and cationic properties. Atttacin, Drosocin, Cecropin and Diptericin are protective against gram-negative bacteria, Defensin against gram-positive bacteria, and Cecropin, Drosomycin and Metchnikowin against fungi. They can act alone or in combination against invaders by damaging its cell membrane (Bulet et al., 1999; Meister et al., 2000).

Toll signalling is initiated by the cleavage and activation of the Toll ligand spätzle in response to gram-positive bacteria and fungi (Levashina et al., 1999; Weber et al., 2003). The binding of spätzle to the Toll receptor induces a cascade, via several mediators, to the degradation of the repressor Cactus that consequently leads to the release of the NF-κB-like proteins Dorsal and Dif, and

nuclear translocation of these proteins (Imler and Hoffmann, 2002; Ip et al., 1993; Nicolas et al., 1998).

The Imd pathway does not exclusively affect the innate immune response, as it is also involved in the JNK (c-Jun N-terminal protein kinase) pathway. However, Imd signalling is induced in response to gram-negative infection and shows similarity to the vertebrate TNF-α (tumor necrosis factor alpha) pathway (Hoffmann, 2003). Binding of bacterial DAP-type peptidoglycan by the Peptidoglycan recognition receptor (PGRP) initiates a signalling cascade with several mediators (including imd, dFADD, dAK1, IKK), leading to the activation of the downstream target Relish (NF-κB family protein) by phosphorylation and cleavage (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000).

Both pathways can be induced separately, but can also act together to combat bacterial invaders (Rolff and Reynolds, 2009).

6.1.3 Hormonal regulation of the innate immune system

Hormones can affect diverse biological mechanisms, including metabolism, reproduction, development and immune response. In humans, the use of glucocorticoids (steroid hormones) for medical and immunological applications is very common (Necela and Cidlowski, 2004). Their immunosuppressive effect via the glucocorticoid receptor (GR) compensates the effects of diseases caused by an overactive immune response (allergy, sepsis, autoimmune disease). Further, human hormone nuclear receptors like the vitamin D receptor (VDR), retinoid X receptor (RXR), liver X receptor (LXR) or the estrogen receptor (ER) as downstream components of hormone metabolism were shown to regulate innate immune system and expression of pro-inflammatory cytokines (Baeke et al., 2010; Hong and Tontonoz, 2008; Nunez et al., 2010).

In *Drosophila*, Juvenile hormone (JH) and 20-Hydroxyecdysone (20E) modulate cellular and humoral innate immunity (Flatt et al., 2008a; Meister and Richards, 1996). Both hormones are required for insect development, physiology and reproduction (Kozlova and Thummel, 2000; Riddiford, 1993). Effects of JH on the innate immunity occur through 20E regulation (Flatt et al., 2008a).

20E binds to the Ecdysone Receptor and induces its dimerization with ultraspiracle, the ortholog of LXR and RXR, and its transcriptional activity.

Transcription of early genes, like the zinc binding factor *Br-c*, and the nuclear hormone receptors *Eip75B, Eip78C* and *Hr46* (*dhr3*), are induced in a time and hierarchical dependent manner. Dependent on the developmental stage, JH can induce or suppress 20E induced ER/USP signalling (Flatt et al., 2008a; King-Jones and Thummel, 2005; Mugat et al., 2000; Thummel, 1996).

20E treatment of *mbn-2* cells (derived blood cell line) or larval *Drosophila* induces phagocytic activity and expression of the AMP genes *Diptericin* and *Drosomycin* (Dimarcq et al., 1997; Lanot et al., 2001). Moreover, 20E is required for development of tissues or components that are important for cellular innate immunity, including the lymph gland and hematopoiesis (Sorrentino et al., 2002). In contrast, 20E treatment of blowfly (*Calliphora vicina*) revealed a down-regulation of the innate immune signalling during the diapause of larvae, suggesting that developmental stage is an important criterion (Flatt et al., 2008a). However, in most other stages and conditions, 20E acts as an inducer and JH as a suppressor (Flatt et al., 2005). In adult *Drosophila*, 20E regulates AMP gene expression through two different mechanisms (Rus et al., 2013). Both mechanisms are mediated through downstream target genes of the EcR/USP complex. Five early genes, including *Br-c, Eip93F, Eip74EF, Eip78C* and *Hr46*, and two dGATA factors, *Serpent* (*srp*) and *Pannier* (*pnr*), regulate PGRP-LC activity. As PGRP-LC initiates the Imd signalling cascade, its regulation represents a critical basis for the transduction of the Imd immune response. In a second mechanism, gene expression of a subset of AMP genes, including *Diptericin*, *Metchnikowin* and *Drosomycin*, is regulated by PNR, SRP, and BR-C. This effect might occur downstream of Relish activity (figure 6.1.1).

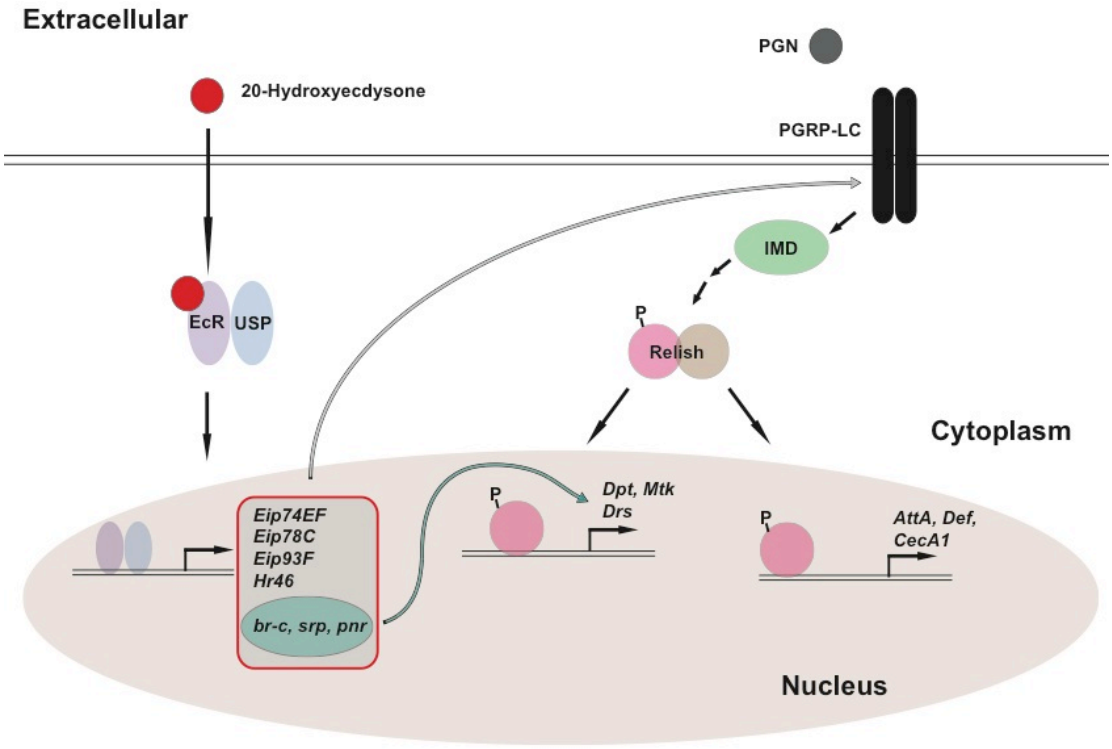


Figure 6.1.1 Model for hormonal regulation of innate immune pathway in *Drosophila* (modified from Rus et al (2013)). 20-Hydroxyecdysone can modulate expression of AMP genes through ecdysone signalling by two mechanisms: (1) Ecdysone-inducible transcription factors (red box) control expression of the peptidoglycan receptor (*PGRP-LC*). (2) Transcription of a subgroup of AMP genes is regulated independently of PGRP-LC.

6.1.4 Immunity and ageing in *Drosophila*

Drosophila represents an excellent model to study the relationship between innate immunity and ageing. Underlying biological mechanisms, including the Imd and Toll pathways, or IIS signalling, are conserved across species. Diverse genetic tools that are very well established in flies, and a short generation time, provide a broad range of possible studies that would be difficult in vertebrates due to longer lifespans and their additional adaptive immune system. In vertebrates, as in *Drosophila* (see below), although immunity declines with age, as aged organism are more susceptible to pathogen infection, inflammatory markers increase with age too (Cevenini et al., 2013; DeVeale et al., 2004).

In aged flies, immune response is impaired and neutralization of pathogens is negatively affected. Interestingly, expression levels of AMP genes, including Attachin and Defensin, and other effectors in the innate immune response such as Relish are up-regulated in old flies. As the pathway is continuously induced,

aged flies suffer from chronic inflammation (Eleftherianos and Castillo, 2012). This phenomenon is called “inflammaging” and conserved between organisms (Salminen et al., 2012).

Over-expression of *PGRP-LE* in the fat body induces Imd signalling and increases resistance against pathogens. However, this chronic induction of innate immune response by Relish, which is equivalent to chronic inflammation, reduces lifespan (Libert et al., 2006). Moreover, inhibition of NF-κB by pharmacological treatment extends lifespan, suggesting that a lowered basal innate immunity might be beneficial and reduce chronic inflammation (Moskalev and Shaposhnikov, 2011). These studies indicate that chronic inflammatory signals might affect lifespan more than an impaired immune response to pathogens, at least in sterile laboratory conditions.

Given this link between immunity and ageing, many studies have focused on the effect on immunity in lifespan-altered flies. *Chico* mutants are long-lived and show an increased resistance to pathogens. Surprisingly, there is no change in AMP gene expression. Moreover, dietary restricted flies do not show improved survival to bacterial infection, but AMP gene expression is induced (Libert et al., 2008). These results indicate that the underlying biological mechanisms for innate immunity are complex, and pathogen resistance is not necessarily coupled to longevity. So far there is no evidence that dFOXO regulates pathogen resistance, but interestingly dFOXO can bind to the AMP gene regulatory region and induce AMP expression when insulin signalling is reduced, and the Imd and Toll pathways are blocked (Becker et al., 2010). Thus, AMP gene expression is co-regulated by metabolism and the innate immune response, and dFOXO regulation of AMP gene expression might represent compensatory effects upon energy poverty.

Comparing transcript levels of AMP genes over the whole fly life span between wild type and caloric restricted flies revealed that the age-dependent up-regulation of innate immune effectors is delayed in caloric restricted flies (Pletcher et al., 2002). That would suggest that a key link between ageing and immunity is the time point in which basal innate immunity is up-regulated. Caloric restriction slows down the mechanism that induces age-related chronic inflammation

The relationship between DHR96 and IIS signalling requires more studies. But that there is an IIS dependency on DHR96 was shown for xenobiotic resistance. Although longevity was independent from each other, other phenotypes were similar, such as the reduced fecundity or the increased oxidative stress. The question arose, if they might be also linked to immunity since the expression levels of *Mtk* were drastically down-regulated in *dhr96-lbd* over-expressing females. As dFOXO regulates the expression of AMP genes, but DHR96 might act downstream of dFOXO, the potential link between DHR96 and immunity might occur through other mechanisms.

6.2 Materials and methods

6.2.1 *Drosophila* stocks

Dhr96-lbd was over-expressed in female flies by using the *daughterless* Gene-Switch driver. Crossings and maintenance were performed the same way as described in chapter 3. As a standard, 10 day old females were used for bacterial infection studies.

6.2.2 Pathogen and culture conditions

The gram-negative strain *Ecc15* (Erwinia carotovora carotovora or Pectobacterium carotovora carotovora (PCC)) were provided by Prof. Hoch labarotary (Bonn) to infect flies. This strain was modified from wild type *Ecc* bacteria by inducing a GFP-marker for tracking use (Basset et al., 2000).
Ecc15 bacteria were grown in 100 ml overnight LB culture at 29°C at 220 rpm. OD was measured to identify bacterial density. Bacteria were centrifuged at 3.000 rpm for 15 minutes at 4°C (Beckmann centrifuge, JA-10). The pellet was dissolved in 10% Sucrose in H_2O_2, and the OD was adjusted to 100 (Neyen et al., 2014).

6.2.3 Pricking

Very fine needles (0,1mm; Plano) were used to inject bacteria in the thorax of *dhr96-lbd* over-expressing flies and control flies (driver control on RU and *dhr96-lbd* non-induced form). Female flies were infected under carbon dioxide anaesthesia treatment. Gas treatment was timed to be equal between genotypes. Flies were pricked with 10% Sucrose without bacteria as a further control. 10 female flies for each genotype and condition were stabbled using bacterial or sucrose contaminated needle. Flies were transferred back to vials with 1x SYA food.

6.2.4 CFU - counting colony-forming units per fly

4 hours after infection, flies were transferred to tubes filled with glass beads and 1ml of magnesium sulphate (10mM) on ice, and homogenized using the

FastPrep®-24 instrument (MP Biomedical) at maximum speed for 20 seconds. Different dilutions were prepared to plate flies on LB-plates containing the antibiotic rifampicin (100 µg/mL) as the *Ecc15* strain carries genomic resistance to rifampicin.

Diluted lysates were plated in two different ways. In the first approach, 3 µl of a serial dilution were spotted in a line per fly (1:10, 1:25, 1:50, 1:75). Five flies were spotted on one plate. Every line represents one fly with serial dilutions. Plates were stored at 25°C after spots were dried, and transferred to 4°C when colonies were visible.

To quantify the number of bacterial colonies, lysates were plated on LB-plates containing the antibiotic rifampicin. In this second approach, three dilutions were chosen (1:10, 1:100, 1:1000). To reduce the number of plates, two flies lysates were combined on one plate (100µl each fly sample).

6.2.5 ImageJ counting

Plates were imaged by a gel documentation system (G-BOX, Syngene). The software ImageJ (Java based program) processed pictures for automated counting of colonies. Images were converted from 16-bit to 8-bit images and inverted by the command “watershed”. Colony counts per image were obtained by the command “analyse particles”.

6.2.6 Data analysis

Data displays counting colony-forming units per female fly in case of the spotting approach and two flies as a unit in case of the second approach. All quantitative data are reported as the mean ± SEM (standard error of mean). P-values were determined by Student’s *t*-test.

6.3 Results

6.3.1 *Dhr96-lbd* over-expressing flies showed increased bacterial density

Dhr96lbd over-expressing and control flies were infected with the pathogen *Ecc15*, and different dilution of each lysate was spotted on LB-plates containing rifampicin.

Comparing bacterial spot colonies of *dhr96-lbd* over-expressing females to spots of its ethanol control or the driver control on RU revealed that *dhr96-lbd* over-expressing flies gave more bacterial colonies than did the controls. The 1:10 dilution spots of *dhr96-lbd* over-expressing flies showed dense colonies (figure 6.3.1 A), whereas control spots contained moderate and little colony density (figure 6.3.1 B and C). In higher diluted spots (1:25 and 1:50), more colonies grew in the *dhr96-lbd* over-expressing flies than in control flies. Spots of control flies contained none or few colonies in these dilutions. No colonies grew on plates that contained the 10% Sucrose control.

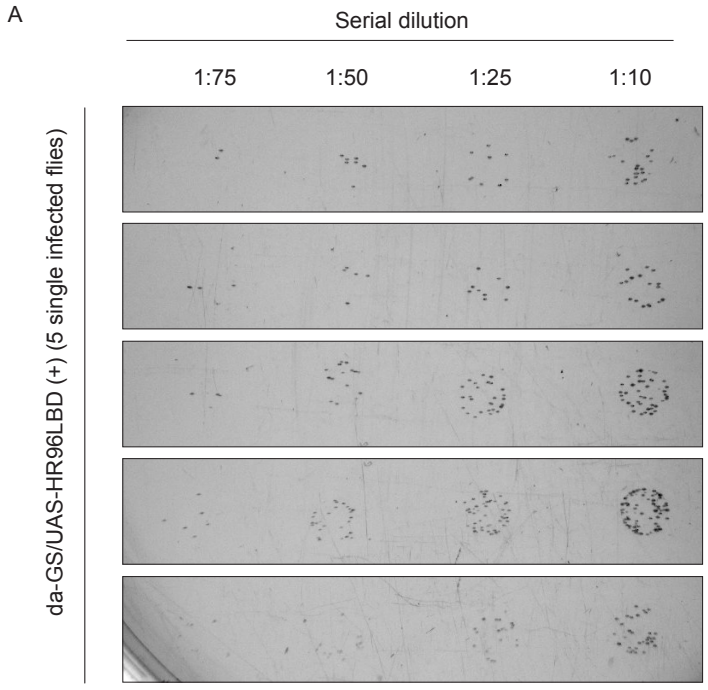


Figure 6.3.1. Serial dilutions of *dhr96-lbd* over-expressing and control fly lysates 4 hours after infection with *Ecc15* bacteria. (A) *Dhr96-lbd* over-expressing flies showed increased number of bacterial colonies than in (B) control flies on ethanol or in (C) driver control flies on RU. (A) The 1:10 and 1:25 dilutions of fly lysates from *dhr96-lbd* over-expressing show dense or moderate dense colonies in lysate spots. It is noticeable that these dilutions in control flies (B and C) show reduced numbers of colonies.

6.3.2 Quantification of bacterial colonies in *dhr96-lbd* over-expressing females

Samples diluted to 1:100 showed that *dhr96-lbd* over-expressing flies gave significantly increased colony numbers compared to driver control flies on RU or control flies on ethanol (p-value **<0.01, Student's *t*-test) (figure 6.3.2). Control plates with sucrose samples did not show any colonies. No bacterial colonies grew on plates, which contained samples from 1:1000 dilutions.

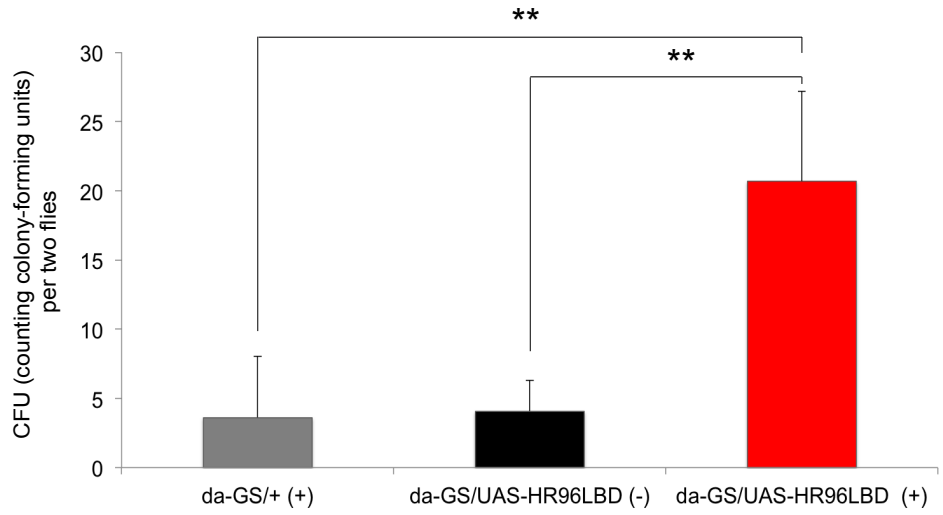


Figure 6.3.2 *Dhr96-lbd* over-expressing flies are sensitive to bacterial infection (1:100). *Ecc15* gram-negative bacteria colonies of infected flies were counted by using ImageJ software. *Dhr96-lbd* over-expressing flies showed enhanced bacterial colony numbers than control flies (da-GS (+) and da-GS/UAS-HR96LBD (-)) (p-value **<0.01, Student's *t*-test).

Plates containing 1:10 diluted fly homogenates were analysed as well (figure 6.3.3). Quantification of these data confirmed that *dhr96-lbd* over-expressing flies have significantly increased bacterial growth compared to driver control flies on RU (p-value **<0.01, Student's *t*-test) or to UAS-control flies on ethanol (p-value *<0.05, Student's *t*-test).

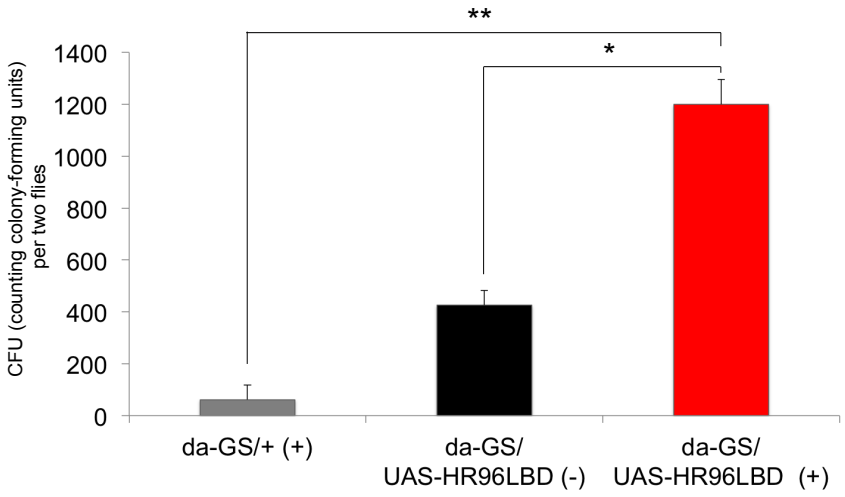


Figure 6.3.3 *Dhr96-lbd* over-expressing flies were sensitive to bacterial infection (1:10). Counting of *Ecc15* gram-negative colonies of infected flies in a dilution of 1:10 revealed that *dhr96-lbd* over-expressing flies showed enhanced bacterial numbers than control flies (da-GS (+) and da-GS/UAS-HR96LBD (-)) (p-value **<0.01, Student's *t*-test).

6.4 Discussion

Over-expression of *dhr96-lbd* in females caused a decrease in expression of the AMP gene *Mtk*, and functional analysis of *dhr96-lbd* over-expressing flies infected with gram-negative *Ecc15* revealed that the innate response after 4 hours of infection is suppressed in these flies. Infected *dhr96-lbd* over-expressing flies contained more bacteria than did the control flies. However, these results support only the first time point after infection and it is not proven if survival or the immune response at a second time point would differ between *dhr96-lbd* over-expressing flies and controls. Thus, the next steps would be survival assays of infected transgenic flies, and bacterial counting for several time points after infection.

Although *dhr96-lbd* over-expressing females have beneficial effects in xenobiotic resistance, oxidative stress response and lifespan, the negative effect on bacterial immune response implies that DHR96 has both positive and negative effects on life history traits. Interestingly, a contrary link between oxidative stress and the innate immune response is shown in transcriptional profiling studies of wild type flies treated with the oxidative stressors paraquat and H_2O_2 (Misra et al., 2011). These flies reveal induced expression of genes

mediating oxidative stress response, but suppressed expression of many genes involved in the innate immune response. AMP genes, including *Mtk*, *Def*, *dro3*, *Drs*, and 3 *cecropin* genes, and additional main effectors in the innate immune response, were down-regulated. Moreover, studies with peroxiredoxin 5 (dPrx5), a redox-sensing enzyme, revealed that over-expression of this gene increase lifespan and oxidative stress response, whereas the null mutation caused sensitivity to oxidative stress and shortened lifespan (Radyuk et al., 2010). Interestingly, short-lived mutants exhibit an enhanced resistance to bacterial infection, but long-lived dPrx5 over-expressing flies show decreased resistance to bacteria (Radyuk et al., 2010). These results are very similar to DHR96 phenotypes, and might indicate that a negative correlation between ageing or oxidative response and the innate immune response is possible.

A negative correlation is also shown for the *dhr96* homolog *PXR* in humans. Rifampicin is a drug, which induces the activity of PXR, and patients that were treated with rifampicin in clinical trials, showed immunosuppressive effects. In mice, induced PXR activation decreases activity of NF-κB protein, whereas knock out PXR mice show increased expression of NF-κB target genes (Ihunnah et al., 2011).

The suppressed immune response in *dhr96-lbd* over-expressing flies might be caused by hormonal regulation. Juvenile hormone (JH) and 20-Hydroxyecdysone (20E) modulate innate immunity in *Drosophila*. 20E can induce the expression of AMP genes, whereas JH inhibits the 20E induced AMP expression (Flatt et al., 2008a). Interestingly, expression levels of Juvenile Hormone Binding Protein (JHBP) are modulated in *dhr96* null mutants (King-Jones et al., 2006). JHBP ensures the transport of JH and other lipophilic molecules to target tissues and protects this hormone from degradation (Touhara and Prestwich, 1993). *Dhr96* null mutants show induced expression of JHBP and treatment of *dhr96* null flies with phenobarbital shows an additive effect to this induction. Thus, it might be interesting to test if expression levels of these genes are modulated in *dhr96-lbd* over-expressing flies, and if so, whether a suppressed effect of JH on 20E induced AMP gene expression might occur.

Hormones, including JH and 20E, are produced from cholesterol through several steps and catalyzed by cytochrome P450 enzymes. DHR96 regulates cholesterol homeostasis (Bujold et al., 2010). Interestingly, *dhr96* null flies show increased cholesterol and decreased TAG that is caused by down-regulation of the lipase *magro* and increased *npc1b* levels (Sieber and Thummel, 2012). In *dhr96-lbd* over-expressing flies, *magro* was also down-regulated, which might cause changes in cholesterol homeostasis (chapter 5.3.2.1). Interestingly, *Cyp18a1*, which inactivates 20E (Guittard et al., 2011), was also down-regulated in *dhr96-lbd* over-expressing flies. Thus, the suggestion would arise that 20E levels would be increased and might be beneficial for AMP gene expression. But the hypothesis I support is that a decrease of *magro* might cause overall low cholesterol and affect cytochrome P450 genes in a negative feed back loop. Thus, it is of major interest to identify the levels of cholesterol and 20E in *dhr96-lbd* over-expressing flies. Exposing wild type flies on low cholesterol food induced *magro,* but decreased *Cyp18a*1 expression in the whole body (supplement S.6.1). That would suggest that cholesterol starvation might cause a compensatory mechanism, for example by enhancing cholesterol uptake via *magro* and changing the activity of cytochrome P450, as is the case for *Cyp18a1*. It would be interesting to test whether these regulations are mediated via DHR96 by testing *dhr96* null mutant and *dhr96-lbd* over-expressing flies on low and normal cholesterol food for qRT-PCR analysis.

In aged flies the innate immune response is up-regulated and permanently induced (Eleftherianos and Castillo, 2012). Over-expression of PGRP-LE that induces a chronic immune response via induction of Imd signalling shortens lifespan (Libert et al., 2006). In contrast, inhibition of NF-κB by pharmacological treatment extends lifespan (Moskalev and Shaposhnikov, 2011). Thus, inhibitory effects on the innate immunity can be anti-ageing. According to my results, this is very interesting as the basal expression of the AMP gene *Mtk* in *dhr96-lbd* over-expressing flies is reduced. However, the experiments covered only flies at an age of 10 days. As AMP transcript levels are increased in old flies it would be important to test the AMP expression levels at different ages of *dhr96-lbd* over-expressing fly lifespan. Moreover, caloric restricted flies showed in a delay in the up-regulation of AMP genes (Pletcher et al., 2002). Thus, it

would be interesting to test whether this also occurs in *dhr96-lbd* over-expressing flies.

This chapter indicates that expression levels, at least for the AMP gene *Mtk* of *dhr96-lbd* over-expressing flies were reduced and that the innate immune system of *dhr96-lbd* over-expressing flies was sensitive to bacterial *Ecc15* infection. How this effect might correlate to the other phenotypes of *dhr96-lbd* over-expressing flies is so far unclear and requires further studies. But that immunity can be coupled to oxidative stress and to ageing has been shown in different studies. Thus, it represents an interesting field to invest further studies and to understand how the different traits are balanced.

Chapter 7 DHR96 ligand sensor screen with various drugs and *Drosophila* lipid extracts

7.1 Introduction

DHR96 is involved in life history traits of *Drosophila* but the underlying mechanisms are still unclear. It is unknown whether DHR96 acts as an activator, repressor, or both, of target genes. Identifying the natural ligand of DHR96 would facilitate various studies to discover DHR96 mechanism. Thus, the aim of this chapter is to identify the ligand(s) of DHR96.

7.1.1 Function and ligands of the *dhr96* human ortologs *CAR, PXR* and *VDR*

Human constitutive androstane receptor (CAR), pregnane X receptor (PXR), and vitamin D receptor (VDR) are the closest homologs of *Drosophila* DHR96 (King-Jones and Thummel, 2005; Lindblom et al., 2001). Furthermore, the liver x receptor (LXR) shows functional relation to DHR96 activity as both receptors regulate target genes that are involved in cholesterol metabolism (Horner et al., 2009). These hormone nuclear receptors heterodimerize with retinoid X receptor (RXR) to induce transcriptional regulation of target genes (Haussler et al., 2010; Wang et al., 2012). VDR activity is induced by the sterol 1,25-dihydroxyvitamin, and modulated bone mineral metabolism, mammalian hair cycling, xenobiotic response and metabolic pathways that are involved in cancer and immune response (Adorini et al., 2006; Haussler et al., 2008). CAR and PXR share target genes and are both described as xenobiotic sensors (Tolson and Wang, 2010). Interestingly, both HNRs are expressed highly in the liver and intestine, which are important for detoxification, but also crucial for glucose and lipid metabolism (Gao and Xie, 2012).

Studies on CAR revealed diverse biological functions (Molnar et al., 2013). Next to the xenobiotic response, it is involved in lipid metabolism and in endobiotic

metabolism of steroids, bile acid, or hormones for other NHR like thyroid hormone (Halilbasic et al., 2013; Wagner et al., 2010). Moreover, CAR is involved in energy metabolism, and is crucial for hepatic glucose regulation (Gao and Xie, 2012). Cell cycle regulation, hepatic proliferation and response to chemical carcinogenesis are additional functions reported for CAR (Chen et al., 2012; Kohle et al., 2008).

Phenobarbital is a prominent xenobiotic identified to modulate CAR activity (Zelko and Negishi, 2000). PB induces the expression of cytochrome P450 *2B* genes via PB responsive DNA elements (Honkakoski et al., 1998). In CAR null mice these *Cyp2B* genes were not induced upon PB treatment (Wei et al., 2000). Phenobarbital among other compounds were reported as ligands for CAR, but turned out to induce its activity in a ligand-independent manner (Mutoh et al., 2013). CAR under normal conditions is located in the cytoplasm, where it is bound to HSP90 (Yoshinari et al., 2003). PB treatment induces dephosphorylation of CAR by protein phosphatase 1ß and protein phosphatase 2A, and subsequently its translocation into the nucleus (Sueyoshi et al., 2008). The dephosphorylation of CAR occurs by the inhibition of epidermal growth factor receptor (EGFR) signalling (Mutoh et al., 2013).

CAR is constitutively active (Choi et al., 1997), but several identified compounds can modulate its activity. Some activators exhibit a low selectivity and can activate also other TFs, like many pesticides (CAR and PXR) or etrogens (estrogen receptor and CAR). However, the numbers of chemicals that activate specifically CAR directly or indirectly increased and include compounds like steroids, pesticides, synthetic, or natural compounds (Molnar et al., 2013). Among the few direct ligands are TCPOBOP (1,4-bis-[2-(3,5-dichloro-pyridyloxy)]benzene) (Tzameli et al., 2000) and CITCO (6-(4-**C**hloro-phenyl)**I**midazo[2,1-b][1,3]**T**hiazole-5-**C**arbaldehyde**O**-(3,4dichlorobenzyl) oxime) (Maglich, 2003), where ligand binding was shown in crystal structures. Inverse agonists that repress transcriptional activity of CAR have also been identified, like androstanol (Forman et al., 1998).

Interestingly, the list of interaction partners of CAR contains co-activators and co-repressors, suggesting that it can act as an activator or a repressor, possibly

dependent upon an agonist or inverse agonist is interacting with CAR (Molnar et al., 2013).

PXR and CAR overlap in some function, which occur to the overlap in some target gene and ligands (Tolson and Wang, 2010). In contrast to CAR, PXR activity is reported to be fully ligand-dependent. As with CAR, PXR is involved in glucose regulation and lipid metabolism in the liver (Hukkanen et al., 2014).

7.1.2 Reporter assay studies of DHR96 in *Drosophila melanogaster*

The activation pattern of several nuclear receptors, including DHR96, was investigated using an HNR-GFP fusion reporter construct, which showed the time point and location of activation for these transcription factors. No signal was detected for DHR96. However, treatment of embryos with CITCO (6-(4-**C**hlorophenyl)**I**midazo[2,1-b][1,3]**T**hiazole-5-**C**arbaldehyde**O**-(3,4-dichloroben-zyl)oxime), which is a known non-natural ligand for DHR96 homologue nuclear receptor CAR, leads to DHR96 activation (Maglich, 2003).

Of the 18 canonical hormone nuclear receptors in *Drosophila* (King-Jones and Thummel, 2005), only two have known ligands. 20-Hydroxyecdysone is identified as the ligand for Ecdysone receptor(Koelle et al., 1992; Thomas et al., 1993; Yao et al., 1993). For HNR E75, heme is shown to induce E75 activity (Cruz et al., 2012; Reinking et al., 2005). Ligand(s) for the other 16 canonical *Drosophila* hormone nuclear receptors have not been identified yet, and therefore they are referred to as orphan nuclear receptors (Mangelsdorf and Evans, 1995).

Interestingly, studies have revealed possible crosstalk between the Ecdysone receptor and DHR96. In general, nuclear receptors bind to specific promoter regions of target genes, which are called nuclear receptor response elements (Ham et al., 1988; Payvar et al., 1983). DHR96 is known to interact with the *hsp27* response element, which is in turn a target promoter region for EcR (Fisk and Thummel, 1995). Competition between both nuclear receptors for the promoter region could serve as a further layer of regulation for the 20E response. Furthermore, overexpression of *hsp27* leads to lifespan extension and starvation resistance (Wang et al., 2004).

7.1.3 Ligand sensor screen with various drugs and *Drosophila* lipid extracts

Chapter 3 introduced DAF-12 and its regulation by dafachronic acid, the DAF-12 ligand. The identification of the natural DAF-12 ligand (Motola et al., 2006) gave the opportunity to use a similar approach to identify the natural ligand of DHR96 in *Drosophila* (chapter 7.1.4). Although cholesterol binds to DHR96, this has not been proven to be the case in natural conditions. The approach was focused on lipids from *Drosophila*, and to test lipid fractions in a *dhr96* reporter system as it was done for DAF-12. Moreover, *C.elegans* lipids were used in an equivalent *daf-12* reporter system as a control for the approach.

Figure 7.1.1 depicts an overview of the experimental approach, which indicates the different steps of the ligand screen experiment. It shows that lipids from flies were extracted and fractionated and that each fraction has been tested in *Drosophila* S2 cells expressing the reporter system for DHR96 activity. Fractions with an activating effect can then be used to identify the ligand with further fractionation steps and mass spectrum analysis (MS). Furthermore, candidate ligands including CITCO, phenobarbital, tebufonzide, glucose, and dafrachronic acids were tested in a similar manner.

Due to the similarities between DHR96 and DAF-12, we started a collaboration with Dan Magner of the Antebi laboratory, where we aimed to gain an insight into how the activity of these nuclear receptors are regulated and affect ageing. The focus of the proposed project is therefore to identify the hormone(s) controlling DHR96 function.

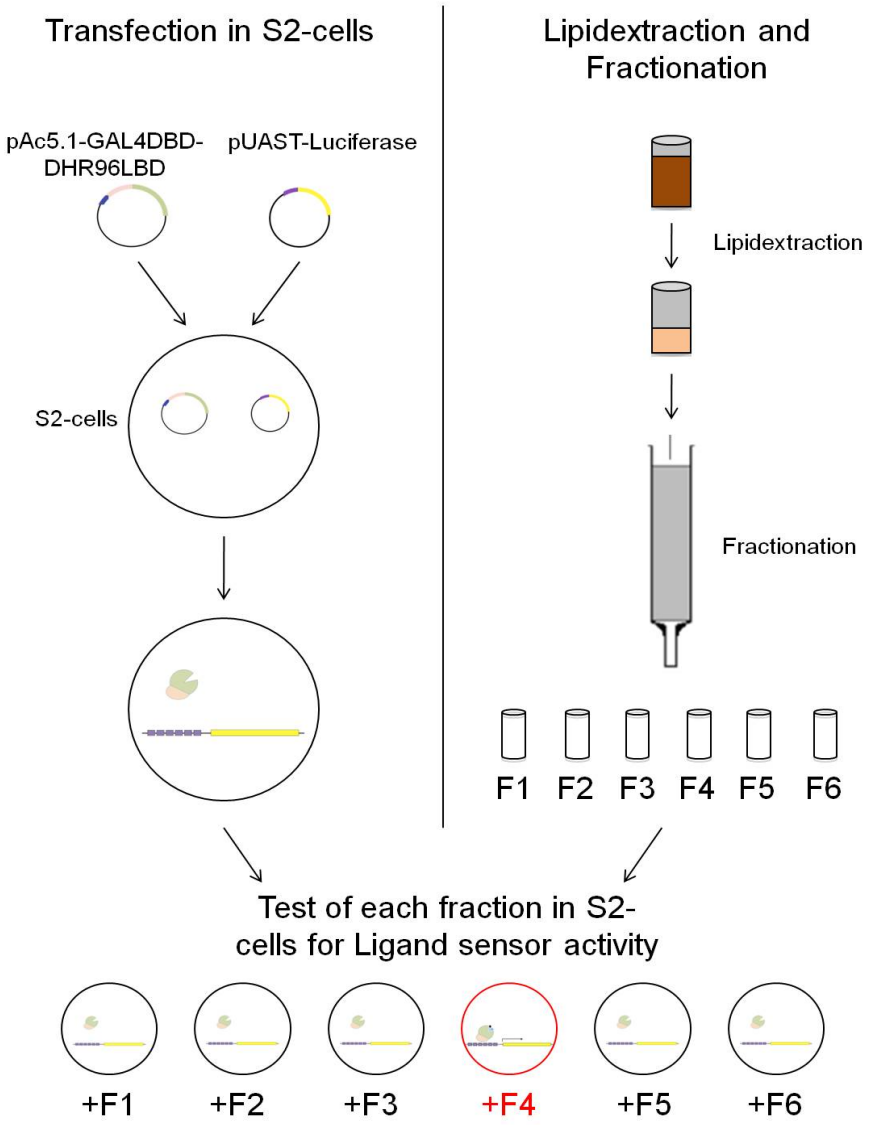


Figure 7.1.1 Ligand sensor screen. The ligand sensor system (left) together with lipid extract fractions from flies (right) and candidate ligands (not shown) were tested for activation in S2 cells. A silica column was used to fractionate lipid extracts through their polarity. All fractions were dissolved in DMSO and tested at 1:1000 dilution for activation in S2 cells.

7.2 Materials and methods

7.2.1 Fly extraction and fractionation

For the first approach of the ligand sensor screen, adult and larvae white Dahomey^{+} (wDah^{+}) *Drosophila* from multiple stages of the life cycle and both genders were collected (adult and non-adult samples). We have used a mixed selection of flies to have all possible sterols contained in the extracts, as lipid composition differs during development and between genders.

For the second approach, female flies were exposed to DDT (275mg/L in 1x SYA) food for 4 hours, or to holidic medium (a chemical defined medium) with a low cholesterol concentration, recipe see supplement S.7.1) for 2-5 days. Flies from the first and second approaches were snap-frozen on liquid nitrogen and then stored at -80°C. Frozen flies were homogenized in ice-cold PBS (Phosphate buffered saline, Invitrogen) using a homogenizer (IKA T10basic). Lipids from fly homogenate were extracted with 2:1 chloroform/methanol (BLIGH and DYER, 1959) and the resulting organic phase was dried with nitrogen, which transfers the methanol of the extract into the gas phase, and an evaporator was used to completely dry the sample.

Dan Magner collected lipid extracts from worms of varying ages and genders, and in addition a pig liver using the above extraction technique. Using a silica coloumn lipid extracts were fractionated through their polarity. Initial fractions possess high polarity, whereas subsequent fractions have decreased polarity.

7.2.2 Gas chromatography/Tandem mass spectrometry (GC/MS/MS) analysis

Fractions were subjected to GC/MS/MS analysis to identify fraction(s) that contains dafachronic acid. This serves as an essential control, on the one hand to verify that lipid extraction worked, and on the other hand, to verify that worm fractions (especially those containing dafachronic acid, as the known ligand) gives DAF-12 activation when applied to the ligand sensor screen.

Analysis was performed with a 7000A Triple Quadrupole GC/MC instrument (Agilent Technologies) that provides an ESO source and a HP-5MS column.

Purified dafachronic acid was used as an internal control and samples were derivatized with diazomethane. The instrument was adjusted in a multiple reaction monitoring (MRM) mode and transition for dafachronic acid (m/z 220 → 250) was used.

7.2.3 Ligand sensor system

Fractions and candidate sterols were tested in a reporter assay for activity. This ligand sensor system, which was developed for different *Drosophila* nuclear receptors and tested on embryo flies (Palanker, 2006), was used in cell culture experiments to identify candidate ligand(s) for DHR96. The system includes two essential constructs: (I) The Ligand-Binding-Domain (LBD) of *dhr96* (624bp-2463bp) (see cloning) was fused to the DNA-Binding-Domain of *gal4*. (2) 5xUAS-*firefly luciferase* was used as a reporter gene to monitor nuclear receptor activity. Ligand(s) binding to the LBD of DHR96 leads to the activation of GAL4 via a conformational change, resulting in an interaction of GAL4 with the UAS-promoter and an induction of *renilla luciferase* expression (see Figure 7.2.1).

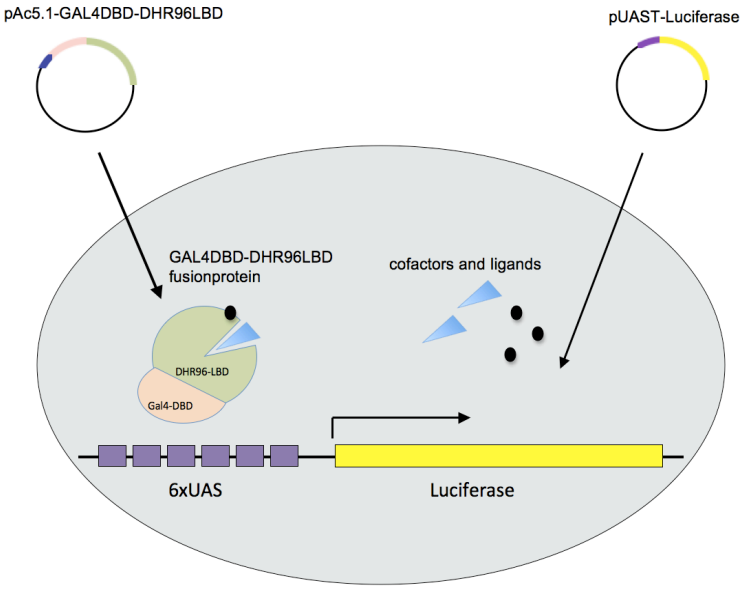


Figure 7.2.1 Schematic representation of the ligand sensor system. S2 cells were transfected with *Gal4DBD-dhr96LBD* together with the *luciferase* reporter construct**.** In the presence of the DHR96 ligand(s) the Gal4DBD-DHR96LBD fusion protein should be able to bind with the Gal4 UAS response element. As a result the expression of the reporter gene *luciferase* is induced.

7.2.4 Cloning of constructs

The following constructs were generated for the screen: The LBD-domain of the three different nuclear receptors, *dhr96*, *daf-12* and *nhr8*, which were identified via alignments between NHR genes (Antebi et al., 2000), were amplified by PCR, fused in frame to the *Gal4*-DNA-Binding Domain, and cloned into a pAc5.1/V5-His B vector (Invitrogen) to generate the constructs *Gal4DBD-dhr96LBD*, ligand-insensitive *Gal4DBD-dhr96LBDmut*, *Gal4DBD-daf12LBD*, and *Gal4DBD-nhr8LBD*. Individual primers and restriction sites are listed in supplement S.2.1. For cloning experiments the In Fusion HD Kit (Clontech) was used according to the manufacturer's instructions.

pUAS-*firefly luciferase* (adgene) has already been tested in insect cell lines (Antebi et al., 2000) and was therefore used as the reporter gene of the ligand sensor system. An additional reporter vector was necessary as a transfection control and for normalization, which was co-transfected in all samples. Normalization is essential for transfection in multiple-wells as transfection efficiency of cells differs between wells. To avoid this confounding factor, RLU values of firefly *luciferase* were normalised to RLU values of the normalisation vector. *Gfp*, *ß-galactosidase* or *renilla luciferase* can be used for normalisation; and as *renilla* is well established for reporter assays, three different *renilla* vectors were used in S2 cells: pRL-*null* (Promega), pSV40-*renilla* (Promega), pact-*renilla* (Prof. Hoch laboratory). Only pact-*renilla* worked efficiently in S2 cells, and was therefore selected for following experiments.

7.2.5 Transfection of S2 cells

Schneider *Drosophila* S2 cells were grown at 25 °C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% heat-inactivated FCS and antibiotics (100 µg/mL streptomycin and 60 µg/ mL penicillin, Roche).

The day before transfection, S2 cells were split and seeded in 96-wells plates (polystyrene black plate, costar) at a density of $1x10^6$ cells/ml and a volume of 100µl per well. S2 cells were transfected using Effectene Transfection Reagent (Quiagen) with a total DNA amount of 0,18µg per well, containing 30ng pact-Renilla, 75ng pAc5.1-*Gal4DBD-TF-LBD* (TF for Transcription factor), 75ng pUAS-*luciferase* and, for diverse controls 75ng pAc5.1/V5-His B as a fill vector.

After 2 days of incubation at 24 °C to allow DNA uptake and optimal protein expression, cells were treated with each *Drosophila* lipid extract fraction or candidate ligands. Luminescence was measured after a 16 hours incubation time (see 7.2.7).

7.2.6 Sterol and fraction supplementation

All dried fractions were dissolved in DMSO, which is a good solubilisation reagent for organic compounds (Balakin et al., 2004). They were used in a 1:1000 dilution for the ligand sensor screen in S2 cells. This dilution activated DAF-12 in preliminary experiments in Human Embryonic Kidney 293 cells (HEK-293) using *C. elegans* lipid extracts (Dan Magner, unpublished data). Commercial available drugs, listed in table 7.2.1, were used at different solvents and concentrations (from 50nM until 100mM), depending on the drug.

Sterol	**Solution**	**Source**	**Positive activation**
CITCO	DMSO	Sigma	CAR
Phenobarbital	Methanol	Sigma	CAR, VDR
Tebufonzide	Ethanol	Sigma	(Ecdysone)
Glucose	Water	Roth	LXR
Dafrachronic acid	DMSO	Antebi laboratory	DAF-12

Table 7.2.1 Sterols used. Sterols were dissolved in the recommended solvents. Most of them have been shown to activate *dhr96* orthologs (CAR, VDR, DAF-12), other closely related nuclear receptors (*LXR*), or nuclear receptor with possible cross talk (ecdysone receptor).

7.2.7 Luciferase assay

For measurement of *firefly* and *renilla* luciferase, the Dual *Glo* Luciferase Kit (Promega) was used according to the manufacturer's instructions. Activity was measured in the form of luminescence by the infinite M200 plate reader (Tecan) for the screen using drugs, and by the BMG microplate plate reader (Omega) for the screen, using lipid extracts from fly, pig and worm. Firefly luciferase values were normalised to renilla luciferase activity, and in both cases their luminescence is reported as Relative light units (RLU), calculated as ([light

units/OD 420] x reaction time in minutes). Results are reported as the means of three independent experiments, each assayed in triplicate. All quantitative data are reported as the mean ± SEM (standard error of mean). P-values were determined by Student's *t*-test.

7.3 Results

7.3.1 Ligand sensor screen

7.3.1.1 Dafachronic acid activated DAF-12 but not DHR96 in S2 cells

The *Gal4DBD-daf12LBD* fusion construct was generated as a positive control for the ligand sensor screen, to test if their purified ligand, dafachronic acid (DA), induces activation of DAF-12 fusion protein in an S2 cell culture system. In figure 7.3.1, it is shown that treatment with the DAF-12 ligand led to a significant induction of GAL4DBD-DAF12LBD reporter gene transcription in a dose dependent manner (4-fold at 100 and 200nM DA, 10-fold at 500nM DA). Thus the *gal4DBD-Tf-LBD* system works in S2 cells. Moreover, no activation of *luciferase* transcription was observed for DHR96.

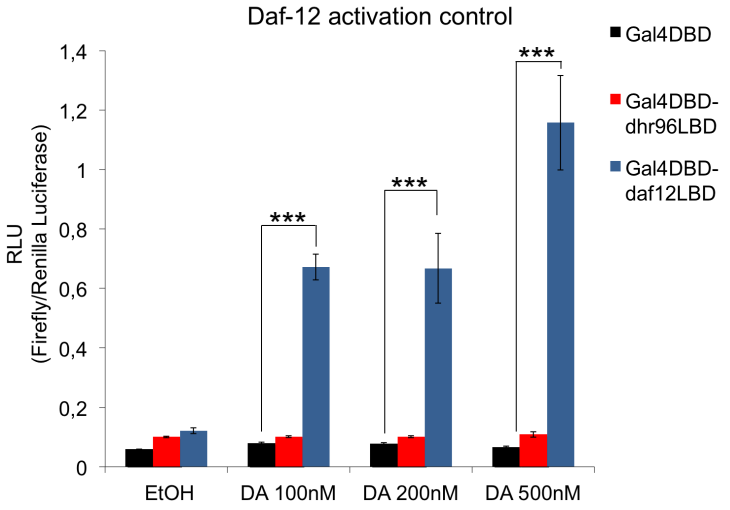


Figure 7.3.1 Reporter assay with dafachronic acid (DA) in S2 cells. In the presence of dafachronic acid, GAL4DBD-DAF12LBD fusion protein was able to interact with the *Gal4* UAS response element. As a result the expression of the reporter gene *luciferase* (RLU) was induced. In contrast, dafachronic acid did not affect activity of GAL4DBD-DHR96LBD.

7.3.1.2 Ligand sensor screen with various drugs

The drug CITCO, which activates the DHR96-GFP-reporter system in *Drosophila* embryos (Palanker, 2006), failed to induce expression of *firefly luciferase* in S2 cells at the concentrations of 1, 10 and 100µM (figure 7.3.2 A). Remarkably, DMSO (Dimethyl Sulfoxide) alone affected DAF-12 activity (significant induction of 2-3 fold) and also possibly stimulated DHR96 to a lesser extent (not significant, approximately 1,5 fold).

Phenobarbital, tebufonizide, or glucose did not activate DHR96 or DAF-12 fusion proteins at any tested concentrations (figure 7.3.2 B, tebufonizide and glucose see supplement S.7.2). At a dose of 500µM, phenobarbital (PB) treatment caused a significant activation of GAL4DBD-DHR96LBD, when compared to the GAL4DBD control at 500µM PB treatment, or to the GAL4DBD-DHR96LBD methanol control (p-value *<0.05, Student's *t*-test). The activation amounted about ~20%. However, this increase was not significant when compared to the Gal4DBD control on methanol.

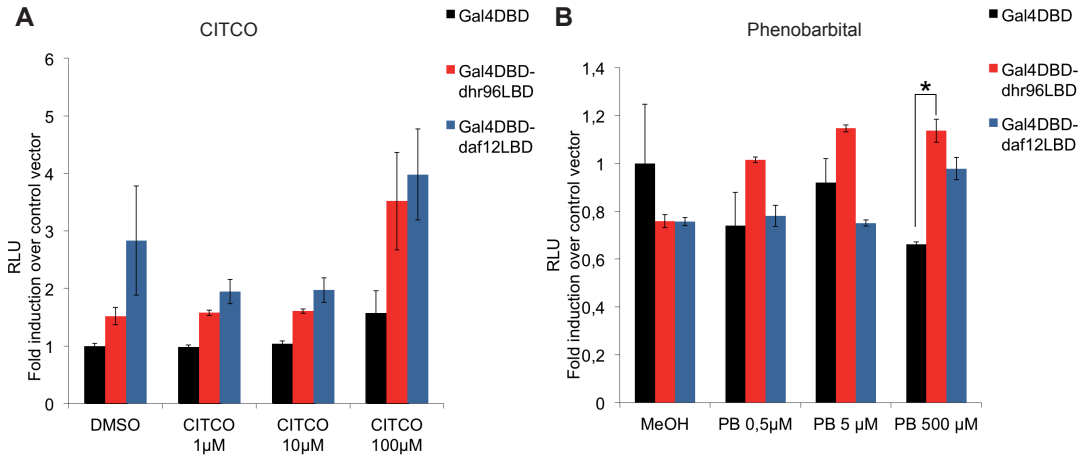


Figure 7.3.2 Effect of (A) CITCO and (B) Phenobarbital on *GAL4DBD-DHR96LBD* and GAL4DBD-Daf-12LBD activity in transiently transfected S2 cells. *Gal4DBD-dhr96LBD* or *Gal4dbd-Daf-12LBD* fusion constructs and the pAct-*luciferase* reporter were over-expressed in S2 cells. After 2 days to allow expression, cells were treated by (A) CITCO and (B) phenobarbital (PB) at various concentrations. Activity in form of luminescence (RLU) was measured 16h after treatment. RLU values were normalised to the values of the control pAct-*renilla*. (A) DMSO had an effect on the reporter system. No activation was detected for CITCO. (B) A significant increase of luminescence was observed in cells treated with PB at a concentration of 500µM. No significant changes were observed using 0.5 and 5µM PB.

The experiment was repeated with higher doses of phenobarbital as the 500µM treatment gave a significant increase. Interestingly, concentrations of 2mM PB significantly induced *luciferase* transcription when compared to all controls (figure 7.3.3) (p-value ***<0.001, Student's *t*-test). The activation amounted 3.5 fold when compared to the Gal4DBD control at 2mM PB concentration. However, longer treatment or higher concentrations of PB induced cell lethality and no significant *luciferase* transcription (data not shown). The experiment at 2mM was performed once and has to be repeated.

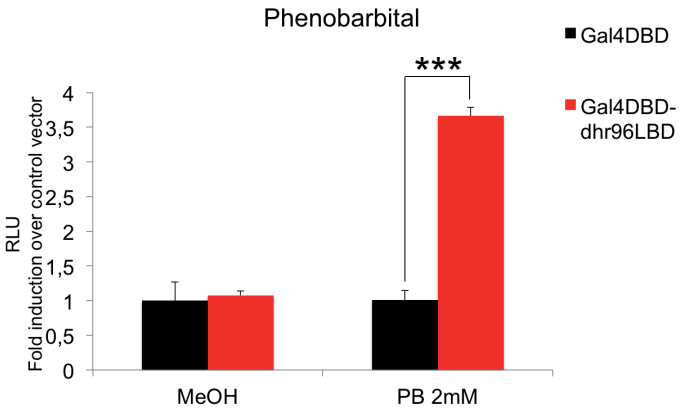


Figure 7.3.3 2mM Phenobarbital treatment induced DHR96 ligand sensor system in S2 cells. Activity of Gal4DBD-DHR96LBD was significantly induced by 2mM phenobarbital treatment (p-value ***<0.001, Student's *t*-test).

7.3.2 Ligand sensor screen with wild type *Drosophila, C. elegans* and pig liver lipid extracts

7.3.2.1 GS/MS/MS analysis of lipid fractions

GS/MS/MS analysis of lipid extract fractions identified *C. elegans* fraction 58 as containing dafachronic acid (DA) (figure 7.3.4), thus the lipid extraction worked. However, it does not indicate that all lipids were successfully extracted and solubilized, as sterols differ in their properties. No DA was detected in pig liver or in *Drosophila*.

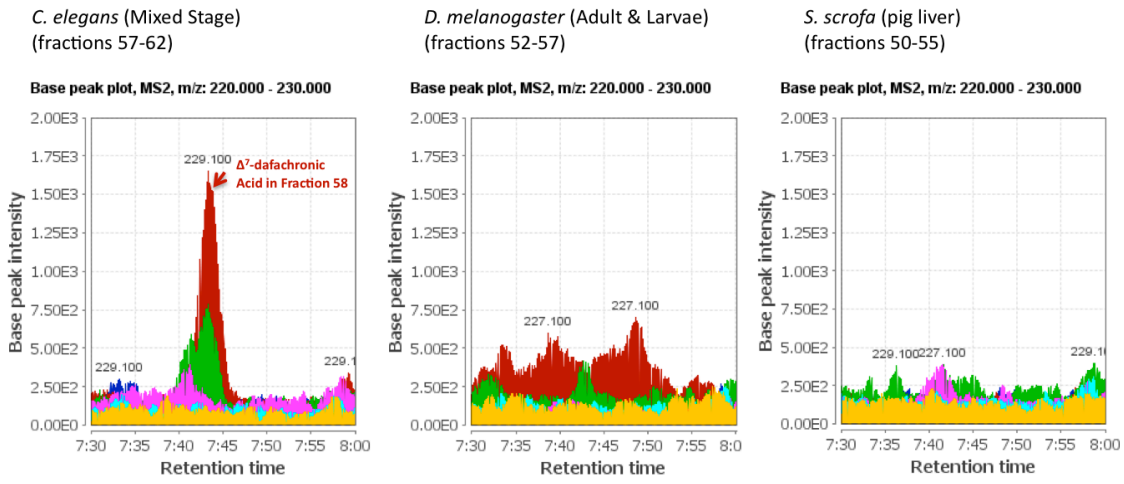


Figure 7.3.4 GS/MS/MS analysis of fraction from *C. elegans, D. melanogaster, S. crofa* (Dan Magner). Fractions were prepared by trimethylsilyldiazomethane derivitization and analyses were performed by GC-MS-MS on a 15 m HP5-ms column for dafachronic acid (retention time of Δ^7-dafachronic acid is reported at 7:43). Analysed peaks were chosen based on the co-migration of DA. Fraction 58 (marked in red) is identified to contain the dafachronic acids in worms.

7.3.2.2 Analysis of wild type *Drosophila, C. elegans* and pig liver whole lipid extracts

Whole lipid extract of each organism was tested for activation using the S2 cell system (figure 7.3.5). No notable induction using whole lipid extracts (1:1000) was observed for any extract. Only the DA control (1mM) showed a three-fold induction for DAF-12 when compared to the GAL4DBD-DAF12LBD DMSO control, which was lower than seen in previous experiments due to the high concentration of the dafachronic acid (figure 7.3.1, 10-fold induction with 500µM DA). Notably, no activation for DAF-12 was observed by using *C. elegans* whole lipid extract although dafachronic acid was identified in fraction 58 and was thus present in the whole lipid extract.

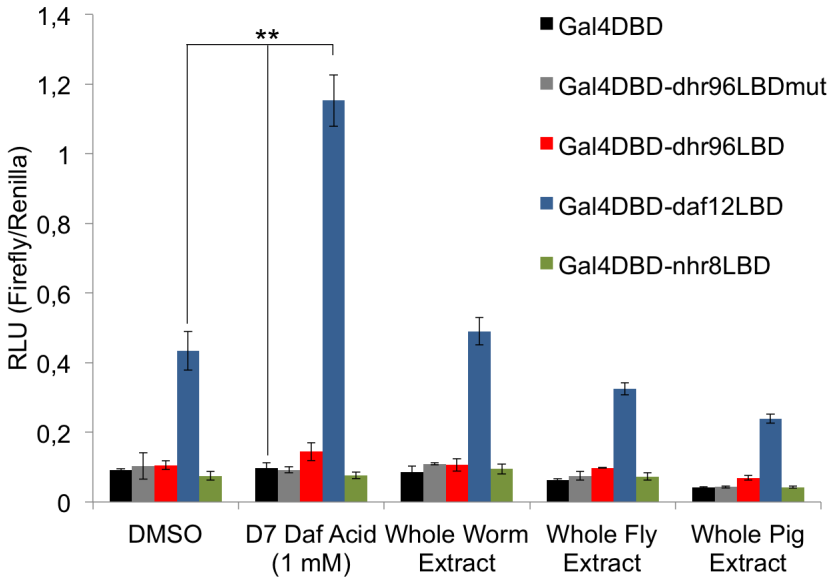


Figure 7.3.5 Reporter Assay with whole lipid extracts of *C. elegans, D. melanogaster, S. scrofa.* Whole lipid extracts were used in a 1:1000 dilution for the reporter assay. DMSO was included as the control. Only the purified dafachronic acids showed an approximately three-fold activation on DAF-12.

7.3.2.3 Ligand sensor screen with wild type *Drosophila, C. elegans* and pig liver lipid fractions

Dhr96, ligand-insensitive *dhr96*, *daf-12* and *nhr-8 Gal4DBD* fusion constructs were tested in HEK-293 and in S2 cells using lipid fractions from *Drosophila*, worm and pig liver. Results are summarized in figure 7.3.6 and supplement S.7.3.

Figure 7.3.6 A depicts the screen results for DAF-12 using *C. elegans* and *Drosophila* lipid extracts, in which fold changes of fractions were normalised over the control vector Gal4DBD. In contrast to the whole lipid extracts, fraction 58 of worm extract significantly activated the DAF-12 reporter system (figure 7.3.6 A1). There was no obvious DAF-12 activation notable around these fraction numbers (shown not to contain DA) in screen results of *Drosophila* fractions (figure 7.3.6 A2).

Results for the ligand screen of DHR96 using *Drosophila* lipid extract fractions were normalised to the Gal4DBD controls (figure 7.3.6 B1) and additionally to ligand-insensitive DHR96 (figure 7.3.6 B3). Results revealed certain fractions showing increased or decreased luminescence compared to average luminescence. To determine candidate fractions, statistics were defined that accept fractions as candidates where luminescence was above or below a statistically specified value. Fractions with fold change values greater than 2 standard deviations from the mean of all fractions after log transformation were selected. Log transformation is required, as a 2-fold increase and a 2-fold decrease are represented by 2 and 0.5 respectively, but just taking an average and SD without log-transformation gives more weight to the 2-fold increase. Using these settings, about 64 from 330 fractions from *Drosophila* and *C. elegans* significantly increased or decreased reporter activity, and were therefore selected for a repeat reporter assay (see supplement S.7.4). Results from the equivalent screen in HEK-*293* cells were included in this data set (Dan Magner). The HEK-*293* system seemed to be more sensitive than the S2 cell system, as the control induction of DAF-12 activation was much higher than in S2 cells. Overlapping candidate fractions between S2 and HEK-293 cells have been designated as high priority candidates (see supplement S.7.4).

When normalised to a control vector, the strongest candidate for an activator of the ligand-insensitive DHR96 is *Drosophila* fraction 26 (figure 7.3.6 B2), for an activator of the wild type it is the non-polar *Drosophila* fraction 174 (figure 7.3.6 B1). Normalising wild type DHR96 to ligand-insensitive DHR96 reporter system identified, in addition, *Drosophila* fraction 64 and *C. elegans* fractions 113-116, 146 and 147 as strong candidates (figure 7.3.6 B3).

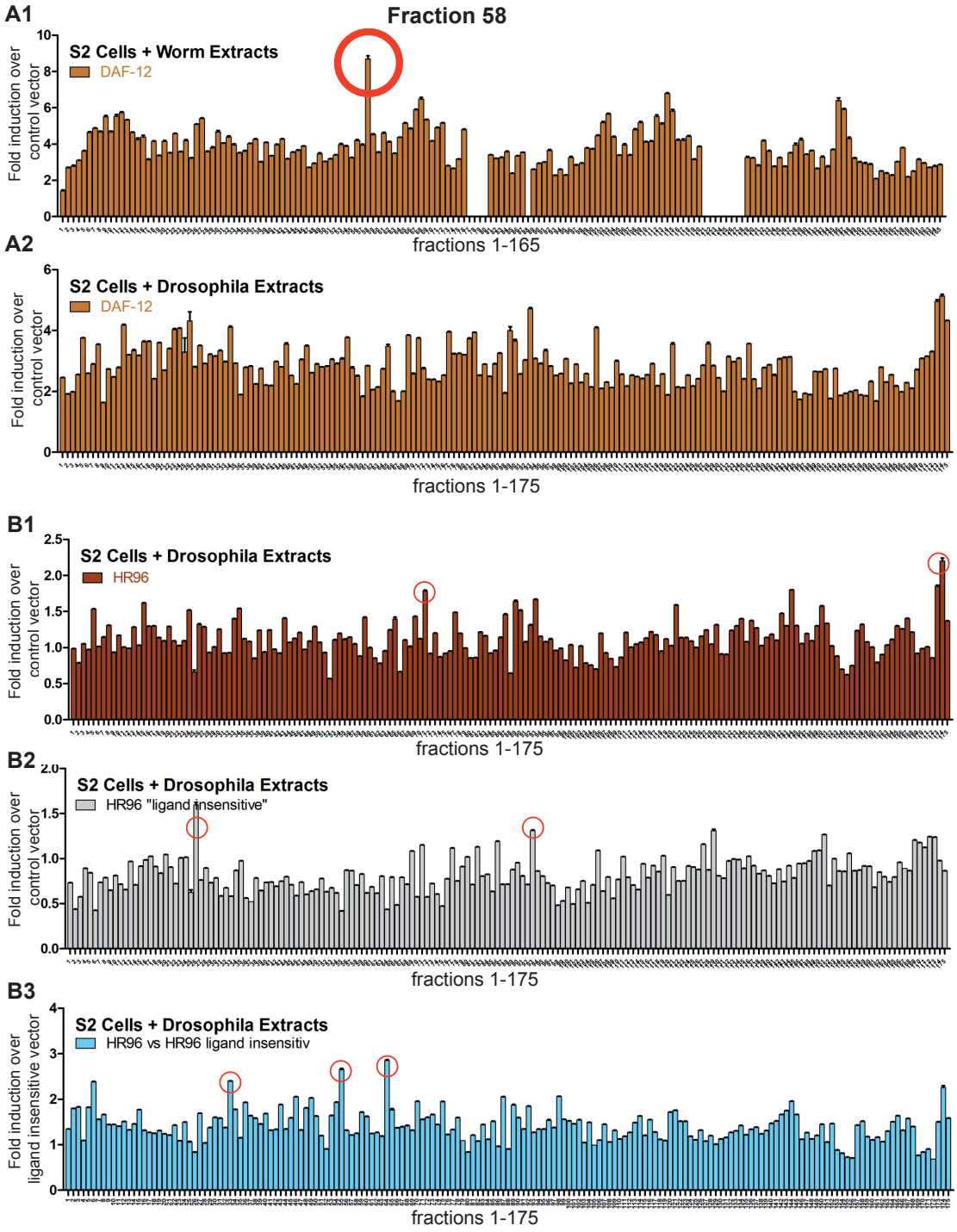


Figure 7.3.6 Ligand sensor screen for *dhr96* and daf-12 in S2 cells by using fractions from lipid extracts of *C. elegans or D. melanogaster* (A1) Activation of GAL4DBD-DAF12 was observed for *C. elegans* lipid fraction 58, which contained dafachronic acid. In *Drosophila* lipid fractions, no DA was detected around these fraction numbers and (A2) also no notable activation was observed around these fractions. (B1-3) Activity of (B1) GAL4DBD-DHR96LBD and (B2) ligand-insensitive GAL4BD-DHR96LBD was tested using *Drosophila* lipid fractions and mathematical statistics were defined to determine candidates. (B3) In addition, GAL4DBD-DHR96LBD was normalised over ligand-insensitive GAL4DBD-DHR96LBD. Strongest candidates were marked by a small red circle.

Candidates listed in these data sets (see supplement S.7.4) were repeated in a second screen. Unfortunately, fraction responses were variable and not consistent with the first screen and therefore no conclusions could be drawn from this. A third screen with only the strongest candidates and with fractions around these candidates is planned.

7.3.3 Ligand sensor screen with lipid extracts from wild type *Drosophila* females exposed to DDT and a low cholesterol diet

As screen results using wild type flies did not give any positive results so far, the suggestion arose that the ligand might not be synthesised in normal wild type flies. Thus, a second approach was performed, in which lipid fractions from female *Drosophila* treated with DDT or exposed to low cholesterol diet were tested. DDT was chosen as *dhr96* and *dhr96-lbd* over-expressing flies were resistant to DDT, suggesting that DDT might activate a DHR96 response in wild type flies. Low cholesterol was selected, as expression of DHR96 downstream targets was modulated under this condition (Supplement S.6.1).

Whole fly lipid extracts from females treated with DDT or exposed to a low cholesterol diet were fractionated into 20 DDT (D1-D20) and 19 low cholesterol (C1-C19) lipid fractions and tested in the ligand sensor system.
In a first screen the same settings were used as in previous experiments (1:1000 dilution of fractions, 16h incubation). RLU values (firefly/renilla) resulting from GAL4DBD-DHR96LBD reporter system were normalised over RLU values (firefly/renilla) of the GAL4DBD control in the resulting data sets. As shown in figure 7.3.7 A, fractions C12-C19 revealed induced *luciferase* expression for DHR96. Remarkably, these fractions also showed a high toxicity in the form of cell death (observation using a microscope). The screen was repeated with an incubation time of 12 hours, rand gave similar data (7.3.7 B). In a third screen, dilution was increased from 1:1000 to 1:10.000 and fractions which were induced in the previous screens, did not show obvious changes under these conditions (7.3.7 C). DDT fractions did not show any obvious and consistent changes.

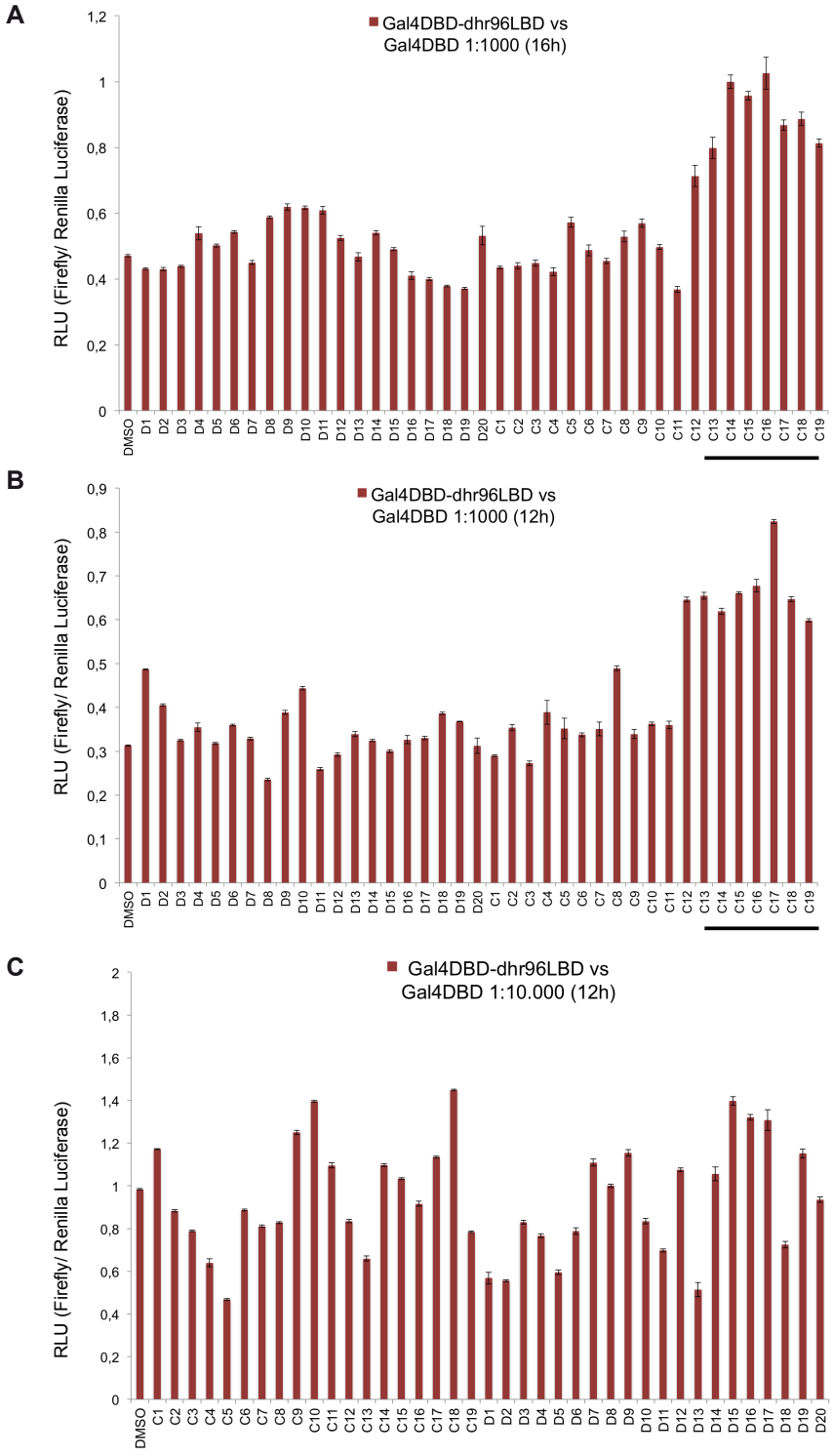


Figure 7.3.7 Ligand sensor screen for DHR96 in S2 cells with lipid fractions from female *Drosophila* flies treated with DDT or exposed to low cholesterol food type. (A1) GAL4DBD-DHR96LBD activity is induced in fraction C12-C19 (flies exposed to a low cholesterol diet) when dilutions of 1:1000 and an incubation time of 16 hours were used. These fractions showed high toxicity. (A2) A repeat screen with 12 hours incubation time of fractions on cells gave a similar phenotype, (A3) but using higher dilution revealed a decrease of toxicity and no obvious induction in fractions C12-C19.

7.4 Discussion

7.4.1 Internal GAL4DBD-DAF12LBD control was affected by DMSO

The use of *Gal4DBD-Daf12LBD* ensured a positive control for the ligand sensor system and for the lipid extraction. Treatment with purified dafachronic acid induced the activity of GAL4DBD-DAF12LBD and the control was therefore successful. Interestingly, DMSO itself induced transcription of *luciferase* for the GAL4DBD-DAF12LBD sensor system, whereas the other GAL4DBD-TF fusion proteins did not show a significant response. Indeed, DMSO is known to change gene expression of some specific cytochrome genes, so that activation may arise from components that are involved in the ligand production pathway. However, for DAF-12, this might appear unexpected, as no dafachronic acid was identified in the fractions tested in the GS/MS/MS analysis, suggesting that *Drosophila* might not synthesise this compound. Thus, it might be that a *Drosophila* lipid structure with similar DA, synthesized upon DMSO treatment, or a indirect regulation induced DAF-12 activity to a small extent.

7.4.2 Phenobarbital induced GAL4DBD-DHR96LBD activity

CITCO and phenobarbital were used as possible positive controls for the GAL4DBD-DHR96LBD sensor system. Although CITCO was shown to induce activity of DHR96-GFP-reporter system in *Drosophila* embryos (Palanker, 2006), treatment of three different CITCO concentrations failed to induce activity of GAL4DBD-DHR96LBD in S2 cell culture. It might be that induction would require another dose of this drug. Another reason might be that CITCO can not induce GAL4DBD-DHR96LBD activity in a cell system as the regulation of the xenobiotic response between a cell system and an *in-vivo* system can differ (Baum and Cherbas, 2008).

Experiments with the drug phenobarbital revealed that the concentration of ligand(s) might be a critical criterion for DHR96 activation in this assay. Too low or too high concentrations of phenobarbital failed to induce expression of the reporter gene. This is in agreement with studies showing that too low as well as too high concentrations of tested compounds can lead to different results (Thorne et al., 2010). However, 2mM phenobarbital treatment succeeded to

activate the DHR96 reporter system (figure 7.3.3). This finding would overlap with studies in which DHR96 is shown to regulate many genes, which are also induced by phenobarbital in *Drosophila* (King-Jones et al., 2006) and would suggest that PB modulates activity of DHR96. These results would also indicate that DHR96 could act as an activator, as it induced luciferase transcription. However, a repressive function might be also possible, as for some nuclear receptors both a repressive or activating response are observed (Aranda and Pascual, 2001).

Interestingly, phenobarbital was primarily identified as a ligand for both mammalian *dhr96* orthologs *CAR* and *PXR*, resulting in the induction of cytochrome genes (Moore et al., 2000). However, recent results uncovered that the effect of phenobarbital on CAR is mediated through an indirect mechanism, in which the epidermal growth factor receptor (EGFR) signalling is involved. The actual activation is induced by the dephosphorylation of CAR, which is caused by the inhibition of (EGFR) signalling (Mutoh et al., 2013).

In case a DHR96 ligand would be identified, binding assays are required to test if a direct interaction causes activation, or whether indirect pathways are involved. According to this, it would be of major interest to test if activity of ligand-insensitive GAL4DBD-DHR96LBD is affected by phenobarbital treatment. This would provide a useful control if the mutation in the LBD domain is required for the transcriptional response. Moreover, posttranslational modifications, for example by phosphorylation, represent further regulatory tools and are not studied for DHR96 so far.

7.4.3 Ligand screens using lipid fractions from *Drosophila* did not uncover the natural ligand

Using fractions from wild type flies did not reveal an obvious ligand for DHR96. As shown for activation of DHR96 by PB, a critical aspect in this assay is to have the right concentration of agonists that induce activation. In whole worm lipid extract, the low concentration of DA is the likely reason that DAF-12 activation is not induced upon treatment although dafachronic acid was present.

A strategy that was tried to enrich the possible ligand for DHR96 in flies was to use flies in which the probability was higher that DHR96 is active. As this transcription factor is involved in xenobiotic response and cholesterol metabolism (King-Jones et al., 2006), the second approach was focussed on females that were treated with DDT or exposed to a low cholesterol diet. 20 fractions of each condition were used, as this number of fractions was sufficient to bring DA to an appropriate concentration for DA activation (Motola et al., 2006). But the problem that arose in the performed experiments was that concentrated lipids were toxic seeming strongly in fractions C12-C19 (judget by cell membrane integrity). Interestingly, these toxic effects seemed to induce the reporter system of DHR96, and would suggest that DHR96 activity and possibly ligand synthesis might be regulated in response to toxicity as a cytoprotective mechanism. Moreover, DHR96 is suggested to interact with the *hsp27* response element *in-vitro*, which is in turn a target promoter region for EcR (Fisk and Thummel, 1995). Although over-expression of *dhr96-lbd* females did not show any changes in the transcript level of *hsp26* and *hsp27*, it would be interesting to test whether stress conditions induce *dhr96*, and subsequently *hsp26* and *hsp27*.

The ligand sensor screen has to be optimized and adjusted to the DHR96 reporter system. Although internal controls were successful, it represents a challenge to find the right conditions to identify the DHR96 ligand(s). The main problem is the treatment of the lipids. The choice of dilution, fraction numbers, solvent, and incubation time are critical points for this approach. Higher fraction numbers reduce toxic effects, concentrate present sterols, and would present a better approach. Although DMSO generally provides effective solubilisation of lipids, some lipids might require other detergents. Thus, a further step would be to solubilise the lipids in different solvents.

In addition, ligands can enter target cells as a precursor or active ligand through the haemolymph to modulate nuclear receptor activity. The question for DHR96 is if the ligand is produced in the target cell from cholesterol or if it is synthesized in endocrine tissues. The exchange of cholesterol, cholesterol derivates or other hormones is provided through the haemolymph. The use of

haemolymph for the reporter assay is therefore another tool to identify DHR96 ligand(s).

7.4.4 Does DHR96 repress target gene expression?

Studies have characterized several transcription factors that exert inhibitory regulation via negative hormone response elements that allows NRs to bind and inhibit transcription (Bodenner et al., 1991; Carr and Wong, 1994). A main challenge is thus to figure out if DHR96 is a transcription factor which induces or inhibits the expression of target genes, or both.

In a ligand sensor system, transcriptional repression might be difficult to analyse; the use of target genes in contrast would monitor DHR96 activity and repression in the form of up-regulation and down-regulation. An alternative approach would be to analyse changes in the expression level of direct target genes, once they are identified by ChIP sequencing, in response to treatment with diverse sterols. However, further controls would be necessary to ensure that expression changes are only due to DHR96 activity, as studies have suggested a crosstalk between DHR96 and ecdysone receptor target genes (Fisk and Thummel, 1995).

Of the many hormone nuclear receptors in *Drosophila*, only two have been identified as having a ligand, whereas the others are defined as orphans. Uncovering ligands would give useful tools to understand the mechanism of nuclear receptors. For DHR96, the mechanism of activation is unclear, and studies of the ortholog CAR indicated that posttranslational modification might also be important for function. Thus, further studies are necessary to identify regulatory processes that might modulate DHR96 activity and to test which of them are required for longevity.

Chapter 8 Conclusion and future perspectives

DHR96 is a hormone nuclear receptor that regulates diverse biological functions, including TAG metabolism, cholesterol homeostasis, and xenobiotic response (King-Jones et al., 2006; Sieber and Thummel, 2012). In cooperation with Janne Toivonen and Matt Piper, I identified roles of DHR96 in *Drosophila* lifespan, fecundity and immunity, and dissected a DHR96 xenobiotic function downstream of the IIS pathway.

The DHR96 homolog DAF-12 in *C. elegans* regulates life history traits and ligand-insensitive *daf-12* mutants are long-lived (Antebi et al., 2000; Fisher and Lithgow, 2006). According to a possible gain-of-function in a ligand-insensitive form, over-expression of wild type and ligand-insensitive *dhr96* were used for the experimental approaches. The induced expression of *dhr96* and *dhr96-lbd* using a ubiquitous *daughterless* GS driver extended lifespan, enhanced stress response, and reduced fecundity for both *dhr96* and *dhr96-lbd* over-expressing females. Moreover, induction levels and/or expression pattern were critical factors whether DHR96 had beneficial or non-beneficial consequences on lifespan and stress response. *Dhr96* expression was more than two times higher when the *tubulin* GS driver was used to over-express *dhr96-lbd*. Higher induction levels correlated with reduced fecundity, and an increased sensitivity to bacterial infection. Thus, *dhr96* over-expressing flies were long-lived, but effects in other traits diminished survival when *dhr96* induction was too strong. This hypothesis also agrees with studies of the last chapter in which *dhr96* over-expressing flies showed sensitivity to infection with the pathogen *Ecc15*. The diminished innate immune response might occur due to the reduced expression of AMP genes, which was shown in qRT-PCR analysis to be the case for the AMP gene *mtk*. Interestingly, in spite of bacterial sensitivity, the immune-suppressive effects of *dhr96-lbd* over-expressing flies might be causal for enhanced longevity. Studies in *Drosophila* revealed that immunosuppressive effects induced by pharmacological treatment with the drug pyrrolidine

dithiocarbamate that inhibit NF-κB signalling extends lifespan (Moskalev and Shaposhnikov, 2011).
According to this, it is of major interest to investigate how *dhr96-lbd* over-expression suppresses immunity and whether this effect causes the enhanced longevity effect. One possible approach to address this hypothesis is to over-express *dhr96*-lbd in female flies treated with immunosuppressive drugs.

One possible explanation for how *dhr96-lbd* over-expression might modulate immunity could be by hormonal regulation, which displays an important role also in lifespan regulation and reproduction (Toivonen and Partridge, 2009). Analysis of qRT-PCR revealed a down-regulation of *magro* in *dhr96-lbd* over-expressing females. *Magro* encodes a lipase, and is involved in TAG and cholesterol metabolism (Sieber and Thummel, 2009). TAG and Cholesterol affect a broad range of biological functions, including energy metabolism and cell homeostasis. Furthermore, cholesterol affects sterol metabolism, cell signalling, cell membrane function, and is a precursor of steroid hormones (Tabas, 2002). As *Drosophila* is not able to produce cholesterol, the regulation of cholesterol homeostasis is a crucial issue (Hoog, 1936). Due to altered expression of *magro,* TAG and cholesterol levels might be altered upon *dhr96-lbd* over-expression. Part of future experiment is therefore to test TAG and cholesterol levels in transgenic lines and their relevance to DHR96 phenotypes.
The candidate Cytochrome *Cyp18a1* was down-regulated in *dhr96-lbd* over-expressing females. This Cytochrome protein is involved in sterol metabolism as it inactivates 20-Hydroxyecdysone, the ligand for ecdysone receptor (ER) (Baker et al., 2000; Guittard et al., 2011). Interestingly, ER signalling also affects *Drosophila* lifespan and immunity (Rus et al., 2013). Thus, there might be a cross talk between these hormone nuclear receptors via 20-Hydroxyecdysone (20E). Epistatic analysis between ER and DHR96 would discover whether longevity and immunity phenotypes are affected by each other. Furthermore, the use of 20E on lifespan and immunity assays of *dhr96* null or *dhr96* over-expressing flies might be additional steps to investigate interactions.

Dhr96 is a target gene of the transcription factor dFOXO (Alic et al., 2011), a key regulator of the IIS pathway. Reduced IIS signalling, resulting also in transcriptional activation of dFOXO, extends lifespan, reduces fecundity, increases stress response, and affects metabolism (Partridge et al., 2011). Thus, DHR96 and dFOXO modulates similar life history traits. Although *dhr96* expression was not affected in long-lived IIS mutants, it could be that expression was altered in specific tissues or at different time points, or that co-factors are modulated in long-lived IIS mutants. Thus, dependency in lifespan and xenobiotic response between IIS-reduced flies and DHR96 phenotypes was tested. Longevity effects were independent from each other, whereas the increased xenobiotic response for DDT and phenobarbital of IIS-reduced flies was dependent on DHR96 function. Thus, DHR96 mediates some stress responses of IIS signalling. Cytochrome *Cyp6g1* transcript levels were increased upon *dhr96-lbd* over-expression in the gut of females, which likely contributes to the increased DDT resistance of *dhr96-lbd* over-expressing flies. It might be of interest to test if this is the case also in the gut of long-lived IIS mutants.

However, the enhanced longevity of DHR96 did not seem to be caused by improved detoxification in *dhr96-lbd* over-expressing flies. Although *dhr96-lbd* over-expressing male flies and tagged *dhr96* over-expressing females revealed increased xenobiotic resistance, their lifespan were not affected. However, the induction of *dhr96* transcript levels in these flies needs to be tested for a distinct conclusion.

Next to the link between longevity and immunity, another suggestion arose that reproductive signalling might be crucial for longevity of *dhr96-lbd* over-expressing flies. Also here, hormonal regulation by Juvenile Hormones and 20E are required for reproduction function (Toivonen and Partridge, 2009).

Fecundity is a cost-energetic process, which is ensured by fat reservoirs (Hansen et al., 2013). A reduced fecundity in *dhr96-lbd* over-expressing females might contribute from a shift of energy storage to processes involved in survival and somatic maintains. To test this hypothesis future plans include over-expression of *dhr96-lbd* in long-lived germ-line ablated flies and analysis of fat metabolism. Another approach includes the over-expression of tagged *dhr96*

over-expressing females. As they did not show an effect in longevity, it would be interesting to test their fecundity as well.

In the last chapter, I tried in cooperation with Dan Magner und Shruti Chreti to identify the DHR96 ligand using a ligand sensor system in S2 cells. Testing fractions of *Drosophila* lipid extracts did not give any robust positive results. Treatment of fractions (dilution and solubilisation) represented a critical challenge and might be a possible reason for no positive activation of the ligand sensor system. The second screen using flies treated with DDT or exposed to a low cholesterol diet showed toxic effects, as fractionation was restricted to 20 fractions, in contrast to the ca. 180 fractions in the first screen. Fractions that induced cell death also activated DHR96 ligand sensor activity. Thus, DHR96 might represent a xenobiotic sensor that would agree with its function in the xenobiotic response.

In addition, I could show that phenobarbital activated the DHR96 ligand sensor activity. The concentration of 2mM was very high compared to other used chemicals. The ligand sensor activation might therefore occur due to toxic effects. On the other hand, phenobarbital is shown to activate CAR via an indirect and ligand-independent regulation pathway. Interestingly, PB treatment induces CAR activity by dephosphorylation (Sueyoshi et al., 2008) as a result of PB inhibition of epidermal growth factor receptor (EGFR) signalling (Mutoh et al., 2013). As PB also induced DHR96 activity in the ligand sensor system, it is of major interest to investigate possible posttranslational modifications of DHR96.

Interestingly, analysis of DHR96 using NetPhosk 2.0 server program (Blom et al, 1999**)** predicts several phosphorylation sites of DHR96 by PKC. Four sites, S26, T55, T91 and T641, have a score higher than 0.8, and might represent interesting candidates for phosphorylation sites that could modulate DHR96 function. PKC is regulated by PDK1, a key kinase of the IIS cascade, and so this could represent a link to IIS. However, none of these sites are conserved in DHR96 orthologs. Analysis of posttranslational modifications of DHR96, using bioinformatics, or directly analysing protein modifications on DHR96 by mass spectrometry of immunoprecipitated protein, could uncover a mode of regulation.

One last future perspective is the study of the DHR96 orthologs CAR and PXR in mice. These hormone nuclear receptors are in main focus of drug screens with a high relevance in pharmacological use, indicating their importance in health and diseases (Gao and Xie, 2012; Halilbasic et al., 2013). Thus, many research are progressed on CAR and PXR function in mice, showing them to be key regulators in detoxification, energy metabolism and lipid homeostasis (Gao and Xie, 2012; Tolson and Wang, 2010), but no longevity studies were performed. A correlation for increased xenobiotic resistance was already shown for induced PXR activity (Xie et al., 2000a). Thus, it would be of major interest to test over-expression of CAR or PXR for longevity studies in mice.

Supplement

S.1 Supplement Buffer and Solution

S1.1 Squishing Buffer (pH 8.2), 1 L

10 mM Tris Base

1 mM EDTA

25 mM NaCl

S1.2 Phosphate buffered saline (PBS) (pH 7,4), 1L

137 mM NaCl

2,7 mM KCl

10 mM Na2HPO4

1,8 mM KHPO4

S1.3 TBS Buffer (pH 7,6), 1L

50 mM Tris Base

150 mM NaCl

S1.4 TBS-T (pH 7,6), 1L

1 L TBS-T

0,05% Tween 20

S.1.4 TAE Buffer (pH 8,2)

40 mM Tris acetate

1 mM EDTA

S.1.5 LB Broth, 1L

10 g Tryptone

10 g NaCl

5 g Yeast extract

Ingredients were dissolved in 1 liter distilled water, and autoclaved.

S.1.6 LB Agar

0.75 g Agar

5 g Tryptone

5 g NaCl

2.5 g Yeast extract

Ingredients were dissolved in 1 liter distilled water, autoclaved, and cooled to 55 °C. Appropriate antibodies (ampicillin, kanamycin) were added to the solution, and immediately dispensed into sterile petri dishes.

S.1.7 Grape fruit plates

Ingredients	**Volume**
Distilled water	1000 ml
Agar	50 g
Grape Juice	600 ml
Extra water after boiling	100 ml
Nipagin (10%)	42 ml

Water, agar and grape juice were mixed and boiled. Extra water was added and solution was cooled down to 55°C. Nipagin was added, and grape fruit mix was dispensed into sterile petri dishes.

S.2 Supplement Chapter 2

S.2.1 Oligonucleotide primer list

Name	**Sequence**	**Application**
pAC-NotI-GAL4DBD	cagcacagtggcggccgcatgaag ctactgtcttctatcgaaca	Cloning Gal4DBD-TF-LBD in pAC5.1 vector
pAc5B-EcoRI-Gal4DBD	cagtgtggtggaattcatgaagctact gtcttctatcgaac	Cloning Gal4DBD-TF-LBD in pAC5.1 vector
EcoRIGal4DBD	gaattcatgaagctactgtcttctatcg aac	Cloning Gal4DBD-TF-LBD in pAC5.1 vector
Gal4DBDend-EcoRI-HR96LBDFor	Tgtatcgccggaattcggcgaggaa agggatcacaaa	Cloning Gal4DBD-*dhr96*LBD in pAC5.1 vector

DHR96end-NotI-pAc5Brev	tctagactcgagcggccgcctagtgattttcaaatcgaatatttcac	Cloning Gal4DBD-*dhr96*LBD in pAC5.1 vector
pACend-XbaI-NHR8	aaccgcgggccctctagatggttaataaatggttattcaaaagaac	Cloning Gal4DBD-*nhr8*LBD in pAC5.1 vector
pACend-XbaI-Daf12	aaccgcgggccctctagatcaaatttatattcattagttttgacaagatactgttgg	Cloning Gal4DBD-*daf12*LBD in pAC5.1 vector
pAc-Gal4controlfor-NotI-Infus	cagcacagtggcggccgcatgaagctactgtcttctatcg	Cloning Gal4DBD-empty in pAC5.1 vector
Gal4DBDcontrolrev-Xba-Pac	aaccgcgggccctctagatcgatacagtcaactgtctt	Cloning Gal4DBD-empty in pAC5.1 vector
Ligandinsensitive R539C_antinense	tggccattttgataaggcacttgatggccaccgcc	Site directed mutation primer
Ligandinsensitive R539C	ggcggtggccatcaagtgccttatcaaaatggcca	Site directed mutation primer
cDNA-NHR8	aaccgcgggccctctagattattcaaaagaacataattctcgtatcagagg	Sequencing primer
cDNA-Daf12	aaccgcgggccctctagactatttgatttttgaaaaattctcctggcag	Sequencing primer
HR96 begin seq primer (inv/com)	tcgagtgaccacagtgatgtcgca	Inserted vector sequencing primer
HR96 middle seq primer (inv+comp)	agtgtcgtcgggcttaatgcgatc	*Dhr96* sequencing primer
HR96 end seq primer	ttgacgagaagtggcgcatgga	*Dhr96* sequencing primer
Gal4 N-term	gagtagtaacaaaggtcaa	Sequencing primer (GAL4)
Lucnrev	ccttatgcagttgctctcc	Sequencing primer (*luciferase*)
AC5_primer	acacaaagccgctccatcag	sequencing primer

		(pAc5.1)
BGH_rev_primer	tagaaggcacagtcgagg	Sequencing primer
T3-Promoter	gcaattaaccctcactaaagg	Sequencing primer
ttt BglII-ATG_HR96start	Tttcgagatctatgtcgccgccgaagaactg	Cloning *dhr96*-FLAG
ttt HR96end-FLAG-Stop-NotI	Tttgcggccgcctacttgtcatcgtcatccttgtaatcgtgatttttcaaatcgaatatttcacgcag	Cloning *dhr96*-FLAG
InFus-pUASattbHR96-revNotI-FLAG	taccctcgagccgcggccgcctacttgtcatcgtcatccttgtaatcgtgattttt caaatcgaatattt cacgcag	Cloning *dhr96*-FLAG in pUAST-attb vector
InFus-pUASTattb-BglII-ForDHR96	Ggaattcgttaacagatctatgtcgccgccgaagaactg	Cloning *dhr96*-FLAG in pUAST-attb vector
Sol 236	ttgccgctgacaattatgatcaag	*Dfoxo* null genotyping Primer
Sol 237	aaggtagtgcctatgatccag	*Dfoxo* null genotyping Primer
eGFP Rev	cacgaactccagcaggaccatg	*Dhr96* null genotyping Primer
eGFP For	atgccacctacggcaagctga	*Dhr96* null genotyping Primer
Exon 5 P2000 rev	acactctccagatatcttcgcagaag	*Dhr96* null genotyping Primer
Exon 4 Primer rev	gtcacggaatgctgtaatcttcttgg	*Dhr96* null genotyping Primer

Table .2.1 List of used oligonucleotide primers.

S.2.2 Taqman Probes used for qRT-PCR analysis

Gene name	Assay ID
Ribosomal protein L32	Dm02151827_g1
Hormone receptor-like in 96	Dm02151379_g1
Cytochrome P450-18a1	Dm01813939_g1
forkhead box, sub-group O	Dm02140212_g1
Thor	Dm01842928_g1
Cytochrome P450-18a1	Dm01813937_m1
Ecdysone Receptor	Dm01811601_m1
Cyp6g1	Dm01819890_g1
Mtk	Dm01821460_s1
Ecdysone Receptor	Dm01811601_m1
Zwischenferment	Dm01813969_g1
Defnsin	Dm01818074_s1
IIP5	Dm01798339_g1
IIP3	Dm01801937_g1
IIP2	Dm01822534_g1
Npc1b	Dm01799742_g1
Npc2c	Dm02138599_g1
Magro	Dm01807059_g1
Zw	Dm01813969_g1
Cyp6g2	Dm01819891_g1
Pgd	Dm01841976_m1
Cyp12a4	Dm02142149_g1
S6K	Dm01822188_g1
Hsp27	Dm01822485_s1
Keap-1	Dm02141390_g1
alphaTub84	Dm02361072_s1

Table S.2.2 List of used Taqman probes.

S.3 Supplement Chapter 3

S.3.1 The six classes of *daf-12* alleles shown by Antebi et al. (2000)

(The figure is taken unmodified from Antebi et al. (2000))

Table 1. *Sites of mutations, and summary of dauer and heterochronic phenotypes of* daf-12 *alleles*

Class	Allele	Mutation	Location	Dauer phenotype	Heterochronic phenotypes: Extra-gonadal (L3 seam)[a]	Heterochronic phenotypes: Gonadal (L4 distal tip)[b]
WT	+			Normal	++++	++++
Class 1	*rh61*	Q618stop	LBD beta turn	Defective	+	+
	rh84	R688stop	LBD H9			++
	rh257	Y699splice acceptor	LBD H9			+
Class 2	*rh62rh157*	S70stop, R564C	before DBD, LBD, H3	Defective	+	++++
Class 3	*m583*	Q59stop	before DBD	Defective	+++	++++
	sa156	C121Y	DBD Zn1			
	m421	S137F	DBD Zn1			
	m116/m423	R143K	DBD Zn1			
	rh61rh411	L147stop, Q618stop	DBD after Zn1, LBD beta turn			
	m422	C154stop	DBD Zn2			
	m20/m425	W187stop	DBD after Zn2			
	rh61rh412	R195frameshift,Q618stop	DBD after Zn2, LBD beta turn			
	m524::Tc1	Y239insertion	DBD after Zn2			
	m419	Q501stop	LBD before H1			
	rh258	n.d.				
	[c]*m420*	A125V	DBD Zn1		++++	++++
	m424	R197K	DBD Zn1			
	m25	M562I	LBD H3			
Class 4	*rh193*	G582K	LBD H4	Normal[d]	++	++
	rh285	Q707stop	LBD after H9			+
Class 5	*rh284*	P746S	LBD H12	Normal	++++	+
	rh286	C461Y	Hinge		+++	+++
Class 6	*rh62/rh274*	R564C	LBD H3	Constitutive	++++	+
	rh273	R564H	LBD H3		+++	

(+) 0%–33% wild type; (++) 34%–66% wild type; (+++) 67%–99% wild type; (++++) 100% wild type.
[a]Percentage of seam cells at larval stage L3 that execute stem cell division (S3) versus equational division followed by stem cell division (S2) as described in Fig. 5 legend. ca. 10 animals (120 cells) were scored by DIC microscopy as described (Antebi et al. 1998).
[b]Percentage of distal tip cells that migrate dorsally by larval stage L4 as described (Antebi et al. 1998). ca. 100 animals (200 cells) were scored by DIC microscopy or dissecting scope.
[c]Missense alleles *m420* and *m424* have no observable heterochronic phenotype in seam cells, but have impenetrant heterochronic phenotypes in the intestine (not shown). *m25* had no observable heterochronic phenotypes.
[d]A small fraction of partial dauer animals formed at 25° and 15°C for *rh193* and *rh285*, respectively. Phenotypes for alleles *rh61, rh84, rh257, rh62rh157, rh258, rh61rh411, m20, m583, rh193, rh285, rh284, rh286, rh62, rh274,* and *rh273* are adapted from Antebi et al. 1998.

Table S.3.1 Six classes of daf-12 alleles by Antebi et al. (2000). The table includes the location of the mutations and whether they show phenotypes.

S.3.2 *Dhr96*, *daf-12* and *nhr8* alignment

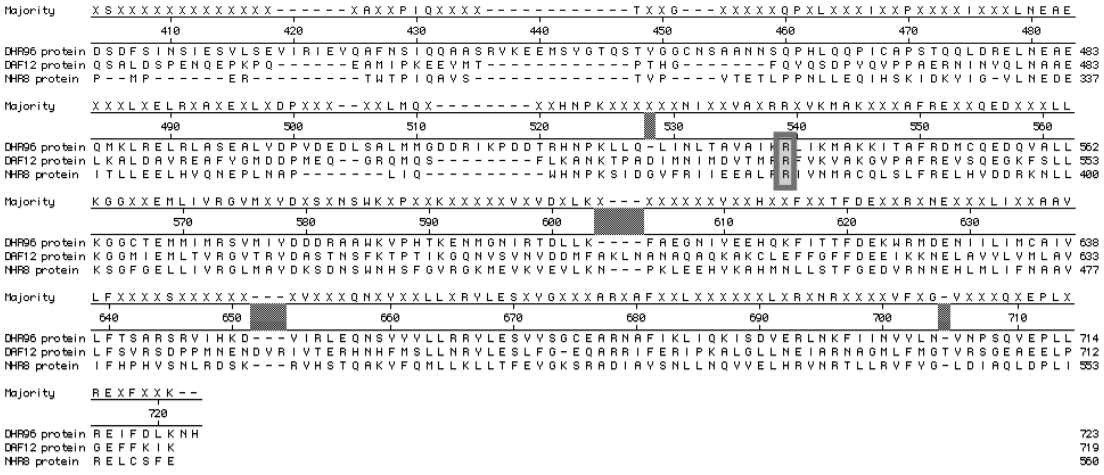


Table S.3.2 Protein alignment of *dhr96*, *daf-12*, and *nhr-8*. Ligand-insensitive *daf-12* mutants carry a single mutation in the ligand-binding domain. The corresponding amino acid is conserved between *dhr96*, *daf-12*, and *nhr-8* (grey box).

S.3.3 Lifespan of *dhr96* and *dhr96-lbd* overexpressing flies using the *tubulin*-GS driver on 1xSYA food (experiment performed and graph provided by Janne Toivonen).

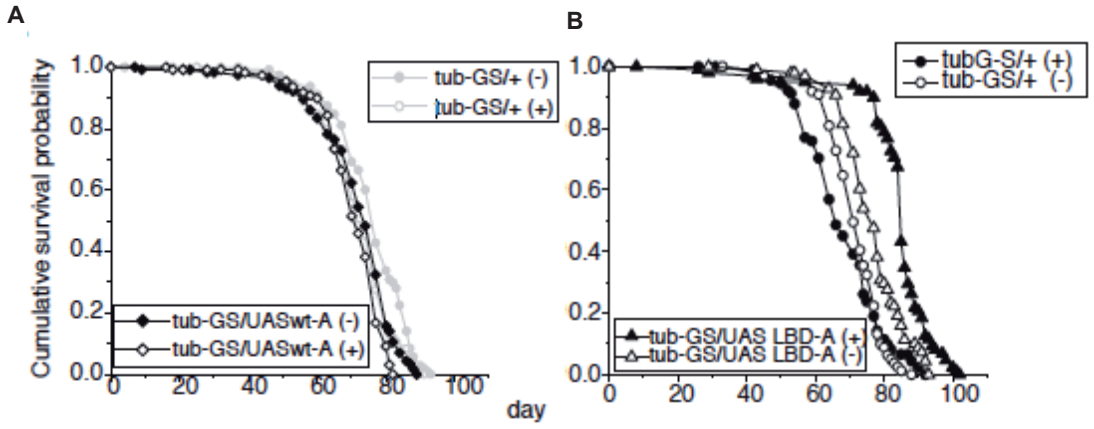


Figure S.3.3 Lifespan of *dhr96* and *dhr96-lbd* over-expressing females using the tubulin GS driver. (A) Over-expression of *dhr96* had no effect on lifespan in female flies, whereas (B) *dhr96-lbd* over-expression extended lifespan.

S.3.4 Western Blot analysis of *dhr96-lbd* over-expressing flies using the *daughterless* and *tubulin* GS driver.

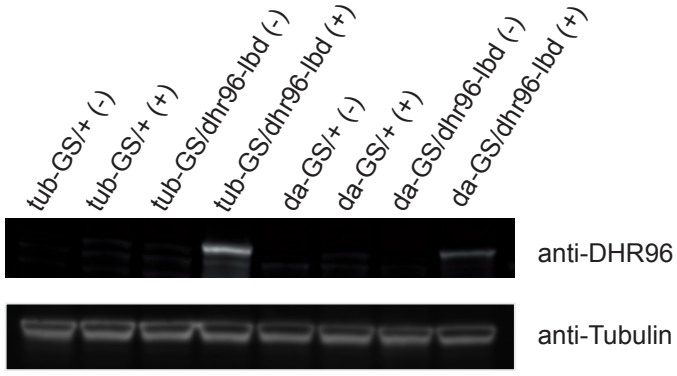


Figure S.3.4 Western Blot analysis. DHR96 protein of *dhr96-lbd* over-expressing flies was induced using two different driver lines (*daughterless* and *tubulin* GS driver). Induction was higher in *tubulin* GS-driven *dhr96-lbd* over-expressing flies compared to *daughterless* GS-driven *dhr96-lbd* over-expressing flies (polyclonal anti-DHR96 serum was provide by Janne Toivonen).

S.3.5 Cloning of *flag-dhr96-lbd* into attP-flanked UAS-vector

As expression levels differed between *dhr96* and *dhr96-lbd* over-expressing females, and as ChIP sequencing was not possible using the DHR96 antibody (antibody provided by Janne Toivonnen, ChIP sequencing by Nazif Alic), new

transgenic *dhr96* fly lines were generated. The pUAST-*dhr96* and pUAST-*dhr96-lbd* (provided by Janne Toivonen) were used as templates for the *dhr96* cloning procedure. The FLAG-tag was introduced in frame at the *dhr96* C-terminus, and by shifting the stop codon after the FLAG sequence. Primers used to introduce the FLAG-tag to the gene by PCR are listed in S.2.1. FLAG-*dhr96* and FLAG-*dhr96lbd* PCR products were cloned into the pUAST-attb vector via the *NotI* and *BglII* restriction sites using the InFusion cloning procedure. Positive sequenced constructs were injected into the second chromosome via the attp40 locus.

S.3.6 N-terminal tagged *dhr96* and *dhr96-lbd* over-expressing flies

Over-expression of N-terminal *Flag-Strep-6xHis-dhr96* and *Flag-Strep-6xHis-dhr96lbd* (Teresa Nicoli) females increased significantly resistance to DDT ($P<0.001$, Log Rank Test) (Figure S.3.5.1), but had no effect on lifespan (Figure S.3.5.2). The Tag may have interfered with DHR96 function. Induction levels were not tested and might represent another possible reason that over-expression did not extend lifespan.

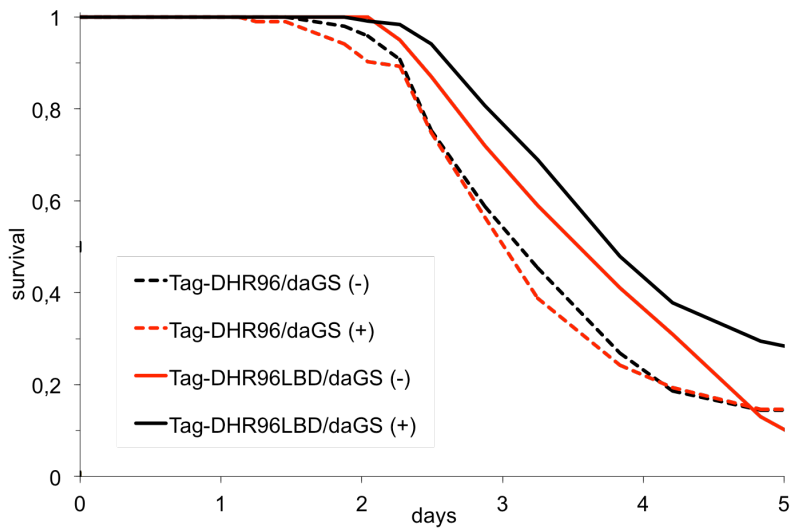


Figure S.3.5.1 DDT Assay. *Flag-Strep-6xHis-dhr96* and *Flag-Strep-6xHis-dhr96lbd* over-expressing females show an significant increase in DDT resistance ($P<0.001$, Log Rank Test).

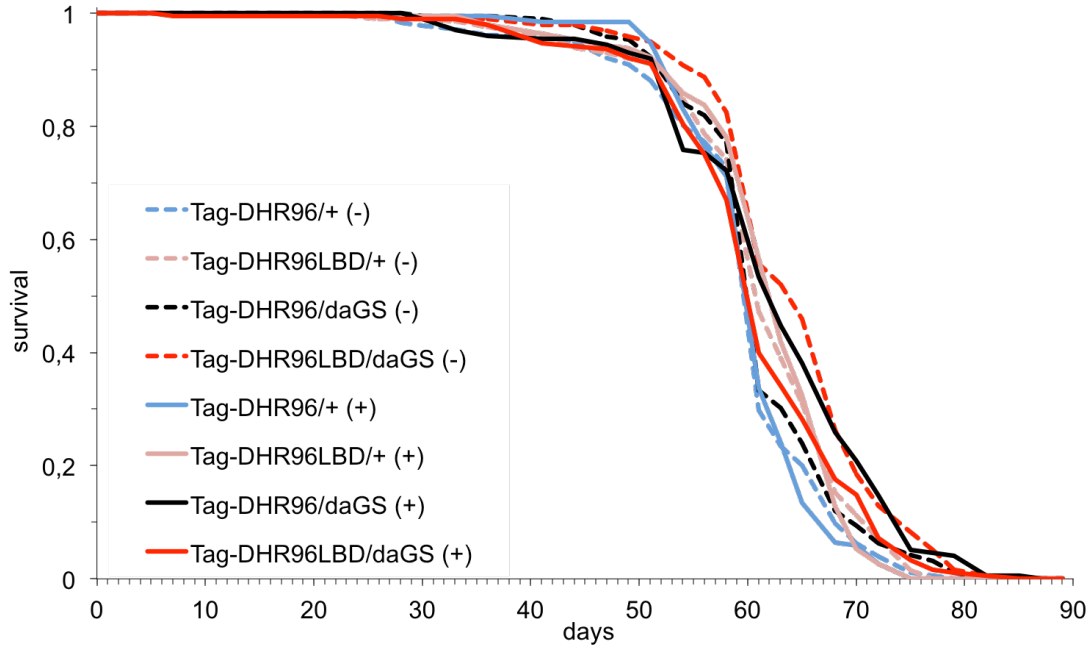


Figure S.3.5.2 Lifespan analysis of tagged *dhr96* transgenic flies. *Flag-Strep-6xHis-dhr96* and *Flag-Strep-6xHis-dhr96lbd* over-expressing females did not extend lifespan in female flies.

S.4 Supplement Chapter 4

S.4.1 PCR of *daughterless* GS in a *dfoxo* null background

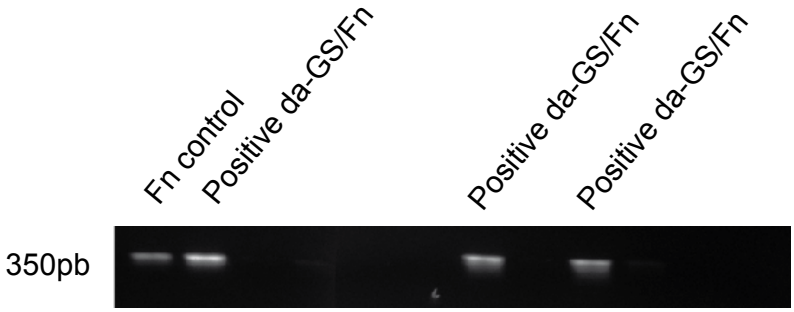


Figure S.4.1.1 Leg PCR for genotyping purpose. Flies with the *dfoxo* null mutation produced a DNA fragment at a size of 350bp using Sol 236 and Sol 237 primers.

25 positive flies (balanced over TM3Sb and tested by *dfoxo* null and *dfoxo* wild type primer) from each gender were crossed together.

S.4.2 Cox Proportional Hazards statistics of epistatic analysis between DHR96 and IIS-reduced flies

Relevant Figure	Experiment	Coefficient	Estimate	SE	p-value
Figure 4.3.1	Lifespan assay	*Dhr96* status *dfoxo* oe status *Dhr96* status > dfoxo oe status	0.2009 0.2583 0.0662	0.0362 0.0362 0.0356	<0.001 <0.001 0.0909
Figure 4.3.3	Lifespan assay	*Dhr96* status MNC ablation status *Dhr96* status > MNC status	0.1518 -0.309 -0.0377	0.0369 0.0383 0.0368	<0.001 <0.001 0.3055
Figure 4.3.4	Phenobarbital stress assay	*Dhr96* status MNC ablation status *Dhr96* status > MNCablation status	0.3911 -0.0199 0.1672	0.0524 0.0522 0.0522	<0.001 <0.001 0.0012
Figure 4.3.5	Lifespan Assay	*dfoxo* status *Dhr96* oe status *Dhr96* oe status >dfoxo status	2,0408 0,4531 -0.0208	0.0751 0.0421 0.042	<0.001 <0.001 0.6215

Table S.4.2 Cox Proportional Hazard (CHP) statistical analysis. P-values, standard errors (SE), and estimate were calculated for each epistasis assay using CHP survival analysis.

S.4.3 DDT assay of *dhr96-lbd* overexpression in *dfoxo* null flies on 275 mg/L

Over-expression of *dhr96-lbd* in a *dfoxo* null background did not increase xenobiotic resistance in females when experiment was conducted on DDT food with a concentration of 275mg/L.

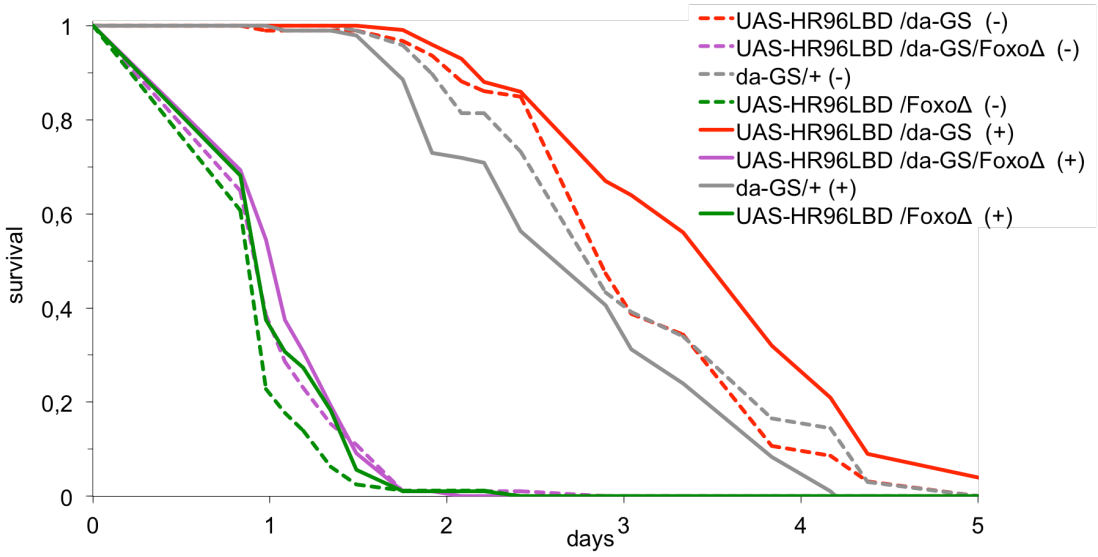


Figure S.4.3.1 DDT assay of *dhr96-lbd* over-expressing flies in a wild type and *dfoxo* null background using a DDT concentration of 275mg/L. Over-expression of *dhr96-lbd* in a wild type background significantly increased DDT resistance (P<0.001, Log Rank Test). Resistance was not enhanced when *dhr96-lbd* was over-expressed in a *dfoxo* null background using a DDT concentration of 275mg/L.

S.4.4 Daf-2 alleles

S.4.4.1 Table is taken unmodified from Gems et al (1998) and shows the two pleiotropic classes of daf-2 mutations.

	Adult life span					
	15°			22.5°		
Allele[a]	Median	Maximum	N[b]	Median	Maximum	N[b]
+	24.0 ± 1.6	31.0 ± 3.0	304 (5)	16.3 ± 1.3	22.2 ± 2.4	306 (6)
daf-12(m20)	16.7 ± 0.6	24.8 ± 4.1	80 (4)	10.6 ± 1.4	16.0 ± 1.2	80 (4)
Class 1						
daf-2(e1365)	28.2 ± 2.9	36.5 ± 2.5	224 (2)	29.2 ± 3.0	39.7 ± 8.3	257 (3)
daf-2(m577)	25.8 ± 1.1	34.0 ± 4.3	269 (4)	25.9 ± 3.8	40.3 ± 6.6	246 (4)
daf-2(sa193)	33.5 ± 1.2	40.5 ± 1.5	41 (2)	26.0 ± 2.0	35.0 ± 1.0	39 (2)
daf-2(e1371)	31.8 ± 2.8	45.5 ± 2.2	273 (4)	21.6 ± 0.5	36.3 ± 5.6	247 (4)
daf-2(e1368)	33.2 ± 3.2	47.5 ± 5.3	263 (4)	29.5 ± 4.2	43.3 ± 8.6	223 (4)
daf-2(m41)	27.0 ± 1.3	35.0 ± 1.4	71 (3)	29.4 ± 1.2	35.0 ± 0.0	60 (2)
daf-2(m212)	48.4 ± 1.4	64.0 ± 0.0	40 (2)	34.0 ± 3.0	47.5 ± 4.5	38 (2)
daf-2(e1369)	52.8 ± 2.8	74.5 ± 1.5	213 (2)	38.2 ± 2.4	54.8 ± 3.1	232 (4)
daf-2(e1365); daf-12(m20)	18.2 ± 1.5	29.7 ± 3.8	64 (3)	15.2 ± 2.3	24.0 ± 4.7	77 (4)
daf-2(m577); daf-12(m20)	21.6 ± 3.2	32.7 ± 1.9	61 (3)	18.5 ± 7.5	33.8 ± 1.1	89 (4)
daf-2(sa193); daf-12(m20)	26.7 ± 2.4	42.0 ± 9.0	41 (2)	18.4 ± 2.6	29.5 ± 3.0	91 (4)
daf-2(m41); daf-12(m20)	24.6 ± 3.9	32.8 ± 6.0	89 (4)	17.9 ± 4.8	41.3 ± 4.7	88 (4)
daf-2(m212); daf-12(m20)	25.3 ± 5.6	51.1 ± 7.0	78 (4)	34.0 ± 7.0	52.5 ± 3.6	80 (4)
Class 2						
daf-2(m120)	32.3 ± 1.8	50.0 ± 3.0	252 (2)	31.6 ± 4.4	57.8 ± 7.2	257 (4)
daf-2(e1370)	33.8 ± 0.2	42.0 ± 1.4	66 (3)	20.4 ± 2.4	45.0 ± 0.0	39 (2)
daf-2(m596)	36.8 ± 5.3	56.5 ± 4.5	208 (2)	35.4 ± 3.8	54.3 ± 4.6	257 (3)
daf-2(m579)	44.3 ± 1.3	63.5 ± 1.5	239 (2)	40.0 ± 3.2	64.3 ± 15.0	266 (3)
daf-2(e1391)	63.4 ± 5.0	91.3 ± 10.9	259 (3)	29.2 ± 9.7	68.0 ± 10.0	142 (3)
daf-2(e979)	50.3 ± 2.8	69.0 ± 3.0	105 (4)	28.3 ± 1.7	56 ± 12.0	57 (2)
daf-2(sa223)	58.7 ± 2.7	92.0 ± 2.0	36 (2)	41.0 ± 2.0	53.0 ± 3.0	37 (2)
daf-2(e1370); daf-12(m20)	30.4 ± 2.0	41.7 ± 2.1	65 (3)	21.6 ± 4.6	62.5 ± 1.7	88 (4)
daf-2(e1391); daf-12(m20)	58.7 ± 8.0	81.3 ± 9.6	69 (3)	71.8 ± 2.2	94.0 ± 7.0	94 (4)
daf-2(e979); daf-12(m20)	45.6 ± 4.8	91.5 ± 13.5	37 (2)	43.3 ± 8.7	72.0 ± 2.9	86 (4)

[a] *daf-2* alleles ordered in increasing severity of L2d and dauer arrest phenotype, within each class.
[b] Sample size; number of trials in parentheses.

S.5 Supplement Chapter 5

S.5.1 Quantitative RT-PCR analysis of *dhr96-lbd* over-expressing whole bodies

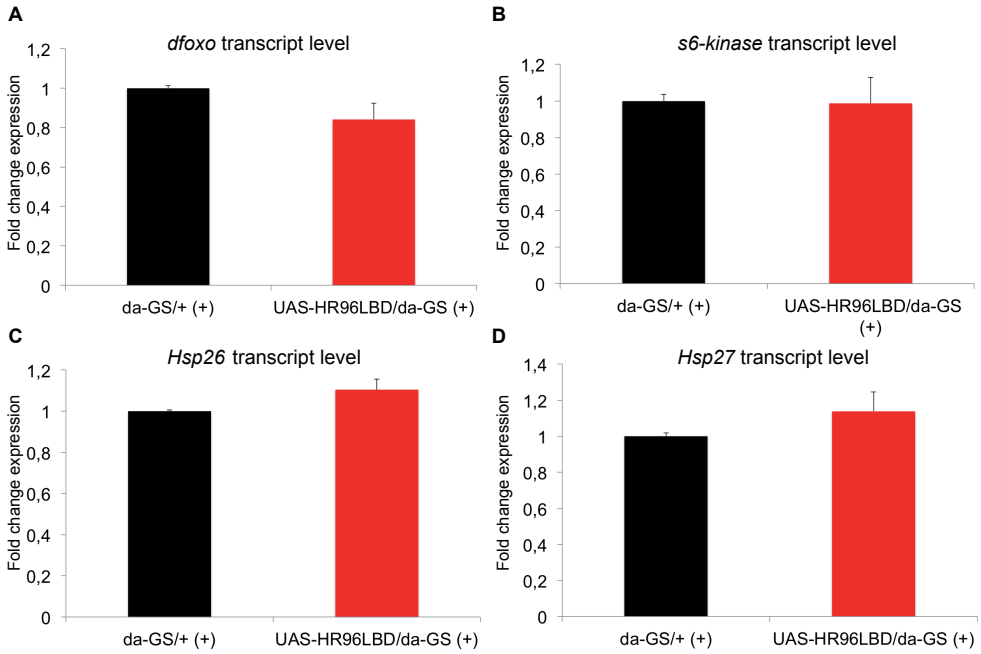


Figure S.5.1.1 Transcript levels of candidate genes in whole bodies of *dhr96-lbd* over-expressing flies. Expression levels of (A) *dfoxo*, (B) *S6-kinase*, (C) *Hsp26*, and (D) *Hsp27* were not significantly changed in whole bodies of *dhr96-lbd* over-expressing flies

S.5.2 Quantitative RT-PCR analysis of dissected ovaries from *dhr96-lbd* over-expressing female flies

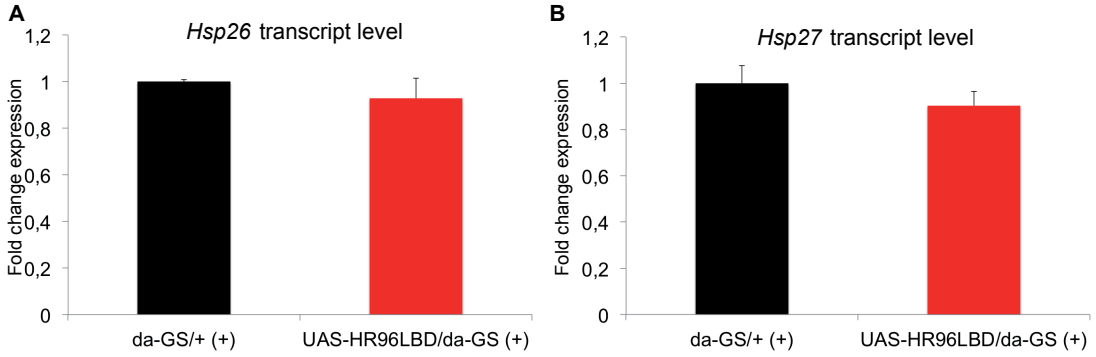


Figure S.5.2.1 Transcript levels of candidate genes in ovaries of *dhr96-lbd* over-expressing flies. Expression levels of (A) *Hsp26* and (B) *Hsp27* were not significantly changed in ovaries of *dhr96-lbd* over-expressing flies

S.6 Supplement Chapter 6

S.6.1 Quantitative RT-PCR analysis of *magro* and *Cyp18a1* in white Dahomey *Wolbachia* plus flies (wild type strain) on a low cholesterol diet

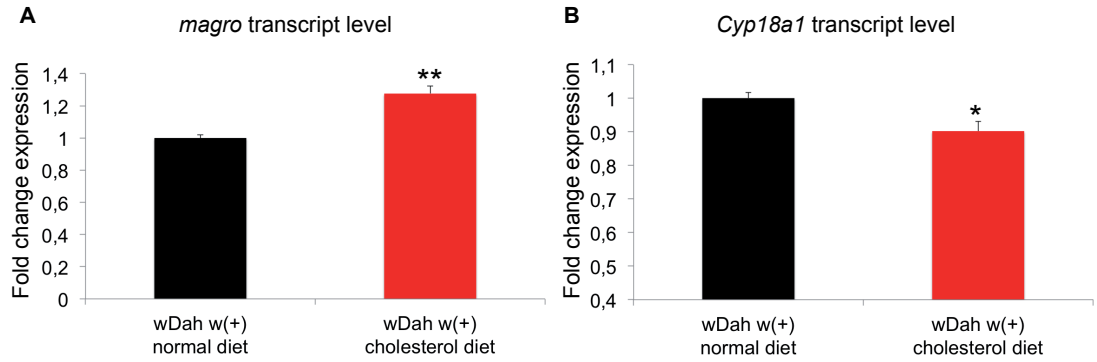


Figure S.6.1.1 Transcript levels of *magro* and *Cyp18a1* in whole bodies of white Dahomey *Wolbachia* (+) wild type flies on a cholesterol diet. (A) Expression levels of *magro* are significantly increased (p-value **<0.01, Student's *t*-test) and (B) expression levels of *Cyp18a1* significantly decreased (p-value *<0.05, Student's *t*-test) when wild type flies are exposed to a low cholesterol diet.

S.7 Supplement Chapter 7

S.7.1 Recipe for 200N YAA holidic medium with normal and low cholesterol concentrations

Normal and low cholesterol holidic food was prepared as shown in table S.7.1.1. Detailed information is available in Piper et al. (2014).

Yaa 200N				
Yeast Amino acid ration			**Normal Cholesterol**	**Cholesterol diet**
			1000 ml	1001 ml
			1x	1x
Added ***before*** autoclaving:				
	agar		20	20
	ile		1,16	1,16
	leu		1,64	1,64
	tyr		0,84	0,84
	sucrose		17,12	17,12
	(20mg/ml) cholesterol		**15**	**5**
	CaCl2	1000x	1	1
	MgCl2	1000x	1	1
	CuSO4	1000x	1	1
	FeSO4	1000x	1	1
	MnCl2	1000x	1	1
	ZnSO4	1000x	1	1
	Total water to add:		642,73	632,73
Added ***after*** autoclaving:				
	Acetate buffer		100	100
Prepare as **Master Mix**:	nucl/lipid soln		8	8
	Yaa soln	Essential AA	60,51	60,51
		Non Essential AA	60,51	60,51
		cys (50mg/ml)	5,28	5,28
		glu (40x)	18,21	18,21
	vit		21	21
	folic acid		1	1
add at the end at 55°C	prop acid		6	6
	nipagin		15	15

Table S.7.1.1 Holidic medium recipe with two different cholesterol concentrations.

S.7.2 Ligand sensor screen with glucose and tebufonizide

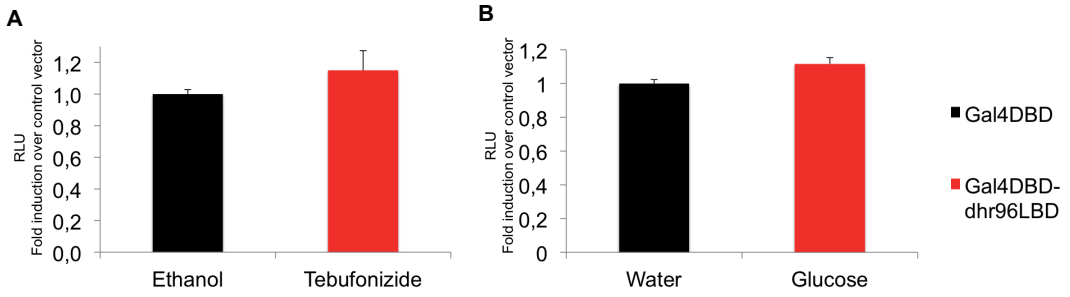
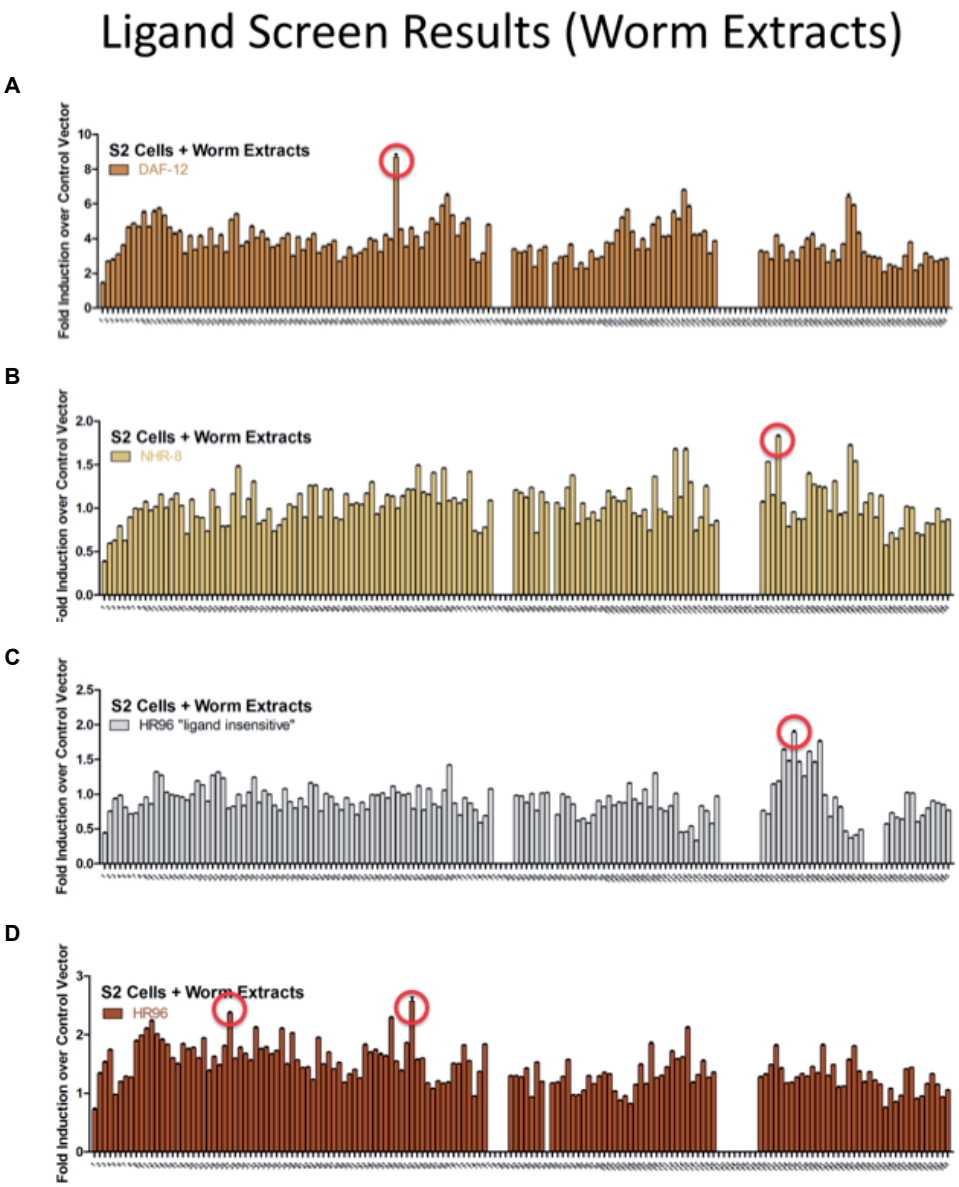
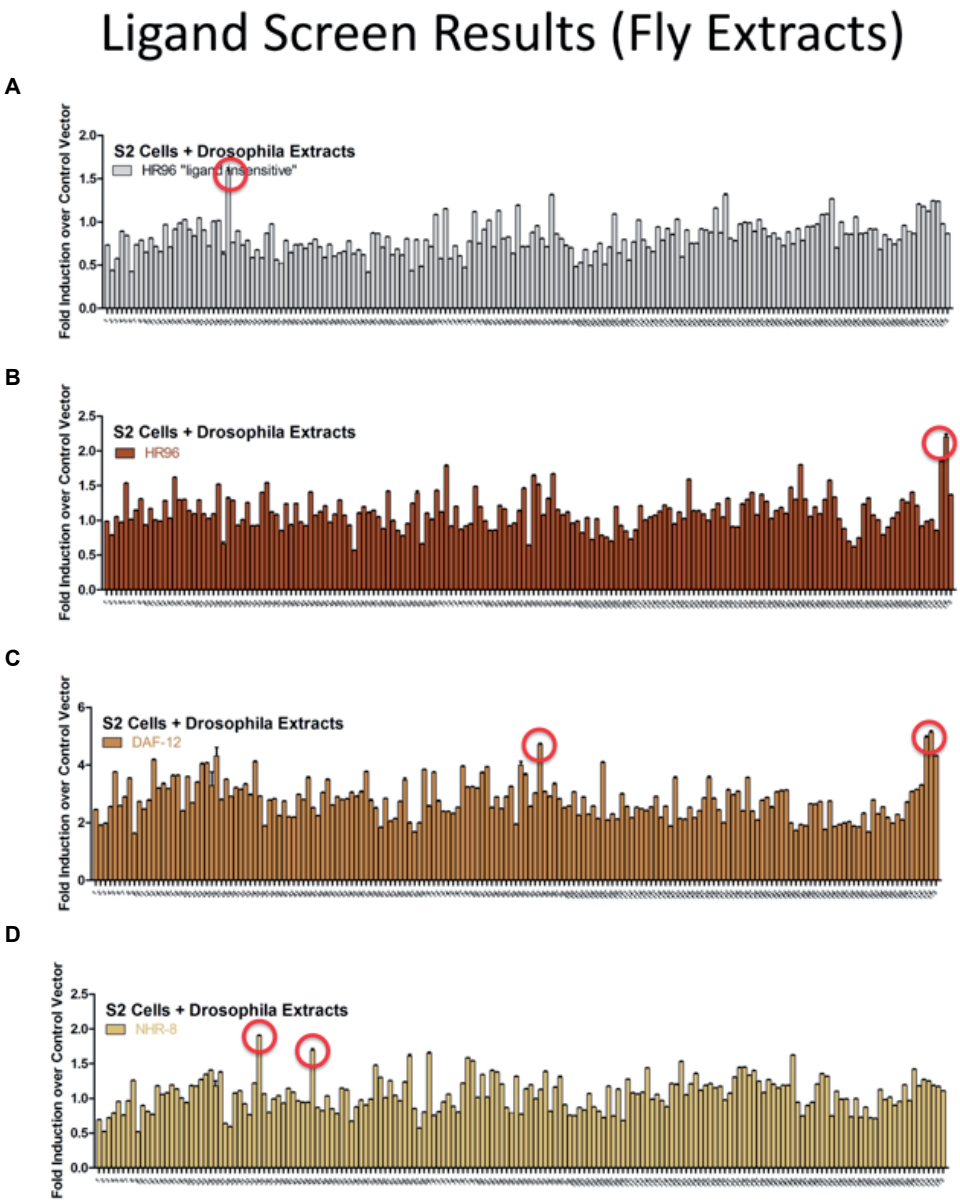


Figure S.7.2.1 Ligand sensor screen for DHR96. (A) Tebufonizide and (B) Glucose did not induce transcriptional activity of the DHR96 ligand sensor system in S2 cells.

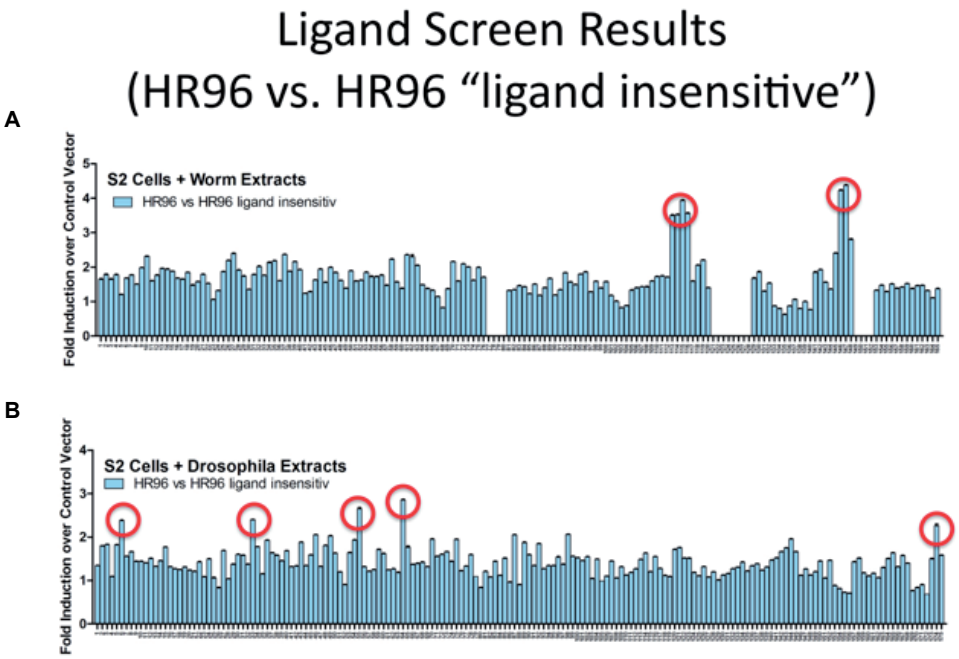
S.7.3 Screen results of ligand sensory system using wild type *Drosophila*, *C. elegans* and pig liver lipid fractions



S.7.3.1 Reporter Assay using lipid extract fractions from *C. elegans* in S2 cells. Lipid fractions from worm were tested at a 1:1000 dilution in a reporter assay with (A) *daf-12*, (B) *nhr-8*, (C) ligand-insensitive *dhr96* and (D) wild type *dhr96*. The LBDs of these NRs were fused to the Gal4DBD, and pUAS-*luciferase* was used as a reporter gene. RLU values are normalised to a control vector. (A) Fraction 58, which contains the dafachronic acids, induces the activation of the Gal4DBD-DAF12LBD sensor. Strong candidate fractions were marked in a red circle. Gaps without any reported luminescence contain cells which died during the experimental process. This may be due to toxicity of lipids or sterols on cells.



S.7.3.2 Reporter assay using lipid extract fractions from *Drosophila* in S2 cells. Each lipid fraction from flies was tested at a 1:1000 dilution on a reporter assay with (A) ligand-insensitive *dhr96*, (B) *dhr96*, (C) *daf-12* and (D) *nhr-8*. The same constructs were used as in the reporter assay using *C. elegans* lipid extracts. Luminescence values (RLU) are normalised to a control vector. (A) Although the LBD mutant should be ligand-insensitive, Fraction 26 shows a highly significant increase in luminescence, and counts therefore as a strong candidate fraction. (B) Strongest activation for DHR96 is shown by end fractions, which contain unpolar lipids.



S.7.3.3 Reporter assay using lipid extract fractions from *Drosophila* and *C. elegans*. Lipid fractions from (A) worms and (B) flies were tested in a 1:1000 dilution on a reporter assay with (A) ligand-insensitive *dhr96* and (B) *dhr96*. RLU values of wild type tested *dhr96* were normalised to ligand-insensitive *dhr96*.

S.7.4 High priority fraction candidates from the ligand sensor screen (S.7.3)

Fractions with fold change values greater or less than 2 standard deviations from the mean of all fractions

Number of Fractions to Retest				
18	18	36	40	25

HEK Cells			S2 Cells	
Worm	Fly	Pig	Worm	Fly
20	1	6	1	2
43	2	17	12	6
58	3	23	27	9
81	4	38	58	13
83	31	39	62	26
86	33	42	68	33
87	34	43	77	34
93	42	50	78	45
97	69	51	79	52
99	70	53	80	55
100	71	54	88	64
104	72	58	112	65
105	90	63	113	69
115	102	65	114	71
119	105	82	115	77
131	**107**	83	116	89
155	146	97	121	93
156	157	123	122	129
		125	123	144
		131	124	151
		148	125	156
		149	126	172
		150	127	173
		155	128	174
		156	130	175
		163	132	
		164	133	
		165	134	
		166	135	
		167	136	
		168	138	
		169	139	
		170	140	
		171	146	
		172	147	
		173	149	
		174	150	
		175	151	
			152	
			153	

S.7.4 Positive fractions from the first screen. Candidates were selected by statistical analysis from the first Ligand sensor screen using fractions of worm, fly, and pig lipid extracts. Fractions highlighted in red and green were classified as high priority candidates.

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Sprach- und EDV Kenntnisse

Sprachkenntnisse	Deutsch und Englisch fließend in Wort und Schrift
EDV-Kenntnisse	MS (Excel, Word, Powerpoint), Photoshop, Adobe, GENtle