FUNCTIONAL CHARACTERIZATION OF GENE REGULATION BY NHR-49

by

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Abstract

Nuclear hormone receptors (NHRs) are transcription factors that regulate a wide variety of developmental and physiological processes. NHRs are targets of numerous drugs. However, due to limited knowledge on NHR specificity, many such drugs activate multiple biological pathways downstream of NHRs, leading to undesired side effects. To study NHR specificity in vivo, I used the model organism Caenorhabditis elegans. One C. elegans NHR is NHR-49, which regulates various aspects of lipid metabolism. Specifically, it activates genes involved in fatty acid desaturation and fatty acid β-oxidation by binding to a subunit of the Mediator multiprotein complex, MDT-15. Vice versa, NHR-49 represses genes involved in sphingolipid breakdown by heterodimerizing with another C. elegans NHR, NHR-66. Recently, three point mutations in nhr-49 were identified that promote fatty acid desaturation, but whether these alleles act specifically in this pathway or also affect other nhr-49 regulated processes is not clear. To test whether the mutated residues are linked to specific biological functions, I studied how they affect gene expression and protein-protein interactions by real time quantitative PCR and Yeast 2 Hybrid assays. I found that the three point mutations have different effects on nhr-49 dependent metabolic processes. While all three alleles broadly promoted nhr-49 dependent activation, only one allele affected nhr-49 dependent repression. This shows that the mutations and the corresponding amino acid residues have some association with specific nhr-49 dependent biological processes.
Preface

Dr. Stefan Taubert and I designed all experiments performed in this project. I conducted the experiments and analyzed all data generated. Experiments in section 2.4 and 2.5 were conducted with the help of Marcus Wong. Grace Goh generated the worm strains I used in section 2.1, 2.2, 2.3, and 2.7.
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<th>Full Form</th>
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<tbody>
<tr>
<td>NHR</td>
<td>Nuclear Hormone Receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>AF-1</td>
<td>Activation Function-1</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone Response Element</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid Hormone receptor</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>AF-2</td>
<td>Activation Function-2</td>
</tr>
<tr>
<td>HNF4</td>
<td>Hepatocyte Nuclear Factor 4</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-Binding Protein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-Binding Protein</td>
</tr>
<tr>
<td>SRC-1</td>
<td>Steroid hormone Receptor Coactivator</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>NCoA</td>
<td>Nuclear receptor CoActivator</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor CoRepressor</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing Mediator of Retinoid and Thyroid Receptors</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl Choline</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element-Binding Protein</td>
</tr>
</tbody>
</table>
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I would like to thank all members of the Taubert lab, who helped me tremendously throughout my Master’s program. Thank you so much: Forum Bhanshali, Robyn Cullen, Grace Goh, Jennifer Grants, and Naomi Shomer. I thank Grace and Jen for answering all of my questions so kindly whenever I was asking the most basic questions in the worm field and also for much input on this project. Thank you Forum and Robyn for proofreading my thesis. And of course, last but not least, thank you Naomi for helping me seed my RNAi plates for my corepressor screen.

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Dedication

To my family
Chapter 1: Introduction

1.1 NHRs are essential regulators of physiological processes

Nuclear Hormone Receptors (NHRs) are proteins that regulate many developmental and physiological processes in response to internal and external stimuli (Evans, 1988; Mangelsdorf et al., 1995). NHRs are transcription factors (TFs) that bind directly to DNA and modulate gene transcription (Giguère, 1999).

Currently, NHRs are important targets of drugs in the biomedical field. About 10-15% of prescription drugs target NHRs, highlighting their importance for treating numerous and diverse human diseases (Overington et al., 2006). Many drugs are able to alleviate or ameliorate disease symptoms. However, the lack of knowledge about how NHRs specifically activate or repress certain biological pathways causes many drugs to stimulate additional pathways other than the targeted pathway, leading to unwanted adverse effects (Burris et al., 2012; Sever and Glass, 2013). For example, the Glucocorticoid Receptor (GR) has been a target of drugs (e.g., prednisone) to alleviate inflammatory reactions and activate immunosuppressive pathways (Schäcke et al., 2002). Unfortunately, GR activation by such drugs can also cause increased blood glucose levels, peptic ulcers, skin atrophy, osteoporosis, dyslipidemia, and pancreatitis (Schäcke et al., 2002). Thus, it is critical to understand how NHRs target specific pathways in order to produce drugs that do not cause side effects.

1.2 NHRs contain distinct functional regions

Typically, NHRs contain four regions, an N-terminal domain, a DNA binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) (Figure 1) (Giguère, 1999; Mooijaart et al., 2005). The N-terminal domain is highly variable and harbours the Activation
Function 1 (AF-1), which confers ligand-independent transcriptional activation by interacting with the transcription machinery and/or various coregulators (Giguère, 1999; Kumar and Thompson, 1999). Located adjacent to the N-terminal region, the DBD is the most conserved NHR domain and recognizes hormone response elements (HRE), which are regulatory DNA elements situated in the promoters and enhancers of NHR-regulated genes, via two zinc finger motifs (Giguère, 1999; Kumar and Thompson, 1999; Mooijaart et al., 2005). Additionally, the DBD is required for NHR dimerization, enabling the formation of homodimers and/or heterodimers, depending on the NHR subtype. The mode of DNA binding is distinct between different types of NHRs (Table 1). For example, mammalian steroid receptors, such as estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and GR, bind inverted, everted, or direct HRE repeats as homodimers. In contrast, NHRs that heterodimerize with retinoid X receptor (RXR) such as retinoic acid receptor (RAR), thyroid hormone receptor (TR), or peroxisome proliferator activated receptor (PPAR), exclusively bind direct repeat HREs. Lastly, some NHRs, such as RevErbAα and steroidogenic factor-1, bind 5′-flanking A/T-rich sequence HRE as monomers (Table 1) (Giguère, 1999; Khorasanizadeh and Rastinejad, 2001). Thus, while evolutionarily related, the different DBDs of distinct NHR subclasses enable molecularly divergent DNA binding.

The NHR hinge region is highly variable and physically links the DBD and LBD (Giguère, 1999). Although its overall function is not well understood, the hinge seems to be important for diverse aspects of NHR function: e.g., the AR hinge is involved in DNA binding and nuclear translocation (Haelens et al., 2007) and the TR hinge is responsible for binding to a transcriptional corepressor (Hörlein et al., 1995). Lastly, the LBD performs several critical NHR
functions, such as ligand binding, NHR dimerization, and activation/repression (Giguère, 1999). The C-terminus of the LBD harbors the Activation Function 2 (AF-2). Like the AF-1, the AF-2 mediates interaction with coactivators/corepressors; however, unlike the AF-1, interactions with the AF-2 occur in a ligand-regulated manner (Giguère, 1999; Kumar and Thompson, 1999). Upon ligand binding, a conformational change occurs. During this process, the AF-2 forms a hydrophobic surface and this new interface provides a site to bind the LXXLL motif that occurs in many NHR coactivators (Giguère, 1999; Pawlak et al., 2012; Renaud et al., 1995). Even small changes in ligand structure can substantially affect the forming of a hydrophobic groove. This can be problematic as ligands that target NHRs could cause various unexpected effects based on slight structural structure changes (Pawlak et al., 2012).

The above-described mechanism of transcriptional activation, involving reversible ligand-induced conformation changes, is not identical for all NHRs. For example, Hepatocyte Nuclear Factor 4 (HNF4) is a transcription factor that constitutively and tightly binds fatty acid ligands; as a result, HNF4 is kept in an active state (Wisely et al., 2002).

**Figure 1. Schematic of NHR structure.**
The figure represents four distinct regions of NHR, including N-terminal domain, DBD, Hinge region and LBD. AF-1 is situated at N-terminal domain while AF-2 is situated at LBD. Adapted from (Giguère, 1999; Mooijaart et al., 2005).
Table 1. List of HREs classified by dimerization mechanism, and corresponding examples. The table shows the different types of NHR dimerization, target HRE sequences for the each type of dimerization mode, and examples. Adapted from (Aranda and Pascual, 2001; Giguère, 1999; Khorasanizadeh and Rastinejad, 2001).

<table>
<thead>
<tr>
<th>Dimerization</th>
<th>Sequence</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>A/T-rich</td>
<td>RO, TLX, RevErb</td>
</tr>
<tr>
<td>Homodimer</td>
<td>Inverted</td>
<td>ERR, GR-GR, AR-AR</td>
</tr>
<tr>
<td></td>
<td>Direct</td>
<td>HNF4, RXR, TLX, RevErb</td>
</tr>
<tr>
<td>Heterodimer</td>
<td>Direct</td>
<td>RXR-RAR, RXR-PPAR, RXR-VDR, RXR-TR, RXR-LXR</td>
</tr>
</tbody>
</table>

1.2.1 Mechanisms of transcriptional regulation by NHRs

Multiple inputs and mechanisms regulate NHR activity including ligand binding, covalent post-translational modification (e.g. phosphorylation), and interactions with NHR and non-NHR partner proteins (Giguère, 1999). Ligands are lipophilic molecules such as steroid hormones (e.g. estradiol, cortisol, and progesterone), vitamins, and/or intermediary metabolites such as fatty acids and bile acids (Nagy and Schwabe, 2004). The sequence and structure of the LBD determine which ligand(s) bind to individual NHRs. A ligand-bound LBD can form a
heterodimer, frequently with RXR, which serves as a heterodimer partner for many mammalian NHRs. Therefore, ligand binding to the LBD initiates a conformational change in NHRs, which then leads to the binding of other partner NHRs and coregulators to modulate transcription.

NHR activity can also be regulated by post-translational modifications such as phosphorylation, acetylation, and sumoylation (Anbalagan et al., 2012; Poukka et al., 2000; Smirnov, 2002). These modifications affect specific residues within the LBD and alter the recruitment of coregulatory proteins by NHRs. For example, phosphorylation of the N-terminal domain of GR causes the formation of a more stable tertiary structure within this domain. This in turn leads to the binding of coregulators such as TATA-Binding Protein (TBP), CREB-Binding Protein (CBP) and Steroid hormone Receptor Coactivator (SRC-1), which alters transcriptional output (Garza et al., 2010; Pawlak et al., 2012).

1.2.1.1 **NHR outputs can be regulated by interacting coactivators and corepressors**

NHR activity is fine-tuned through the interactions with non-NHR proteins such as TFs or coregulators (Giguere 1999). Coregulators can be either coactivators or corepressors. Many NHR coactivators substantially increase the activity of NHRs by binding to ligand bound NHR, thus linking NHRs to RNA polymerase II machinery. Coactivators can also be histone modifying proteins such as Histone Acetyltransferases (HATs) which can acetylate the promoters of NHR target genes (Dasgupta et al., 2014; Rosenfeld et al., 2006; Tetel and Acharya, 2013). On the other hand, corepressors interact with either unliganded or antagonist bound NHRs, and recruit repressive complexes to downregulate target genes (Dasgupta et al., 2014; Tetel and Acharya, 2013). Important NHR coregulators include: the SRC family, nuclear receptor coactivators (NCoAs), the Mediator complex, the nuclear receptor co-repressors (NCoRs), and the Silencing
Mediator of Retinoid and Thyroid Receptors (SMRTs) (Heinzel et al., 1997; Lazar, 2003; Malik and Roeder, 2005; McKenna et al., 1999; Myers et al., 1999; Nagy et al., 1997; Smirnov, 2002; Wong et al., 1998).

Both the AF-1 and the AF-2 domain play important roles for the recruitment of coactivators. In regards to the role of AF-1 function in coactivator recruitment, it seems that the AF-1 domains in different NHRs act distinctively from each other (Lavery and McEwan, 2005). For example, hydrophobic amino acids within the AF-1 in AR are important for both the receptor’s activity and for protein-protein interactions. In GR, the AF-1 provides structural importance, allowing for a solvent-exposed surface for transactivation (Lavery 2005). As described above, the AF-2 also plays a crucial role in coactivator recruitment. Upon ligand binding, helix 12, which resides within the LBD and the AF-2 of NHRs, undergoes a conformational change (Nettles and Greene, 2003). This exposes hydrophobic surfaces that allow coactivator LXXLL motifs to interact with NHR LBDs (Aranda and Pascual, 2001; Nettles and Greene, 2003; Smirnov, 2002). For example, following ligand administration, two LXXLL motifs within the Mediator subunit MED1 interact with numerous NHRs, including ERα, ERβ, PPARγ, and TR, to regulate gene transcription (Ge et al., 2008; Kang et al., 2002; Ren et al., 2000).

Not only does helix 12 plays a crucial role in coactivator binding, but it also plays a role in corepressor recruitment (Lazar, 2003). Deletion of helix 12 strengthened both target gene repression and corepressor binding to NHRs such as TR, RAR, and RXR (Lazar, 2003). NHRs can recruit corepressors through LXXLL motifs as well as through the related ‘CoRNR box’ (Lazar, 2003; Loinder and Söderström, 2004; Perissi et al., 1999). However, the AF-2 is also required for the dissociation of corepressor, suggesting a dynamic role of AF-2 in corepressor
dissociation and coactivator recruitment (Nagy et al., 1999). Thus, the AF-2 acts as a molecular switch, i.e. it is involved in both the activation and the repression of NHR target genes. Overall, the binding of coregulators provides important insight into how NHRs achieve specific gene regulation.

![Diagram showing the role of coactivators and corepressors in gene expression regulation.](Image)

**Figure 2. Coregulators play a crucial role in regulation of gene expression.**
The NHR DBD binds to HREs in genomic DNA, whereas the LBD binds to coregulators. When an NHR binds to a coactivator, it leads to increased gene expression, whereas binding to corepressors represses gene expression.

1.2.2 **HNF4 is important for many hepatic functions and various human diseases**

HNF4 is an NHR known as a regulatory hub in liver, pancreas, and intestinal cells. Since its discovery, HNF4 has been linked to numerous hepatic functions including hepatocyte differentiation and liver development, morphogenesis, glucose and lipid metabolism, xenobiotic detoxification, cholesterol, and steroid hormone metabolism (Babeu and Boudreau, 2014; Chen...
et al., 1994; Cooper et al., 1997; Giguère, 1999; Ibeanu and Goldstein, 1995; Ladias et al., 1992; Legraverend et al., 1994; Walesky and Apte, 2015; Watt et al., 2003; Yokomori et al., 1997).

Because of its involvement in numerous metabolic pathways, HNF4 has been studied extensively. Interestingly, HNF4 is one of the most highly conserved NHRs. It is found in almost every animal species studied to date, including sponges, corals, invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*, vertebrates, and mammals including mice and man (Sladek, 2011).

There are three subtypes of HNF4: HNF4α, HNF4β, and HNF4γ (Giguère, 1999). Human HNF4α is expressed in liver, kidney, intestine, pancreas, and testes, with high levels in liver and intestine and lower levels in testes (Drewes et al., 1996). HNF4α is important for the metabolic homeostasis in liver and intestinal cells (Babeu and Boudreau, 2014). Because of its involvement in liver related function, HNF4α is linked to many diseases. Mutations in HNF4α are associated with diabetes, atherosclerosis, haemophilia, and cancer (Yuan et al., 2009, Bolotin et al., 2012). In particular, dominant mutations in *HNF4A* are linked to maturity onset diabetes of the young (MODY), a heritable form of non-insulin dependent diabetes (Ellard and Colclough, 2006; Yamagata, 2014; Yamagata et al., 1996). Many distinct *HNF4A* mutations have now been identified, and most are located within or in proximity to the AF-2, implicating that disease originates from dysregulated transcriptional activation and/or ligand binding (Bulman et al., 1997; Furuta et al., 1997; Hani et al., 1998).

HNF4β is expressed in stomach, lung, ovary, and testis of the amphibian *X. laevis*. In contrast to the role of HNF4α in metabolism-related hepatic functions, HNF4β is responsible for encoding genes required for oogenesis and embryogenesis (Holewa et al., 1997). HNF4γ is expressed in
the same tissues as HNFα except in liver, a distinct difference from HNF4α (Drewes et al., 1996). Even though the exact function of HNF4γ has not been fully uncovered, its high sequence similarity with one of HNF4α isoforms suggests its function might be similar as well (Gerdin et al., 2006). Due to the linkage of HNF4α to MODY, HNF4γ was also investigated for a putative role in this disease, but available data suggest that HNF4γ is not related to the etiology of this type of diabetes (Plengvidhya et al., 1999).

1.2.3 PPAR plays a vital role in lipid metabolism

The three human PPARs (PPARα, PPARβ/δ, and PPARγ) were first identified as the target for peroxisome proliferating agents in rodents. After the identification of the PPARs, numerous fatty acids have been identified as PPAR ligands, e.g. prostaglandins, which are important in many human tissues. PPARs have also been identified as targets for dyslipidemia and diabetes drugs, in line with their central role in lipid metabolism (Berger and Moller, 2002).

As noted, the human genome encodes PPARα, PPARβ/δ, and PPARγ, each of which has a discrete expression pattern and function (Berger and Moller, 2002; Giguère, 1999; Grygiel-Górniaik, 2014). PPARα is expressed in tissues that are metabolically active such as: heart, liver, kidney, and brown fat (Berger and Moller, 2002; Braissant and Wahli, 1998; Giguère, 1999). In line with PPARα’s expression, it is responsible for regulating fatty acid β-oxidation genes, e.g. those encoding fatty acid transporters or and lipoprotein lipases (Grygiel-Górniaik, 2014). In agreement with PPARα’s role in the regulation of fatty acid homeostasis, lipid metabolism, and glucose metabolism, PPARα null mice have reduced fatty acid catabolism and display hypoglycemia, hyperlipidemia, and fatty liver (Aoyama et al., 1998; Djouradi et al., 1998; Evans et al., 2004; Lee et al., 1995).
PPARβ/δ is expressed in most adult tissues, but most strongly in the gastrointestinal tract (Grygiel-Górniak, 2014). PPARβ/δ is responsible for the regulation of cell proliferation, glucose metabolism, adipocyte differentiation, inflammation, and myelination (Abbott, 2009). Additionally, PPARβ/δ activates genes involved in lipid uptake and metabolism (Grygiel-Górniak, 2014). *In vivo*, PPARβ/δ promotes β-oxidation and triglyceride catabolism in both adipocytes and myocytes (Wang *et al.*, 2003). Furthermore, when PPARβ/δ knockout mice were fed a high fat diet, their energy expenditure decreased and they developed obesity. This suggested PPARβ/δ is responsible for the regulation of fatty acid β-oxidation and fat burning (Wang *et al.*, 2003).

PPARγ is mainly expressed in white and brown adipose tissue, and, to a lesser extent, in other tissues such as intestine, liver, kidney, bone marrow, lymphocytes, and macrophages (Grygiel-Górniak, 2014). PPARγ is involved in lipid synthesis, storage, adipocyte differentiation, and fatty acid binding, transport, and translocation (Braissant and Wahli, 1998; Giguère, 1999; Grygiel-Górniak, 2014; Pol *et al.*, 2015). Synthetic PPARγ agonists are currently used as a drugs to treat type 2 diabetes (Grygiel-Górniak, 2014). PPARγ ligands can either promote or inhibit cancer, depending on the cellular environment and the activation of different signaling pathways (Grygiel-Górniak, 2014).

1.3 The genome of *C. elegans* encodes an unusually large NHR family

The model organism *Caenorhabditis elegans* provides an excellent opportunity to study NHR function because of its experimental tractability and its completely sequenced genome (C. elegans Sequencing Consortium, 1998). Surprisingly, *C. elegans* encodes an extraordinarily large family of 284 *nhr* genes. This is an unexpectedly large number of *nhr* genes in comparison to 48
NHRs in humans, 49 in mouse, and 18 in the fruit fly *Drosophila melanogaster* (Maglich *et al*., 2001). This huge expansion of *C. elegans* NHR provides an opportunity to study both the conservation and divergence of *nhr* gene function.

Of the 284 *C. elegans* NHRs, 15 are related to existing NHR subfamilies, as classified based on the sequence of DBD and LBD (Van Gilst *et al*., 2002; Sluder and Maina, 2001; Sluder *et al*., 1999). Based on their DBD and LBD sequences, the other 269 NHRs in *C. elegans* are thought to have arisen from an HNF4-related ancestor. Gene duplication likely contributed to the explosive *nhr* gene expansion during evolution (Antebi, 2006; Van Gilst *et al*., 2002; Laudet, 1997; Taubert *et al*., 2011). To date, only few of the HNF4-like NHRs of *C. elegans* have been studied; collectively, these studies have revealed prominent roles for this class of NHRs in the regulation of lipid metabolism and of longevity (Van Gilst *et al*., 2005a, 2005b; Pathare *et al*., 2012; Ratnappan *et al*., 2014).

1.3.1 **NHR-49 plays a vital role in lipid metabolism and longevity in *C. elegans***

The best characterized HNF4-like NHR in *C. elegans* is NHR-49. Although NHR-49’s sequence is homologous to HNF4, it is functionally similar to PPARα (Van Gilst *et al*., 2005a). NHR-49 regulates various aspects of fat metabolism, such as fatty acid desaturation, fatty acid β-oxidation, and sphingolipid breakdown (Van Gilst *et al*., 2005a, 2005b; Pathare *et al*., 2012). The *nhr-49* null mutant *nhr-49(nr2041)* shows high fat and decreased lifespan phenotypes, whereas NHR-49 overexpression increases the lifespan of wild-type worms (Folick *et al*., 2015; Van Gilst *et al*., 2005a; Ratnappan *et al*., 2014). The reduced lifespan of *nhr-49(nr2041)* mutant is caused partially by reduced fatty acid desaturase expression, while the high fat phenotype of the *nhr-49(nr2041)* is likely the result of reduced levels of β-oxidation genes, which impairs fat
breakdown (Van Gilst et al., 2005b). nhr-49 is also required for the long lifespan of the germline-less mutant glp-1 by increasing expression of fatty acid desaturation and fatty acid β-oxidation (Ratnappan et al., 2014). Moreover, nhr-49 is implicated in dietary restriction driven longevity (Chamoli et al., 2014), although it is not yet clear whether this requirement for nhr-49 is also linked to its ability to modulate lipid metabolism.

As described, nhr-49 is essential for the regulation of fatty acid desaturation. NHR-49 is required to express the fatty acid desaturase genes fat-5, fat-6, and fat-7, whose protein products catalyze the conversion of saturated fatty acids to monounsaturated fatty acids. To activate fatty acid desaturase genes, NHR-49 appears to bind to a subunit of the Mediator complex, MDT-15, which is also required to express these genes (Taubert et al., 2006). The Mediator complex consists of about 20-30 protein subunits, and is an essential transcriptional coregulator that links regulatory elements such as HREs to RNA polymerase II (Malik and Roeder, 2005). In addition, two other C. elegans NHR, NHR-13 and NHR-80, also physically bind with NHR-49, and are required for the activation of NHR-49 dependent fatty acid desaturase genes (Pathare et al., 2012). Thus, NHR-49 seems to regulate fatty acid desaturation by binding other NHRs and the Mediator coactivator through the subunit MDT-15.

Another process that nhr-49 activates is fatty acid β-oxidation. NHR-49 activates fatty acid β-oxidation genes such as acs-2 and cpt-5. These genes are required for the catabolism of fatty acids and the products ultimately enter the citric acid cycle. In particular, fatty acid β-oxidation genes are highly induced upon fasting, and this upregulation requires nhr-49 (Van Gilst et al., 2005b) and mdt-15 (Taubert et al., 2006).
NHR-49 also regulates lipid metabolism by gene repression. NHR-49 represses sphingolipid breakdown genes including *lips-6*, *tag-38*, and Y65B4BR.1. In *C. elegans*, sphingolipid metabolism is important for many biological processes such as development, lifespan, and mitochondrial surveillance (Cutler et al., 2014; Deng and Kolesnick, 2015; Liu et al., 2014). In gene repression, NHR-49 appears to cooperate with NHR-66, as NHR-66 is also required for the repression of sphingolipid breakdown genes and physically interacts with NHR-49 (Pathare et al., 2012). Although no corepressor is yet known for NHR-49 and/or NHR-66, it is likely that loss of either NHR-49 or NHR-66 prevents a putative corepressor from inhibiting the expression of these genes.

In addition to fatty acid desaturation, fatty acid β-oxidation, and sphingolipid breakdown genes, *nhr-49* also regulates other metabolic genes as well as genes not directly linked to metabolism. These include lipid binding genes (*e.g.* *lbp-1*, *lbp-8*), non-lipid metabolism genes (*e.g.* glucose and amino acid metabolism genes such as *sodh-1*, *ddo-2*, *dhs-18*), stress response genes (*e.g.* *fmo-2*, K05B2.4, *ttr-37*, *gst-29*), and the isocitrate lyase/malate synthase homologue *icl-1* (a key enzyme in the glyoxylate cycle that converts fatty acid β-oxidation products to intermediates metabolites for sugar synthesis) (Goh et al., unpublished, Van Gilst et al., 2005a, 2005b; Pathare et al., 2012). This suggests there are different inputs that stimulate NHR-49 to either activate or repress specific sets of genes, and provides an exciting opportunity to study how NHR-49 might regulate specific biological processes.
1.3.1.1  *nhr-49* point mutations provide resistance to cold sensitivity by activating fatty acid desaturation genes

When an organism is confronted with a temperature change, it needs to restructure its cellular membrane; for example, cold causes the cellular membrane to be stiffer (Hazel, 1995). To adapt, organisms can increase membrane fluidity by changing membrane lipid composition (Brock *et al*., 2007; Hazel, 1995; Ohtsu *et al*., 1998; Overgaard *et al*., 2005). In accordance, *C. elegans* fatty acid desaturases *fat-5, fat-6*, and *fat-7* are required for survival at low temperatures (Brock *et al*., 2007).

In *C. elegans*, a *paqr-2/adiponectin receptor* mutant displays temperature sensitive (cold-induced) lethality (Pilon and Svensk, 2013; Svensk *et al*., 2013). Svensk and colleagues isolated several suppressor mutations from a screen that rescued this phenotype. The genes harboring suppressor alleles are all involved either in phosphatidylcholine (PC) biosynthesis, in fatty acid metabolism pathways, or serve as transcriptional regulators of these processes. Mutated genes include *nhr-49, mdt-15*, and *sbp-1*, the *C. elegans* homologue of mammalian Sterol Regulatory Element-Binding Protein (SREBP). SBP-1/SREBP is an essential regulator of lipid homeostasis, with important functions in the regulation of biosynthesis and trafficking of cholesterol and fatty acids (Walker *et al*., 2011). Interestingly, MDT-15 can bind to both NHR-49 and SBP-1 to regulate lipid metabolism (Taubert *et al*., 2006; Yang *et al*., 2006).

As noted, cold adaptation in membranes can be achieved through an increase in unsaturated fatty acids. In line with the importance of membrane fatty acid composition for cold resistance, several *paqr-2* suppressor mutations cause an increase in unsaturated fatty acid levels. For example, of three *nhr-49* point mutations isolated in the screen, *nhr-49(et8)* (S432F) promoted
an increase in fat-7 expression and in unsaturated fatty acid levels; two other mutations, nhr-49(et7) (P479L) and nhr-49(et13) (V411E), were not investigated, but presumably act through a similar mechanism. Additionally, the suppression of paqr-2 phenotype by a loss of function allele in a PC biosynthesis gene was dependent on fat-6 and fat-7 expression, illustrating that suppression of the cold-sensitivity of paqr-2 mutants relies on the synthesis of unsaturated fatty acids.

The three nhr-49 point mutations identified by Svensk et al. provide an exciting opportunity to study nhr-49 specificity, as they were isolated in a screen that indirectly selected for nhr-49’s role in fatty acid desaturation. Thus, my work addresses several questions about the nature of nhr-49 point mutation alleles identified in this suppressor screen. Specifically:

1) Do the two other nhr-49 point mutations identified by Svensk et al., nhr-49(et7) and nhr-49(et13), also upregulate the desaturase genes?

2) Do nhr-49 point mutations provide specificity to only a subset of nhr-49 dependent target genes (i.e. the fatty acid desaturase genes), or do they affect all known nhr-49 genes in a non-discriminatory manner?

3) Do nhr-49 point mutations have an effect in nhr-49 dependent repression? If so, what is the relationship with a putative corepressor?

4) What are the molecular mechanisms that cause the specificity to activate certain biological process, if such specificity exists?

My study revealed surprising results, which may help to elucidate the mechanism behind specific impact of nhr-49 on different biological processes.
Chapter 2: Results

2.1 *nhr-49* point mutations have varied conservation across species

To investigate the conservation of the residues affected by the three *nhr-49* missense mutations: *et7(P479L), et8(S432F), and et13(V411E)* across different species, I aligned sequences surrounding these residues to predicted NHR-49 orthologues from several other *Caenorhabditis* species and to HNF4α and HNF4γ orthologues found in other higher organisms including: *Homo sapiens* (human), *Mus musculus* (mouse), *Danio rerio* (zebrafish), and *Drosophila melanogaster* (drosophila).

I found that P479 is conserved across *Caenorhabditis* species (Figure 3A). In non-*Caenorhabditis* species, it is missing in every organism other than drosophila (Figure 3B,C).

S432 is also conserved in all four tested *Caenorhabditis* species (Figure 3). Similar to P479, the residue is not conserved in non-*Caenorhabditis* species (Figure 3B,C).

V411 was conserved in all tested species (Figure 3A-C), as a V in the four tested *Caenorhabditis* species and as an I in human, mouse, zebrafish, and drosophila. This suggests that V411E might alter a conserved function of HNF4 due to a change in a conserved residue.
Figure 3. Alignment of *C. elegans* NHR-49 to orthologues from other *Caenorhabditis* species and from non-*Caenorhabditis* organisms.

Blue arrow indicates V411, green arrow indicates S432, and yellow arrow indicates P479. (A) Alignments of *C. elegans* NHR-49 to other *Caenorhabditis* NHR-49 homologues. (B) Alignments of NHR-49 to HNF4\(\alpha\) homologues in higher organisms including: *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and *Danio rerio*. (C) Alignments of NHR-49 to HNF4\(\gamma\) homologues in higher organisms.
2.2 The nhr-49 point mutations broadly upregulate nhr-49 dependent activated genes

*C. elegans* nhr-49 promotes fatty acid desaturation by strongly activating the fatty acid desaturase genes *fat-5* and *fat-7*, and, more weakly, *fat-6* (Van Gilst et al., 2005a). An *nhr-49* point mutant strain, *nhr-49*(et8), showed increased *fat-7* expression and rescued the cold-sensitive phenotype of the *paqr-2* mutant by inducing membrane lipid desaturation (Svensk et al., 2013). Two other *nhr-49* point mutations were also isolated in this screen but their effects on gene expression were not assessed. Additionally, *fat-7* is only one of many NHR-49 activated genes. It is unclear whether the *nhr-49* point mutations affect other desaturase genes such as *fat-5* and *fat-6* and/or broadly upregulate NHR-49 activated genes.

To address these questions, I used real-time quantitative PCR (qPCR) to quantify the expression levels of NHR-49 activated genes in *nhr-49(nr2041)* null, *nhr-49(et7)*, *nhr-49(et8)*, and *nhr-49(et13)* mutants at ambient temperature (20°C). I found that both *fat-5* and *fat-7* were upregulated by all three *nhr-49* point mutations compared to WT (N2) worms, whereas the point mutations did not affect *fat-6*, which displays weaker *nhr-49* dependence (Van Gilst et al., 2005a) (Figure 4A).

Besides fatty acid desaturases, NHR-49 induces fatty acid β-oxidation genes and non-lipid metabolism genes (Van Gilst et al., 2005a, 2005b). Thus, I next wished to test whether the point mutations broadly affect NHR-49 activated genes other than the fatty acid desaturases. I found that all three point mutations also caused an increased expression of the β-oxidation genes *acs-2* and *cpt-5* (Figure 4B). Moreover, the three *nhr-49* point mutations all upregulated NHR-49 dependent non-lipid metabolism such as glucose and amino acid metabolism (Figure 4C).

Because our lab also found that NHR-49 is a regulator of oxidative stress response genes (Goh et
al., unpublished), I studied oxidative stress response genes such as *fmo-2*, K05B2.4, and Y40B10A.7. I found that all three point mutations caused a similar phenotype, as most tested genes were also upregulated (Figure 5). In all the gene groups I studied, *nhr-49(et13)* caused the highest inductions, followed by *nhr-49(et8)*, and *nhr-49(et7)*. In sum, I concluded that the point mutations’ effects were not limited to the desaturases; instead, they broadly induce NHR-49-driven transcription.
Figure 4. nhr-49 point mutations broadly affect nhr-49 activated genes.

(A-C) Bars represent average mRNA fold changes of (A) nhr-49 dependent desaturases, (B) nhr-49 dependent β-oxidation genes, and (C) nhr-49 dependent non-lipid metabolism genes (compared to N2 worms) (n ≥ 3 for each condition). I normalized fold-changes to act-1, tba-1, and ubc-2. Measurements were made at L4 stage. Errors bars show SEM. * represents p<0.05, ** represents p<0.01, *** represents p<0.001, **** represents p<0.0001 (unpaired Student's t-test).
Figure 5. *nhr-49* point mutations upregulate *nhr-49* dependent stress response genes.

Bars represent average mRNA fold changes in *nhr-49* dependent stress response genes (compared to N2; *n* = 3). I normalized fold changes to *act-1*, *tba-1*, and *ubc-2*. Measurements were made at the L4 stage. Errors bars show SEM. * represents *p*<0.05, ** represents *p*<0.01, *** represents *p*<0.001, **** represents *p*<0.0001 (unpaired Student's *t*-test).

Figure 6. Only *nhr-49(et13)* upregulates *nhr-49* dependent repressed genes.

Bars represent average mRNA fold changes in *nhr-49* dependent repressed genes (compared to N2; *n* ≥ 3). I normalized fold changes to *act-1*, *tba-1*, and *ubc-2*. Measurements were made at the L4 stage. Errors bars show SEM. * represents *p*<0.05, ** represents *p*<0.01, *** represents *p*<0.001, **** represents *p*<0.0001 (unpaired Student's *t*-test).
2.3 Only nhr-49(et13) upregulates NHR-49 repressed genes

From the above experiments I concluded that nhr-49 point mutations broadly induce NHR-49 activated genes, but NHR-49 is also involved in gene repression, likely through an NHR-49 and NHR-66 heterodimer (Pathare et al., 2012). Thus, I wished to test whether the point mutations also affect repression by NHR-49, using nhr-49(nr2041) and nhr-66(ok940) mutants as positive controls. I expected that the point mutations would not affect NHR-49 dependent repressed gene expressions, as they were isolated from the suppressor screen for the cold sensitivity; NHR-49 repressed genes are involved in sphingolipid breakdown, a process not known to affect cold sensitivity.

Consistent with published results (Pathare et al., 2012), NHR-49-repressed genes were strongly induced in nhr-49(nr2041) and nhr-66(ok940) null mutants (Figure 6). Strikingly, the nhr-49(et13) mutant paralleled this expression pattern, effectively presenting a null phenotype in regards to NHR-49 repression (Figure 6). This effect was specific to nhr-49(et13), as nhr-49(et7) and nhr-49(et8) largely failed to affect these genes (Figure 6). Notably, nhr-49(et13) upregulated lips-6 and tag-38 as much as both nhr-49(nr2041) null and nhr-66(ok940) null mutants, whereas Y65B4BR.1, oac-56 and W02B12.1 were not induced to the extent seen in the null mutants. This could have been the result of a few possibilities. First, nhr-49(et13) seems to affect the structure of NHR-49, destabilizing the LBD (data not shown), presumably affecting the binding to coregulators and/or promoter of certain genes through intramolecular communication. This could cause the binding to a different promoter regulatory element, showing a different induction between genes. Another possibility is tissue specificity. While nhr-49 is expressed in different tissues such as muscles, intestines, neuron and hypodermis (Ratnappan et al., 2014), Y65B4BR.1
and oac-56 might be partially expressed in these organs, thus leading to a partial induction compared to lips-6 and tag-38.

2.3.1 nhr-49(et13) and nhr-66(ok940) induce Plips-6::gfp expression in vivo

To validate the qPCR results, I generated a transcriptional GFP reporter to investigate promoter activity in nhr-49(et13), nhr-49(nr2041), and nhr-66(ok940) worms. I chose the lips-6 promoter because lips-6 was the most highly induced gene in these three strains, as judged by qPCR (Figure 6). Upon generating Plips-6::gfp transgenic worms, I noticed that Plips-6::gfp fluorescence in WT worms is much stronger in L1 and L2 larvae than in L3 and L4 larvae, with virtually no visible fluorescence at L4 stage (data not shown). To best study NHR-49 dependent repression of lips-6, I thus decided to assess GFP levels at the L4 stage with low expression in WT worms.

In support of my qPCR data, I found that Plips-6::gfp reporter activity was induced in nhr-49(et13) and nhr-66(ok940) worms; however, I note that induction varied substantially between individual worms, suggesting that for unknown reasons the promoter reporter is not uniformly activated by these mutations (Figure 7). Surprisingly, nhr-49(nr2041) displayed much weaker Plips-6::gfp induction than nhr-49(et13) and nhr-66(ok940) worms, somewhat contradictory to my qPCR data; further investigation is needed to address this issue.

Overall, my gene expression analysis using qPCR and in vivo reporter analysis suggests that all three nhr-49 point mutations broadly induce NHR-49 activated genes whereas only nhr-49(et13), but not the other two missense mutations, causes upregulation of NHR-49-repressed genes.
Figure 7. *Plips-6::gfp* is upregulated in *nhr-49(et13)* and *nhr-66(ok940)* mutants.
The left column shows DIC micrographs and the right column shows fluorescence micrographs of *Plips-6::gfp, Plips-6::gfp;nhr-49(et13), Plips-6::gfp;nhr-66(ok940)*, and *Plips-6::gfp, nhr-49(nr2041)* worms. Red fluorescence represents the *Pmyo-2::mCherry* pharyngeal injection marker.
2.4 The nhr-49 point mutations modify MDT-15 binding

Next, I attempted to identify the underlying mechanism that allows the three point mutations to induce NHR-49 activated genes. All three point mutations localize near the LBD of NHR-49, a domain essential for gene regulation due to its capacity to bind ligands, partner NHRs, and coregulators (Figure 8) (Pathare et al., 2012; Svensk et al., 2013; Taubert et al., 2006). The only known NHR-49 coregulator is MDT-15; a subunit of the Mediator complex that is required for the expression of many NHR-49 activated genes (Taubert et al., 2006). Because the point mutations all upregulate the expression of NHR-49 activated genes and because they are adjacent to the LBD, I hypothesized that they might cause an increase in MDT-15 binding.

To answer this question, I used the Yeast 2 Hybrid (Y2H) system. Specifically, I examined the protein-protein interaction between a Gal4-activation domain (AD)-MDT-15 prey and the Gal4-DBD-NHR-49-LBD baits (Figure 9A). Compared to WT NHR-49-LBD, NHR-49(et7)-LBD showed a modestly increased binding to MDT-15, NHR-49(et8)-LBD did not change binding, and NHR-49(et13)-LBD showed a decreased binding to MDT-15 (Figure 9A). Thus, although all point mutations upregulate the NHR-49 activated genes, only nhr-49(et8) showed a modest increase in MDT-15 binding. Furthermore, although nhr-49(et13) induced NHR-49 activated gene the highest, this mutation actually caused a decreased binding to MDT-15. Thus, the point mutations upregulate NHR-49 activated genes despite their varied binding to MDT-15.

![N-terminal Region, DBD, Hinge Region, LBD, C-terminal Region](image_url)

Figure 8. Point mutations are located within LBD of NHR-49 protein.
Yellow, green, blue arrow indicates nhr-49(et7), nhr-49(et8), and nhr-49(et13) respectively. Adapted from (Svensk et al., 2013).
Figure 9. \textit{nhr-49} point mutations modify the binding to MDT-15 and NHR-66. Bars represent percent average of relative binding strength to (A) MDT-15 and (B) NHR-66, as assessed by Y2H assays (n ≥ 6). Error bars represent SEM. * represents p<0.05, **** represents p<0.0001 (unpaired Student’s t-test).
2.4.1 *mdt-15* is required for the gene inductions in the *nhr-49(et13)* mutant

The above data show that NHR-49(et13) has a decreased binding to MDT-15, suggesting that *nhr-49(et13)* might not require *mdt-15* for gene upregulation. To test this possibility, I depleted *mdt-15* using RNA interference (RNAi) and measured NHR-49 dependent gene expression by qPCR. Upon *mdt-15* depletion, WT worms showed a downregulation of desaturases and β-oxidation gene expressions (Figure 10), as expected (Taubert et al., 2006). Similar effects were observed in *nhr-49(et13)* mutant grown on *mdt-15* RNAi (Figure 10), suggesting that *nhr-49(et13)* still requires *mdt-15* for the upregulation of desaturases and β-oxidation genes despite binding MDT-15 more weakly.

![Figure 10. *nhr-49(et13)* requires *mdt-15* for the regulation of *nhr-49* activated genes.](image)

Bars represent average mRNA fold changes of *nhr-49* dependent activated genes upon *mdt-15* depletion (compared to *ctrl* empty vector RNAi; n=4). Fold changes were normalized to *act-1*, *tba-1*, and *ubc-2*. Measurements were made at L4 stage. Errors bars show SEM. ** represents p<0.01 (unpaired Student’s t-test).

2.5 The *nhr-49* point mutations mildly modify NHR-66 binding

NHR-66 is another binding partner for NHR-49; in contrast to MDT-15, NHR-66 represses the genes also repressed by NHR-49 (Pathare 2012). Because *nhr-49(et13)* upregulates NHR-49-
repressed genes (Figure 6), I speculated that nhr-49(et13) might decrease the physical interaction between NHR-49 and NHR-66.

As seen for the interaction with MDT-15, the point mutations differentially altered binding to NHR-66 (Figure 9B). In contrast to WT NHR-49 LBD, NHR-49(et7) LBD and NHR-49(et13) LBD showed a modest increase in binding to NHR-66. However, although significant, the changes in protein-protein interactions of the NHR-49-LBD are relatively weak and are unlikely to fully explain the observed gene expression changes.

2.6 The nhr-49 point mutations do not affect NHR-49 protein levels or subcellular localization

Besides affecting coregulator binding, missense mutations might affect NHR-49 protein levels or subcellular localization, both factors that can affect NHR activity. Indeed, Ratnappan and colleagues reported that NHR-49 localizes to the nuclei as well as to the cytoplasm, and that its expression is altered in the long-lived mutant glp-1 (Ratnappan et al., 2014), suggesting that modulation of NHR-49 protein levels occurs at least in this context. To test whether the three missense mutations identified by Svensk et al. affect NHR-49 protein levels or localization, I used site-directed mutagenesis to introduce the point mutations into an existing translational NHR-49::GFP reporter (Ratnappan et al., 2014), and then expressed each variant NHR-49::GFP transgenically in WT worms. This approach allowed me to test whether the point mutations alter 1) protein levels, 2) nuclear localization, or 3) tissue-specific expression that conceivably could cause the phenotypes observed in Figure 4 and Figure 5. Unexpectedly, none of the point mutations caused any noticeable differences in protein levels or localization (Figure 11). This suggests that the point mutations act through different molecular mechanisms.
**Figure 11.*** nhr-49 point mutations do not affect protein levels and localization.** Micrographs show hypodermis (top panel) and intestinal nuclei of the *nhr-49::gfp, nhr-49(et7)::gfp, nhr-49(et8)::gfp,* and *nhr-49(et13)::gfp.*

**2.7 NHR-49-driven repression is not activated by NHR-49 dependent desaturases**

NHR-49 heterodimerizes with NHR-66, and both proteins are required for the repression of sphingolipid breakdown genes. This seems to suggest NHR-49 cooperates with NHR-66 for the repression of these genes (Pathare *et al.*, 2012). An alternate possibility is that the sphingolipid breakdown genes are induced in *nhr-49* and *nhr-66* mutants because NHR-49 activated genes, especially *fat-5* and/or *fat-7*, are downregulated, representing an indirect effect; if this were true, *fat-6* and *fat-7* depletion should induce NHR-49 repressed genes. I tested this hypothesis and, as expected, found that NHR-49 repressed genes were unaffected when *fat-6* and *fat-7* were depleted (Figure 12). Overall, the data indicates a modest change in NHR-49 repressed genes.
upon \textit{fat-6} and \textit{fat-7} depletion; this suggests that \textit{fat-6} and \textit{fat-7} are not major regulators of NHR-49 repressed genes.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure12.png}
\caption{Depleting \textit{nhr-49} dependent desaturases does not activate NHR-49 repressed genes.}
Bars represent average mRNA fold changes in \textit{nhr-49} dependent repressed genes upon \textit{fat-6} and \textit{fat-7} depletion (compared to \textit{ctrl} RNAi; n=3). I normalized fold changes to \textit{act-1}, \textit{tba-1}, and \textit{ubc-2}. Measurement was made at L4 stage. Errors bars show SEM. * represents p<0.05, ** represents p<0.01 (unpaired t-test).
\end{figure}

\subsection*{2.8 A candidate screen identifies putative corepressors for NHR-49 and/or NHR-66}

The above results show that \textit{nhr-49(et13)} upregulates NHR-49 repressed genes. This suggests that the \textit{nhr-49(et13)} mutation alters binding to an as yet unidentified NHR-49 corepressor. To identify such putative corepressors, I designed an RNAi screen using the \textit{Plips-6::gfp} reporter as a readout. I expected that loss of a putative coregulator due to RNAi would induce gene expression, akin to what was seen upon loss of NHR-49 and NHR-66. Initially, I tested several candidate corepressors, specifically three coregulators that bind both NHR-49 and NHR-66 in high-throughput Y2H assays (Reece-Hoyes \textit{et al.}, 2013): HDA-11, a histone deacetylase homologous to human HDAC-11; F53H1.4, a predicted subunit of the integrator complex, which is responsible for small nuclear RNA (snRNA) processing; K03B8.4, an uncharacterized protein.
that is homologous to the chromatin remodeling protein SWSN-2.2, SET-1, a predicted function with histone lysine N-methyltransferase and DCP-66, an orthologue of the NuRD (nucleosomal remodeling and deacetylase) element p66. I knocked down these proteins by RNAi and measured gene expression changes of NHR-49 repressed genes by qPCR. I found that depleting any of these three proteins did not induce NHR-49 repressed genes (Figure 13). Thus, I concluded that HDA-11, SET-1, K03B8.4, F53H4.1 and DCP-66 are unlikely to serve as critical NHR-49 corepressors.

Figure 13. Depletion of candidate corepressor does not induce NHR-49 repressed genes. Bars represent average mRNA fold changes in nhr-49 dependent activated (acs-2, fat-5) and repressed genes upon hda-11, set-1, K03B8.4, F53H4.1 and dcp-66 depletion (compared to ctrl RNAi; n=2).

Next, I investigated two other candidate corepressors because they have been reported to interact physically and/or functionally with C. elegans NHRs other than NHR-49. DIN-1 is the only known corepressor for the C. elegans NHR DAF-12 (Ludewig et al., 2004), whereas GEI-8 is the C. elegans homologue of the human NHR corepressor NCoR and binds to C. elegans NHR-
in vitro (Mikoláš et al., 2013). Using the Plips-6::gfp reporter as a readout, I tested whether RNAi against either protein induced fluorescence, as expected from a candidate corepressor. I detected GFP induction upon gei-8 depletion but not in din-1(RNAi) worms. However, the GFP induction appeared to arise from gei-8 RNAi induced larval arrest; as noted above, Plips-6::gfp reporter activity is much higher in L1 and L2 stage larvae, suggesting that the observed fluorescence is not related to a direct role of gei-8 in lips-6 repression but rather represents an indirect effect. I conclude that gei-8 is most likely a false positive and that neither protein serves as a bona fide corepressor for NHR-49 and/or NHR-66. Thus, I decided to assess the Plips-6::gfp signal only in L4 stage in the subsequent RNAi screen.

Because the candidate studies did not result in the identification of an NHR-49 corepressor, I next performed an RNAi screen to identify such factors. Using an RNAi sublibrary targeting 487 putative transcriptional coregulators (e.g. proteins containing chromatin binding domains, helicases, known transcriptional cofactors, RNA binding proteins, etc), I tried to identify a corepressor(s) for NHR-49 and/or NHR-66. Candidate RNAi clones were tested in 6-well format to enhance throughput; to optimize RNAi efficiency, effects on Plips-6::gfp was assessed in the F2 generation (Figure 14).

To date, after testing 487 candidates, I was able to score 292 RNAi clones (195 RNAi clones were excluded as they caused sterility or embryonic lethality, or due to contamination, fasting etc). I have identified nine candidate corepressors for NHR-49 and/or NHR-66 (Table 2). Many of these proteins are uncharacterized. Thus, further experiments are required to determine whether these genes represent bona fide corepressors of NHR-49 and/or NHR-66.
Figure 14. Illustration of the Corepressor RNAi Screen.
Each 6-well plate represents a different generation, P0, F1, and F2. P0 and F1s worms were removed after they laid eggs. F2s were scored at the L4 stage for phenotypes and gfp fluorescence in an upright dissection microscope. In this figure, clone #1 is a positive hit.
Table 2. List of candidate NHR-49 or NHR-66 corepressors.
The table lists *C. elegans* gene names, the known or predicted function of the protein in *C. elegans*, and the name and function of putative human homologues. The ‘?’ indicates a predicted role of the protein. Functions in *C. elegans* were taken from WormBase, whereas functions in human were taken from Uniprot.

<table>
<thead>
<tr>
<th>WormBase Gene ID</th>
<th>Function in <em>C. elegans</em></th>
<th>Function in Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBGene00000271</td>
<td>Molecular bridge between TBP associated factors that functions in TFIIIB?</td>
<td>TFIIIB: a regulator of tRNA transcription</td>
</tr>
<tr>
<td>brf-1 (B-Related Factor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene00009163</td>
<td>Endoribonuclease in microRNA processing?</td>
<td>DROSHA: involved in the initial step of miRNA biogenesis</td>
</tr>
<tr>
<td>drsh-1 (DROSHA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene00019692</td>
<td>Regulates spatial localization or alternative splicing?</td>
<td>FUBP-2: mRNA trafficking for translation and degrading unstable mRNA?</td>
</tr>
<tr>
<td>M01A10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene0000369</td>
<td>Uncharacterized</td>
<td>CNOT7: mRNA degradation, translational repression during translational initiation</td>
</tr>
<tr>
<td>ccf-1 (CCR associated Factor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene0003224</td>
<td>Required for normal germline and larval development, for anteroposterior patterning</td>
<td>EED: methylates Lys-9 and Lys-27 of histone H3, leading to target gene repression</td>
</tr>
<tr>
<td>mes-6 (Maternal Effect Sterile)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene00007534</td>
<td>Regulates spatial localization or alternative splicing?</td>
<td>Highly Similar to FUBP-1 which has a role in MYC expression</td>
</tr>
<tr>
<td>C12D8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene00000438</td>
<td>Required for specification of the AFD thermosensory neurons and for thermotactic behavior</td>
<td>LHX3: transcriptional activator, Involved in the interneuron and motor neuron development</td>
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<tr>
<td>ceh-14 (C.elegans homeobox)</td>
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</tr>
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<td>WBGene0001912</td>
<td>Component of a nucleosome required for DNA packaging?</td>
<td>H4: Main component of nucleosome, packing DNA into chromatin</td>
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<tr>
<td>his-38 (histone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBGene00021902</td>
<td>Necessary for adult lifespan, heat and hyperoxia response</td>
<td>NARFL: downregulates H1F1A expression level?</td>
</tr>
<tr>
<td>oxy-4 (oxygen sensitivity)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3: Discussion and Conclusions

3.1 The three nhr-49 point mutations have distinct properties

My study aimed to investigate whether three point mutations in NHR-49 affect its transcriptional output specificity. Notably, substitutions in the LBDs of two human NHRs, HNF4α and PPARγ, are known to affect their activity: point mutations in AF-2 residues of HNF4α reduce transactivation potential, whereas a point mutation in PPARγ reduces ligand binding (Aggelidou et al., 2004; Causevic et al., 1999; Iordanidou et al., 2005). These studies demonstrate that specific point mutations and residues control gene expression by affecting interactions with e.g. ligands, and provide a rationale for dissecting the phenotypes and mechanisms pertaining to the three nhr-49 missense mutations.

NHR-49 regulates several distinct biological processes (Van Gilst et al., 2005a, 2005b; Pathare et al., 2012). However, it was not known how NHR-49 achieves specificity, i.e. what molecular determinants allow it to regulate these processes separately. The three nhr-49 point mutations were isolated specifically in association with fatty acid desaturation. Thus, I studied these mutations to elucidate how nhr-49 specificity is determined as these mutations could represent specificity determinants. My results indicate that these substitutions broadly affect NHR-49 activated genes, and are not limited to inducing NHR-49 activated fatty acid desaturase genes, as might have been expected due to the origin of these mutations in a screen favoring activation of such enzymes (Svensk et al., 2013). However, I also found that only one of the three mutations was involved in gene repression, highlighting the affected amino acid as a unique specificity determinant for gene repression. I also attempted to elucidate how the point mutations affect the gene expression level using protein interaction and translational GFP reporter assays. I found that
all three point mutations appear to act distinctively from each other. However, future experiments will be needed to determine the molecular mechanism underlying the mode of action of point mutations affect the gene expression level.

3.1.1 The nhr-49(et7) and nhr-49(et8) mutations only affect activation by NHR-49

The effect of all three point mutations on NHR-49 activated genes was broad, i.e., it was not limited to desaturases as might have been expected. I found that nhr-49(et7) showed the lowest level of gene induction for most tested genes. MDT-15 binding and protein expression level remains largely unchanged compared to controls and/or did not provide an obvious explanation for activation, thus leaving open the mode of action of the point mutations. Previous studies showed that NHR-13 and NHR-80 heterodimerize with NHR-49 and are also required to induce fatty acid desaturase and β-oxidation genes (Pathare et al., 2012). It is therefore possible that any of the three point mutations may cause stronger binding to either NHR-13 or NHR-80, and this may cause gene upregulation. This could be addressed by performing assays that test direct interaction between proteins such as Y2H and co-immunoprecipitation (co-IP).

Like nhr-49(et7), the nhr-49(et8) mutation caused upregulation of NHR-49 activated genes, but, unlike nhr-49(et7), it also resulted in mildly increased binding to MDT-15. It is thus possible that the stronger binding of NHR-49(et8) to MDT-15 causes upregulation of NHR-49 activated genes. To validate this observation, I could perform Co-IP to strengthen the Y2H data. At the molecular levels, the S432F substitution of an aromatic group could strengthen MDT-15 binding. For example, Teng and colleagues showed that mutations of amino acids residing in the binding interface altered the binding energy (positive change in binding energy represents an increased binding affinity and/or stability in a protein complex) (Teng et al., 2010). To validate that such a
mechanisms is involved, I could mutate S432 to another aromatic amino acid and test whether this affects binding.

3.1.2 \textit{nhr-49(et13) is unique, playing a role in both NHR-49 dependent activation and repression}

\textit{nhr-49(et13) is particularly interesting as it affects both NHR-49 dependent activation and repression and is the only point mutation to do so. nhr-49(et13) thus possesses characteristics of both a gain- and loss-of-function allele. This was unexpected as nhr-49(et13) is considered a gain-of-function, not a null allele (Svensk \textit{et al.}, 2013). Because it activates NHR-49 dependent genes, I hypothesized that nhr-49(et13) might increase MDT-15 binding. However, NHR-49(et13) instead showed decreased binding to MDT-15. Nevertheless, \textit{mdt-15} was still required for gene activation in nhr-49(et13), suggesting that despite weaker binding MDT-15 remains a critical coregulator. It is important to note, however, that the binding strength of the coregulator is not always directly related to the gene expression levels. This means decreased binding does not have to be correlated with a decreased gene expression and \textit{vice versa}. In my study, decreased binding of MDT-15 to NHR-49(et13) did prevent the up-regulation of NHR-49 activated genes.

NHR-49 binds NHR-66, and both NHRs are required for NHR-49 dependent gene repression, suggesting that upregulation of repressed genes in nhr-49(et13) worms might arise from defective NHR-49:NHR-66 dimerization. NHR-49(et13) binding to NHR-66 was altered only modestly. This suggests that altered NHR-49:NHR-66 interaction unlikely to be the primary cause for the changes in gene expression observed here. Alternatively, because V411 is located within LBD, it could be involved in binding to a putative NHR-49 corepressor. The \textit{nhr-49(et13)}
mutation might cause a decreased binding to such a factor, thus leading to an upregulation of NHR-49 repressed genes.

One caveat of my gene expression study is that I only tested known nhr-49 targets; thus, I may have missed unknown nhr-49 dependent genes, which could have a differential effect in nhr-49 point mutant background, as well as novel genes that are only activated by the missense mutations but not by WT NHR-49. Gene expression profiling by RNA-seq would be a powerful way to address this knowledge gap.

### 3.2 NHR-49 and/or NHR-66 have several putative corepressors

Pathare and colleagues showed that sphingolipid breakdown genes were highly induced in nhr-49 and nhr-66 null mutants, and this led them to propose a model whereby NHR-49 and NHR-66 dimerize and repress these genes in cooperation (Pathare et al., 2012). Repression by NHRs typically requires transcriptional corepressors (Aranda and Pascual, 2001), but, to date, no NHR-49 and NHR-66 corepressors are known. Because knowing the identity of such a corepressor could lead to an increased understanding of the mechanism of the nhr-49(et13) mutation, I tried to identify NHR-49 and/or NHR-66 corepressors.

I used a reverse genetic approach to identify corepressors, screening for RNAi clones that cause the same phenotype as nhr-49 and nhr-66 mutation or RNAi (Figure 7, Figure 13). I limited my candidate list by only screening through genes predicted to act as potential transcriptional coregulators.

During my screen, I encountered some issues. Numerous coregulator RNAi clones caused embryonic lethality or sterility, which prevented me from scoring Plips-6::gfp activity. This
could be addressed by performing RNAi feeding method for a shorter time to reduce lethality or sterility, *i.e.* scoring in the F1 generation (I decided to perform two generations of RNAi to achieve strong RNAi effects).

I used the *lips-6::gfp* reporter as a readout for my screen. While useful, this wasn’t unproblematic: as noted, *lips-6::gfp* induction was far from uniform even in mutant backgrounds, and RNAi studies suffered from similar caveats. In future studies, I would ideally use a different reporter with a gene that is consistently and strongly induced by both *nhr-49* and *nhr-66* RNAi. This would enable me to validate the results I have found and also enable the discovery of additional corepressors. In addition, Y2H could be performed to see whether NHR-49 and NHR-66 physically interact with possible candidate corepressors. Specifically, NHR-49(et13) could be examined to test whether it has a weaker binding to corepressors to elucidate the cause of *nhr-49(et13)* upregulation in NHR-49 repressed genes.

### 3.3 Conclusions

The summary of my study is presented in Figure 15. From my study, I conclude:

- *nhr-49(et7)* and *nhr-49(et8)* affect only NHR-49 activated genes, and this effect is not limited to fatty acid desaturation genes.
- *nhr-49(et13)* has an effect in both NHR-49 dependent activation and repression.
- I identified several candidate NHR-49 and/or NHR-66 corepressors, the first time to identify such proteins.
Figure 15. Summary of the effects of nhr-49 point mutations on nhr-49 dependent gene activation and repression.

The figure provides an overview of the findings of my thesis. Green font indicates upregulation and red font indicates downregulation. nhr-49(et7) and nhr-49(et8) are important in NHR-49 activation only whereas nhr-49(et13) is required for both NHR-49 activation and repression. Several candidate corepressors have been found and further studies should functionally validate these genes.
Chapter 4: Materials and Methods

4.1 Sequence alignments

I used Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to align sequences of NHR-49 and its homologues. The NHR-49 sequence and the sequences for Caenorhabditis homologue species were downloaded from WormBase (WS252). Sequences for NHR-49 homologues in other species were downloaded from UniProt (http://www.uniprot.org/).

4.2 C. elegans strains

I used N2 Bristol (WT) (Brenner, 1974), nhr-49(nr2041) I, nhr-66(ok940) IV, and pnhr-49::nhr-49::gfp worms, as described (Ratnappan et al., 2014). I obtained nhr-49(et7) I, nhr-49(et8) I, and nhr-49(et13) I by backcrossing QC120 nhr-49(et7) I; paqr-2(tm3410) III, QC121 nhr-49(et8) I; paqr-2(tm3410) III, and QC126 nhr-49(et13) I; paqr-2(tm3410) III (23) (Svensk et al., 2013) to N2, i.e. removing the paqr-2 mutation. I generated pnhr-49::nhr-49(et7)::gfp, pnhr-49::nhr-49(et8)::gfp, pnhr-49::nhr-49(et13)::gfp strains by introducing point mutations into pnhr-49::nhr-49::gfp plasmid (Ratnappan et al., 2014) using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs E0554S). I used SP2879/80 for nhr-49(et7), SP2881/82 for nhr-49(et8), SP2883/84 for nhr-49(et13) to introduce point mutations. After obtaining the plasmids with the point mutations, I microinjected total of 10 µg of plasmids that contain point mutations of nhr-49(et7), nhr-49(et8) and nhr-49(et13), co-injection marker SPD 652 and empty vector SPD488.

I obtained the plips-6::gfp strain by amplifying a ~2kb lips-6 promoter using the primers SP3243/44 (Table 3). The PCR product was cloned to TOPO vector using Zero Blunt TOPO PCR cloning kit (#45-0245 Invitrogen). I used restriction enzymes XmaI (New England Biolabs) and PstI (New England Biolabs) to ensure 2kb region was present. After the reporter plasmid
was made, it was sequenced using the primers from Table 3. After generating the Plips-6::gfp strain, I crossed the reporter into the nhr-49(nr2041), nhr-49(et13), and nhr-66(ok940) strains to generate Plips-6::gfp;nhr-49(nr2041), Plips-6::gfp;nhr-66(ok940), and Plips-6::gfp;nhr-49(et13) strains. I used SP1764/65 and SP1773/74 to genotype Plips-6::gfp;nhr-49(nr2041) both outside the deletion and within the deletion region. I used SP2373/74 and SP2375/76 to genotype Plips-6::gfp;nhr-49(et13). I used SP2450/51 to genotype outside of the deletion of Plips-6::gfp;nhr-66(ok940) worms.
### Table 3. List of primers used for cloning and sequencing.

<table>
<thead>
<tr>
<th>SP #</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1764/65</td>
<td>ttccgccgaacagttcttcg</td>
<td>cgctatctgcatctgatcg</td>
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<td>SP1773/74</td>
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<td>cgctgagattttcatcc</td>
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<td>SP2375/76</td>
<td>tgaatccggtttttctgaa</td>
<td>atgaaaagctagagagagaattct</td>
</tr>
<tr>
<td>SP2450/51</td>
<td>ctgcagcaagtcgaggttcg</td>
<td>gtttgctctaccagcatacc</td>
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<td>SP2879/80</td>
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<td>cgtcgaatgatcattgatgc</td>
</tr>
<tr>
<td>SP2881/82</td>
<td>tgaatccggtttttctgaa</td>
<td>attttaegggtttttctgaaatcatt</td>
</tr>
</tbody>
</table>

### Table 4. List of plasmids used for injection.

<table>
<thead>
<tr>
<th>SP #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPD488</td>
<td>pPD95.77</td>
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<tr>
<td>SPD651</td>
<td>pCFJ90</td>
</tr>
<tr>
<td>SPD886</td>
<td>glmEx5 pnhr-49::nhr::GFP</td>
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<tr>
<td>SPD887</td>
<td>Pnhr-49::nhr-49(et7)::GFP</td>
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<tr>
<td>SPD888</td>
<td>Pnhr-49::nhr-49(et8)::GFP</td>
</tr>
<tr>
<td>SPD889</td>
<td>Pnhr-49::nhr-49(et13)::GFP</td>
</tr>
<tr>
<td>SPD892</td>
<td>Plips-6::GFP</td>
</tr>
</tbody>
</table>

### Table 5. List of strains used.

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC120</td>
<td>nhr-49(et7) I; paqr-2(tm3410) III</td>
</tr>
<tr>
<td>QC121</td>
<td>nhr-49(et8) I; paqr-2(tm3410) III</td>
</tr>
<tr>
<td>QC126</td>
<td>nhr-49(et13) I; paqr-2(tm3410) III</td>
</tr>
<tr>
<td>STE68</td>
<td>nhr-49(nr2041) I</td>
</tr>
<tr>
<td>STE69</td>
<td>nhr-66(ok940) IV</td>
</tr>
<tr>
<td>STE108</td>
<td>nhr-49(et7)</td>
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<tr>
<td>STE109</td>
<td>nhr-49(et8)</td>
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<tr>
<td>STE110</td>
<td>nhr-49(et13)</td>
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<td>steEx55(Plips-6::gfp)</td>
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<td>steEx55(Plips-6::gfp);nhr-49(ok940)</td>
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<tr>
<td>STE113</td>
<td>steEx55(Plips-6::gfp);nhr-66(et13)</td>
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<td>STE114</td>
<td>pnhr-49::nhr-49(et7)::gfp</td>
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<tr>
<td>STE115</td>
<td>pnhr-49::nhr-49(et8)::gfp</td>
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<tr>
<td>STE116</td>
<td>pnhr-49::nhr-49(et13)::gfp</td>
</tr>
<tr>
<td></td>
<td>steEx55(Plips-6::gfp);nhr-49(nr2041)</td>
</tr>
</tbody>
</table>
4.3 *C. elegans* growth conditions

I cultured *C. elegans* strains on nematode growth medium (NGM)-lite (0.2% NaCl, 0.4% tryptone, 0.3% KH₂PO₄, 0.05% K₂HPO₄) agar plates. Worms were fed with *Escherichia coli* strain OP50 except for RNAi, for which I used strain HT115 as food source.

To achieve developmental population synchronicity, gravid adult worms were treated with a sodium hypochlorite potassium hydroxide solution to isolate eggs and embryos, which, following extensive washing in M9, were allowed to hatch overnight on unseeded NGM-lite plates. The resulting synchronized L1 larva populations were placed on seeded NGM-lite plates and allowed to develop to the desired stage. The *nhr-49(nr2041)* mutation causes developmental delay; thus, I allowed it to grow for four extra hours compared to WT when studying the larval stage L4; this resulted in overall population developmental staging that closely resembled that of WT worms (data not shown). For RT-qPCR and microscopy experiments, worms were harvested after growing for 40-44 hours at 20°C after L1 larvae were placed on seeded plates.

4.4 *C. elegans* RNA isolation and qRT-PCR

For qPCR analysis, synchronized L4 larvae were harvested in M9 buffer after 40 hours (*nhr-49(nr2041)* was harvested after 44 hours due to slower growth rate) and washed at least two times with M9; then, worm pellets were flash frozen in an ethanol/dry ice bath. For RNA isolation, Trizol was added to frozen worm pellets, which were then sonicated (Fisher Scientific Sonic Dismembrator Model 500, 30 cycles, 0.1 second pulse, 0.5 second rest) to increase RNA yield. Following BCP extraction of the Trizol mix and precipitation of the purified nucleic acids by isopropanol, the RNA was then cleaned up using the RNAeasy Mini Kit (Cat No 74106 Qiagen) and DNAse treatment. cDNA was generated by using Superscript III reverse
transcriptase (Invitrogen Lot No. 1708235), random primers (Invitrogen Lot No: 1597109), 0.1M DTT (Invitrogen Lot No: 1405708), dNTPs (Fermentas #R0186), and RNaseOUT (Invitrogen Lot No. 1685480) on 2 µg of isolated RNA.

For qPCR in 96-well plate format, primer pairs (custom primers from IDT) were diluted with RNAse DNAse free water to a final concentration of 5 µM. Before using primer pairs, they were subjected to a rigorous primer test by performing qPCR reactions on serial dilutions of *C. elegans* cDNA. Primer sequences are listed in Table 6. To perform qPCR, each plate contained 1µl of primer, 1µl of cDNA diluted 10-fold, 5µl of Fast SYBR Green Master Mix (#4385612 Applied Biosystems) and 3µl of RNAse DNAse free water. All qPCR reactions were carried out in triplicate on an Applied Biosystems StepOne and StepOnePlus machine. Data were collected from at least three independent *C. elegans* growths and RNA isolations (i.e. biological repeats).
<table>
<thead>
<tr>
<th>SP #</th>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>tggcggaaaattcataaatcc</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>SP2671/72</td>
<td>clec-209</td>
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</table>

Note: SP2571-SP2578 sequences were from Pathare et al., 2012.
4.5 Yeast two Hybrid (Y2H) Assay Analysis

Plasmids encoding the LBDs of NHR-49(et7), NHR-49(et8), and NHR-49(et13) generated using the NEB Q5 sites directed mutagenesis kit using the published NHR-49-LBD plasmid as template (Taubert et al., 2006); the MDT-15 and NHR-66 plasmids have been described (Taubert 2006, Pathare 2012). All plasmids were sequenced to verify the presence of the point mutations and absence of other mutations.

Plasmid pairs were then transformed into yeast strain Y187 (Clontech) and liquid β-galactosidase assays to estimate interaction strength was performed as described (Taubert et al., 2006) using an Omegastar plate reader (BMG Labtech) to measure β-galactosidase and yeast growth (optical density OD550) for normalization. Each assay were performed at least three or more times using independent yeast cultures (biological repeats), and included at least six technical replicates.

Table 7. List of plasmids used for Y2H.

<table>
<thead>
<tr>
<th>Plasmid #</th>
<th>Description</th>
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<tbody>
<tr>
<td>SPD213</td>
<td>pGBKnewLeu2 NHR-49-LBD1</td>
</tr>
<tr>
<td>SPD257</td>
<td>pGADnew-MDT-15-ΔCT</td>
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<tr>
<td>SPD278</td>
<td>pGBKTK7 newMCS Leu2</td>
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<tr>
<td>SPD392</td>
<td>pPC86 NHR-66</td>
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<tr>
<td>SPD760</td>
<td>pGBKTK7newMCSLeu2-NHR-49 (et7) LBD</td>
</tr>
<tr>
<td>SPD762</td>
<td>pGBKTK7newMCSLeu2-NHR-49 (et13) LBD</td>
</tr>
<tr>
<td>SPD768</td>
<td>pGBKTK7newMCSLeu2-NHR-49 (et8) LBD</td>
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</tbody>
</table>

4.6 DIC and fluorescence microscopy

For microscopy, worms were placed onto 2% agarose pads containing NaN3, as described (Goh et al., 2014). A CoolSnap HQ camera (Photometrics) attached to a Zeiss Axioplan 2 compound microscope was used to acquire microscopy pictures. MetaMorph Imaging Software with Autoquant 3D digital deconvolution was used to obtain images.
4.7 Feeding RNAi

For feeding RNAi, I poured NGM-lite media containing 25 µg/mL carbenicillin, 1 mM IPTG, and 12.5 µg/mL tetracycline into the 6-well culture plates (Costar). Then, wells were seeded with the proper HT115 RNAi bacteria. The nhr-49, nhr-66, and fat-6 clones were taken from the Ahringer library 96-well format; nhr-49: plate 16, well G07, nhr-66: plate 104, well G04, fat-6: plate 113, well D11. Cofactor plasmids used for the NHR-49 and/or NHR-66 corepressor screen were from the Taubert lab cofactor RNAi sublibrary.

4.8 Statistical Analysis

For both qRT-PCR and Y2H results, I performed two-tailed t-tests on at least three independent biological repeats per experiment. I used GraphPad Prism 6 to create Y2H and qRT-PCR graphs and to perform statistical analysis.
Bibliography


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Sluder, A.E., Mathews, S.W., Hough, D., Yin, V.P., and Maina, C.V. (1999). The Nuclear Receptor Superfamily Has Undergone Extensive Proliferation and Diversification in Nematodes. Genome Res. 9, 103–120.


