“Nuclear hormone receptor regulation of microRNAs”

Dissertation

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von Axel Bethke
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Externer Betreuer: Prof. Dr. Adam Antebi  
Baylor College of Medicine  
Houston, Tx  
USA

1. Berichterstatter: Prof. Dr. Helmut Wieczorek

2. Berichterstatter: Prof. Dr. Achim Paululat
Abstract

The progression of multicellular organisms through different genetically defined developmental programs requires temporal control. Endocrine regulation of this temporal control is achieved by small molecules that originate from endocrine tissues and diffuse throughout the whole body of the animal to coordinate program execution by activating cell specific gene expression patterns. These programs then define cascades of successive, distinct developmental stages or the choice between alternative fates for the same stage.

A model for temporal control of development is found in the *C. elegans*, environmental cues signal through insulin and TGF-beta cascades to regulate the hormone synthesis of *daf-12*/nuclear hormone receptor (NHR) that then coordinates organism wide developmental timing and fate choice.

For cell intrinsic aspects of *C. elegans* temporal control of development, microRNAs play an important role for regulation of developmental progression but their connection to organism wide endocrine control is unknown. This work shows how the DAF-12/NHR directly activates let-7 family microRNAs during the L3 stage to actively repress translation of *hbl-1*, a mediator of L2 stage fates, to prevent L2 stage fates from reoccurring during L3 stage. The interaction of upstream transcription factors with the downstream cis-regulatory elements in promoters of the let-7 family microRNAs, are further analyzed in detail and identify potential DAF-12 co-regulators that might connect *daf-12* endocrine signaling also to later stage developmental control.

These observations are the first to integrate microRNAs into the currently established endocrine signaling cascades. In addition they reveal specific details about how organism wide upstream, endocrine signaling pathways connect to downstream cell intrinsic changes of gene expression and developmental progression throughout the organism. Through these observations, this work postulates a “molecular switch” to be active in driving stage transitions. This switch consists of a NHR that directly activates microRNAs to actively repress mediators of old stages while activating translation of genes mediating the new stage.
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Abbreviations

AMH  anti-Muellerian hormone
CFP / cfp  cyan fluorescent protein
CRM  cis-regulatory element
DA  dafachronic acid
Daf-c  dauer constitutive phenotype
Daf-d  dauer defective phenotype
DBD  DNA binding domain of a NR or NHR
DIC  differential interference contrast microscopy
DR  direct repeat
DTC  distal tip cell
EcR  ecdysone receptor
EMSA  electrophoretic mobility shift assay
ER  everted repeat
EtOH  ethanol
gf  gain-of-function mutation
GFP / gfp  green fluorescent protein
HEK  human embryonic kidney cells
20E  20-hydroxyecdysone
IR  inverted repeat
JH  juvenile hormone
L1/L2/L3/L4/Ad  larval stage 1 / larval stage 2 / larval stage 3 / larval stage 4 / Adult
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<tr>
<td>lf</td>
<td>loss-of-function mutation</td>
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<tr>
<td>Mig</td>
<td>migration phenotype of the DTC</td>
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<tr>
<td>miR</td>
<td>microRNA</td>
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<tr>
<td>na</td>
<td>not applicable</td>
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<tr>
<td>NHR</td>
<td>nuclear hormone receptor</td>
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<td>NR</td>
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<tr>
<td>kb</td>
<td>kilo base pair – length of a DNA fragment</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PPAR delta</td>
<td>peroxisomal proliferation factor delta</td>
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<td>super shift</td>
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<td>VPC</td>
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<td>YFP / yfp</td>
<td>yellow fluorescent protein</td>
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1. Introduction

Many examples have been described of how endocrine regulation controls developmental progression. However, it is still not well understood how organism-wide endocrine signals are converted into cell-intrinsic responses and changes of developmental programs. The aim of this dissertation is to investigate hormonal control of intracellular developmental programs and its relation to environmental influences. In this introduction, I will explain the background necessary to understand how nuclear hormone receptors convert these endocrine hormone signals into changes in target gene expression-- which constitute developmental plasticity. I proceed by applying this knowledge to several model systems for development and hormonal control, before I focus on one specific model organism, *Caenorhabditis elegans*. In *C. elegans*, the nuclear hormone receptor DAF-12 together with “dafachronic acid” hormones control developmental progression through different larval stages as well as the choice between two alternative larvae of the same stages. I connect this developmental control to environmental conditions by reviewing how synthesis of dafachronic acids is regulated by several conserved upstream signaling cascades on an organism wide level. Next I examine the developmental stages at the cellular level and explain how a group of genes, called heterochronic genes, regulates timely execution of cell specific developmental programs. I will show that microRNAs, a newly discovered class of regulatory genes, have important functions as heterochronic genes. This will lead to the question of what mechanisms connect the upstream hormonal signals to the downstream cell-specific developmental programs.

1.1 Nuclear Hormone Receptors

In order to understand hormone activity, it is necessary to understand what nuclear receptors are and how they react to their ligands. Nuclear receptors (NRs) are transcription factors (TFs) that bind to lipophilic ligands such as steroids, bile acids or fatty acids to regulate gene expression. The term ”nuclear hormone receptor” more specifically refers to NRs that bind steroid ligands, like the estrogen or the glucocorticoid receptors. The protein structure of NRs contains a DNA binding domain (DBD) near the N-terminus and a LBD at the C-terminus. The DBD of a NR recognizes promoters of target genes by binding to a short DNA sequence, called the “response element” (RE), which typically consists of six nucleotides. NRs can homodimerize
with the same NRs, (e.g. RXR/RXR) or more commonly, heterodimerize with other NRs (e.g. RXR/VDR or RXR/RAR) (Mangelsdorf and Evans, 1995). These NR-dimers can bind repeats that are direct, as well as inverted or everted palindromes (DR, IR, ER). In addition, the choice of dimerization partners can influence the RE that the complex binds to (Mader et al., 1993), but different arrangements of the REs relative to each other influence the nuclear receptor dimer that can bind the promoter, and with it the promoter activation state (Kurokawa et al., 1994). The LBD of NRs consists of 12-alpha helices and ligand binding induces conformation changes that trigger activation (Bourguet et al., 1995; Tanenbaum et al., 1998).

1.1.1 Type I and type II nuclear receptors

Nuclear receptors are grouped into two classes, type I and type II (Mangelsdorf and Evans 1995). Type I NRs bind to steroids and activate transcription in the presence of a ligand while being inactive in the absence of ligands, such as estrogen and glucocorticoid receptors. Type II NR activate in presence, but repress in the absence of a ligand. Examples of these include the thyroid receptor or vitamin D receptor. The regulation of type II NRs is achieved by the NR interacting in a ligand dependent way with co-regulators that enhance transcription of the NR target genes. An example of a co-activator that activates transcription is SRC-1 (Onate et al., 1995) and an example for a co-repressor is N-Co-R (Zamir et al., 1996) that causes transcriptional repression. But how do ligands regulate the interaction of nuclear hormone receptors and their co-regulators?

1.1.2 Nuclear receptor co-regulators

As stated previously, the results of ligand binding to NRs are dependent on co-regulators that interact with multiple areas of the DNA ligand binding domain. These include both co-activator and co-repressor proteins. For example, during ligand binding, the highly mobile helix 12 of the LBD re-arranges to make contact with helix 3, forming a "charge clamp" and exposing charged amino acid residues that interact with LXXLL-motif of a protein structure called "NR box" that is found in co-activator proteins (Savkur and Burris, 2004). The details of this mode of interaction were first described for estrogen (ER) (Shiau et al., 1998) and thyroid receptor (TR) (Darimont et al., 1998) binding to co-activator GRIP1.

Co-activator complexes enhance transcription through a variety of mechanisms including histone modification, chromatin remodeling, and bridging with components of basic transcriptional machinery. Co-activators induce transcription of a target gene after recruitment to
the corresponding promoter by interaction with a bound NHR. Conversely, co-repressor proteins, such as NCoR and SMART, contain LXX I/H IXX I/L motifs called "CoRNR box" that LBDs of NRs interact with. (Rosenfeld et al., 2006). Usually, NCoR and SMART are recruited to type II NRs like TR, RARs in the absence of a ligand and interact with other proteins like SHARP that recruits enzymes such as histone deacetylases HDAC1 and HDAC2 to decrease transcription, (Shi et al., 2001). Other co-repressors like LCor, RIP140 or REA are recruited in the presence of an antagonist molecule in the LBD (Rosenfeld et al., 2006) to repress transcriptional activation. These factors can be further regulated by posttranscriptional modifications-- such as phosphorylation, acetylation, SUMOylation, or ubiquitinylation-- (Rosenfeld et al., 2006) all of which serve to enhance or decrease transcriptional activation of the TFs. One example is increasing the protein turnover-rate of the TF.

1.1.3 Hormonal signaling in development

Hormone signaling during development is complex and multifaceted. The effects of hormone signaling on nuclear hormone receptors and their transcriptional targets are most obvious during metazoan development. Metazoans, which include *C. elegans*, undergo extensive tissue remodeling during maturation from juvenile to reproductive adult. Most of these processes are regulated by NR ligands, such as steroids, thyroids, or retinoic acids, which promote two different kinds of maturation processes, metamorphosis or non-metamorphic transitions (Paris and Laudet, 2008).

Metamorphosis is a drastic remodeling of organs, which often goes along with a change in ecosystem and food source. One example includes frogs, which are land living insectivores, but develop through metamorphoses into a submerged herbivorous tadpole.

Normal development and maturation, in addition to metamorphosis, is subject to endocrine control. Endocrine signals that regulate metamorphosis in insects are ecdysteroids and juvenile hormones, while thyroid hormones drive amphibian metamorphosis. An example of a less defined metamorphic development is mammalian sexual differentiation and maturation driven by steroids like estradiol or testosterone (Gilbert, 2003). The following paragraphs review the hormonal control of development in insects, amphibians and mammals.

1.1.3.1 Control of developmental progression in insects

Insects that have a larvae stage that is very distinct from the adult stage, also called holometabolous insects, transition between larval and adult stages, which involve
metamorphoses and the formation of an immobile pupae, of which the differentiated adult emerges. Hemimetabolous insects develop without a specific metamorphosis stage through a succession of molts from a small larvae into the larger, sexually reproducing adult with similar morphology (Gilbert, 2003).

The general regulation of insect molting and metamorphosis differs between species, but follows the general mechanism: Neurosecretory cells in the brain release a peptide hormone called prothoracicotropic hormone (PTTH) that acts on the prothoracic gland which produces ecdysone. Ecdysone is modified in peripheral tissue into the biologically active 20-hydroxyecdysone (20E) which binds to a heterodimer of the ecdysone receptor (EcR) and ultraspirical, an RXR homolog. (Thomas et al., 1993; Yao et al., 1993). Larval molts are accompanied by a pulse of juvenile hormone (JH) excreted from an endocrine organ known as corpora allata. Inhibition of this JH pulse during the ecdysone pulse leads to pupae formation and induction of metamorphosis (Adam et al., 2003). The 20E diffuses throughout the whole organism, but tissues react very differently to this single signal. For example, imaginal cells that give rise to adult organs are stimulated to grow, whereas larval tissues die. One reason for this diverse response is thought to be the presence of different EcR isoforms in different tissues. These isoforms share the same DBD and LBD, but differ in their N-terminus (Talbot et al., 1993; Truman et al., 1994).

1.1.3.2 Control of developmental progression in chordates

In contrast to insect steroid hormones, thyroid hormones are responsible for the control of development and metamorphosis in amphibians. Thyroid hormones are found only in chordates and are excreted from the thyroid glands (Brown and Cai, 2007) where they are derived from tyrosine (unlike steroid hormones, which are derived from cholesterol). They act on the thyroid receptor to promote metamorphosis from tadpole to frog which involves extensive tissue remodeling, such as limb growth and tail resorption. The most common model for metamorphosis in chordates is amphibian metamorphosis. *Xenopus laevis* is the currently favored model organism: embryogenesis takes 1 week, tadpole stage 6 weeks or more, depending on environmental conditions, metamorphosis takes 8 days, and the adult stage is approximately 6 months (Brown and Cai, 2007). The major endogenous thyroid hormone, called thyroxine, was initially extracted from thyroid glands, and subsequently chemically synthesized (Harington, 1928). This compound was able to induce precocious metamorphosis while removal
of the thyroid gland of tadpoles inhibits it (Allen, 1925). Ectopic expression of a transgene coding for a dominant negative TR suppresses thyroid hormone induced gill and tail resorption, gut remodeling, limb outgrowth and DNA replication in several tissues. This does so by assembling co-repressor complexes to thyroid hormone and TR regulated genes to inhibit target gene transcription (Buchholz et al., 2003; Hsia et al., 2003).

1.1.3.3 Control of developmental progression in mammals

In contrast to metamorphotic development in insects and amphibians, sex determination and maturation in mammals is determined by steroid hormones. During development the gonadal rudiment passes through a bipotential stage, 4 to 7 weeks after fertilization, where it can form gonads of one of either sexes. In absence of a Y chromosome, the gonadal primordium develops into ovaries that produce estrogens, steroid hormones that promote female secondary sex characteristics and enable the “Muellerian duct” to develop into parts of the female sex organs, like uterus and oviduct. In presence of a Y chromosome, testis are formed that secrete two major hormones: “anti-Muellerian hormone” (AMH), a TGF-beta ligand that leads to destruction of the Muellerian duct, and testosterone that promotes formation of male secondary sex characteristics like penis, male duct system and scrotum (Couse et al., 1999; Couse and Korach, 2001; Fisher et al., 1998). This process is initiated by wnt4/Wnt gene (Vainio et al., 1999) activating $\text{dax-1}/\text{nhr}$ (Muscatelli et al., 1994; Zanaria et al., 1994) that competes with Y-chromosomal $\text{sry}/\text{tf}$ and autosomal $\text{sox9}/\text{tf}$ (Nachtigal et al., 1998; Swain et al., 1998) to prevent $\text{sry}/\text{tf}$ and $\text{sox9}/\text{tf}$ dependent formation of AMH.

Production of estrogen or testosterone during puberty drives the maturation of sexual organs as well as other maturation processes, e.g. termination of bone growth. The growth spurt of bones during early puberty is caused by growth hormones activating the epiphyseal growth plates of the long bones to produce insulin-like growth factor-I (IGF-I), which together with the growth hormone increases bone growth by activating chondrocyte proliferation of the proliferative zone of the growing bone (Gilbert, 2003). The increased dose of steroid hormones induces a progression in development of the epiphyseal growth plates into mature chondrocytes that stop producing insulin. The chondrocytes further differentiate into hypertrophic chondrocytes and at this point the growth spurt is terminated through the steroid hormone induced differentiation.
The above are examples of hormonal control of development. However, to study the mechanisms of their action, it often is beneficial to use more rapidly developing organisms than mammals. A useful model organism for this research turned out to be the nematode *Caenorhabditis elegans*.

### 1.2 Nematodes

Nematodes live in many different ecosystems including marine, soil and plant environments or in animals as parasites that pass through one or more different hosts during their life cycle (Anderson, 1984). They are often referred to as roundworms, have a thread like shape, their mouth opening at the front tip is followed by a pharynx that continues into the gut that opens to the exterior near the rear end of the worm. They have a complex nervous system and excretory-secretory systems (Lee, 2002).

Most nematode species develop through four successive larval stages (L1-L4), separated by molts, into a reproducing adult, and can exit the direct development, usually after the second larval stage to form an alternative, arrested larva, that stops in development and is protected by a strong cuticle that shields it from environmental stresses. The formation of this arrested larva is a response to environmental signals. In parasitic nematodes, the arrested larvae is often the infective form and often occurs during the change from a non-infective stage or an intermediate host to the final host (Anderson, 1984). The endocrine regulation of this choice between developmental progression or arrested larva formation is best studied in the model organism *Caenorhabditis elegans* in the case of the dauer diapause (Motola et al., 2006).

### 1.3 Caenorhabditis elegans

*C. elegans* is a soil living nematode with a rapid generation time of 3 days in which a single adult can produce >300 offspring (Riddle et al., 1997). Adults consist of less then 1000 cells but still have defined organ systems as for example the nervous system with more then 300 cell. All cells are transparent and follow an invariable pattern of cell divisions during development, called the cell lineage. All these characteristics are encoded in a small, diploid genome with 100 Mega base pairs. *C. elegans* reproduces mostly by parthenogenesis, but 1 in 1000 worms is male, providing the researcher with the benefits of both sexual recombination as well as inbreeding by self-fertilization. Very successful were genetic screens using mutagenic reagents like e.g. ethyl methane sulfonate (EMS), followed by screening for worms that showed aberrations of the
normal developmental pattern resulting from a single genomic lesion. Classic and modern genetic procedures can be applied to find then the molecular identity of the gene behind the affected function (Hodgkin, 2007). This process lead to major scientific advances in understanding the nematode biology and along the homolog functions in higher animals and humans. This success is reflected in the Nobel Prices that were awarded to scientists working on *C. elegans* in the years 2002, 2006 and 2008.

**Figure 1: C. elegans life cycle from hatching till adulthood**
Development progresses from left to right and arrows symbolize hatching or molts between stages. Worm images are to scale. The hour numbers indicates the hours post-hatching at which the molt to the next stage occurs. Unfavorable environments can cause developmental arrest and dauer larvae formation (L3d stage) that lasts until environmental conditions improve which induces developmental progression and molting into the L4 stage.

**1.3.1 Life cycle**
One of the many processes that *C. elegans* acts as a model for is the regulation of developmental progression – the focus of this dissertation. Previous research led to the discovery of a complex network of signaling cascades and genes involved. I will discuss several aspects of these cascades, and start out with the developmental timeline during wild type development and normal conditions.
Under favorable growth conditions, *C. elegans* develops rapidly from an egg through four different larval stages into an adult (Figure 1). At 25°C, this process takes 48h. Most cell division and organogenesis occurs during embryogenesis. After larvae hatch from the eggs, a subset of tissues undergo further division, differentiation and growth, mostly oriented towards reproduction. The transition from one larval stage to the next is marked by a molt, in which the old cuticle is shed, and the new cuticle formed underneath by the hypodermis. At the transition to adult, hypodermal cells exit the cell cycle and terminally differentiate causing the molt cycle to cease. Adults lay fertilized eggs during a three day period, and continue to live for a maximum of 17 more days while the body morphology progressively deteriorates (Girard et al., 2007; Herndon et al., 2002). During the entirety of development, *C. elegans* cell lineages follow an invariant division pattern (Sulston and Horvitz, 1977; Sulston et al., 1983). This invariant cell division pattern allowed for example the discovery of mutants that lead to cell lineage modifications (called *lin*) and helped elucidate their underlying regulatory mechanisms.

1.3.2 Dauer formation

In contrast to the rapid life cycle described above, *C. elegans* development also includes the option to exit developmental progression and molt into an arrested larval stage, called “dauer,” which occurs during unfavorable conditions such as temperature extremes, starvation or in the presence of a pheromone that induces dauer formation called “dauer pheromone”. This dauer pheromone is an indicator of high population density and is excreted during the L1-L2 stages. In its presence, the *C. elegans* will molt into an alternative third larval stage, called the dauer larvae (L3d). Dauer larvae are arrested in development and are stress resistant and long lived. They can survive for up to six months, while normal developmental progression leads to a maximum life span of approximately two to three weeks (Cassada and Russell, 1975). When the dauer larva senses improved environmental conditions, it resumes developmental progression and within 24h molts into an L4 stage worm that then continues with normal development and reproduction.

1.3.3 How is dauer formation regulated?

Several forward genetics mutant screens have been performed in order to find out about the mechanisms regulating dauer formation. Those screens revealed several classes of genes that when mutated affect “dauer formation”. Also known as "daf"-genes, the members of this gene class are numbered according to chronological order of discovery. Daf-mutants fall into two classes: dauer defective mutants (Daf-d) fail to form dauer larvae, while dauer constitutive
mutants (Daf-c) constitutively form dauer larvae, independent of environmental conditions (Riddle et al., 1981). Identified genes were grouped by epistasis and phenotypical analysis into different classes that correspond to separate signaling reviewed by Fielenbach (Fielenbach and Antebi, 2008).

The most upstream events influencing the dauer formation decision are environmental factors like temperature, food availability and dauer pheromone, which are detected by sensory neurons. During favorable environmental conditions that promise successful reproduction, these neurons express DAF-7/TGF-beta ligand (Schackwitz et al., 1996) and DAF-28/insulin peptide (Zhang et al., 2003), which activate TGF-beta and insulin signaling cascades, respectively, to prevent dauer formation. Both cascades converge onto hormone biosynthetic enzymes regulating the production of “dafachronic acids” - the bile acid-like steroid ligands for the nuclear hormone receptor DAF-12 (Motola et al., 2006). Liganded DAF-12 then promotes developmental progression. Conversely, under unfavorable environmental conditions or mutations that reduce TGF-beta or insulin signaling dafachronic acid production is shut down and the unliganded DAF-12 causes dauer larvae formation and developmental arrest. These two cascades represent the connection of environmental conditions to endocrine regulation of development, and the mechanism of action is known in great detail for both TGF-beta and insulin cascades.

1.3.4 TGF-beta signaling in C. elegans

DAF-7/TGF-beta ligand is expressed in the ASI sensory neuron only during favorable conditions. It promotes developmental progression and the bypass of dauer arrest (Savage-Dunn, 2005). Repression of daf-7/TGF-beta ligand expression during unfavorable conditions, genetic deletion, or ablation of the expressing neuron lead to dauer arrest (Bargmann and Horvitz, 1991). DAF-7/TGF-beta ligand activates TGF-beta receptors DAF-1 and DAF-4 that phosphorylate smad proteins DAF-8 and DAF-14 causing their nuclear import. Transcriptional activity of DAF-8/smad and daf-14/smad during developmental progression is antagonized by daf-3/co-smad (Patterson et al., 1997) and daf-5/sno-ski (da Graca et al., 2004; Tewari et al., 2004). Deletion of daf-1/type I receptor, daf-4/type II receptor, daf-8/SMAD, daf-14/smad inhibit TGF-beta signaling and cause the Daf-c phenotype, while deletions of daf-3/co-smad are daf-5/sno-ski have a Daf-d phenotype (Inoue and Thomas, 2000; Larsen et al., 1995).
1.3.5 Insulin-like signaling in *C. elegans*

The Daf-28/insulin ligand is one of many insulin-like proteins in the *C. elegans* genome and is only expressed in the ASI and ASJ sensory neurons during favorable conditions (Li et al., 2003). It binds to DAF-2/insulin receptor in target tissues, which then activates AGE-1/phosphoinositol-3-kinase and leads to the production of Phosphatidylinositol 3-phosphate (PIP3). The DAF-2/insulin receptor protein of *C. elegans* is 35% and 34% identical to the human insulin receptor and the human insulin-like growth factor-I receptor, respectively (Kimura et al., 1997). This equidistant relation results in this signaling cascade being most accurately referred to as “insulin/IGF signaling” which is to be kept in mind when, for simplicity, daf-2 signaling will in this dissertation be referred to simply as “insulin signaling”.

PIP3, together with kinases PDK-1, AKT-1, AKT-2 and SGK-1, phosphorylate DAF-16/FOXO and cause it to be retained in the cytoplasm (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). Therefore, decreased insulin signaling leads to the de-phosphorylation and subsequent nuclear localization of DAF-16/FOXO where it activates its transcriptional target genes (Paradis and Ruvkun, 1998). Hypomorphic alleles, *e1370* and *e1368*, of the daf-2/insulin receptor have a temperature sensitive Daf-c phenotype. While growth at 25°C causes constitutive dauer formation, growth at the permissive temperature of 15°C does not cause dauer formation—instead development progresses with an increased resistance against heat stress, oxidative stress, UV, hypoxia and pathogen infection. Unchallenged *daf-2(e13768)* or *daf-2(e1370)* mutant worms show a two-fold extended lifespan compared to wild type (Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995). Enhanced stress resistance and increased life span due to decreased insulin signaling mutations were shown to be conserved across taxa (Kenyon, 2005). Loss of DAF-16/FOXO transcriptional activity suppresses these phenotypes, as well as dauer formation, identifying DAF-16/FOXO as the main downstream factor for insulin signaling (Kenyon et al., 1993).

1.3.6 Steroidal ligands for DAF-12/NHR

These two upstream signaling cascades intersect with endocrine regulation of development via their control over a steroid hormone synthetic pathway that produces bile acid-like steroids, called “dafachronic acids”, which bind to and activate the nuclear hormone receptor DAF-12. DAF-9/CYP p450, the most downstream enzyme of this pathway, is expressed in two neurons
called XXX. In addition, DAF-9 is expressed in the hypodermis in a manner dependent on environmental conditions and insulin and TGF-beta inputs (Gerisch et al., 2001; Jia et al., 2002). Two DAF-12 ligands were identified, Δ4-dafachronic acid and Δ7-dafachronic acid, which differ by the position of a double bound (Motola et al., 2006) (Figure 2) and their potency in activating DAF-12 dependent transcription (Figure 41 A and following page: Figure 42 D). The more potent Δ7-dafachronic acid was chemically synthesized later (Giroux and Corey, 2007); therefore, experiments in this study were initiated with Δ4-dafachronic acid. Structurally related compounds were also tested for in vitro and in vivo activation of DAF-12, but were found to be not as potent as the previously identified (Figure 41) (Giroux et al., 2008).

![Image: Structures of dafachronic acids](Motola et al., 2006)

**Figure 2: Structures of dafachronic acids**

(3-Keto-4-cholestenolic acid) (3-Keto-7,5α-cholestenolic acid)

### 1.3.7 Dauer signaling converges on a nuclear hormone receptor

After discussing the DAF-12 ligand and its regulation, I proceed to compare the nuclear hormone receptor DAF-12 itself to the paradigms of nuclear receptor signaling. DAF-12 is a class-II-like nuclear receptor with a predicted 12 helix ligand binding domain homologous to known steroid receptors (Antebi et al., 1998; Antebi et al., 2000). DAF-12 regulates dauer formation, developmental progression, fat metabolism and life span. For dauer formation, null alleles of *daf-12* are Daf-d which suppress all other known Daf-c loci as well as natural dauer formation; it is therefore considered the most downstream factor of dauer signaling. Null alleles for *daf-9* constitutively form dauers, suggesting that the unliganded receptor promotes the dauer state. Accordingly, this Daf-c allele class is suppressed by *daf-12* null alleles as well as null alleles of the nuclear receptor co-repressor *din-1/sharp*, identified as a *daf-12* interacting factor (Ludewig et al., 2004). This suppression supports the hypothesis that a complex of DAF-12 and DIN-1 works together to repress transcriptional targets, in the absence of DAF-9 biosynthetic products. *Daf-9;din-1* double null mutants allow bypassing dauer arrest even in the presence of unliganded DAF-12. Thus, hormone availability regulates the state of the *daf-12-dauer-switch*
(Gerisch and Antebi, 2004; Mak and Ruvkun, 2004), with the liganded receptor promoting reproductive development and the unliganded receptor promoting dauer formation.

### 1.3.8 Dauer formation overview

Taken altogether, the data suggest the following model for environmental control of developmental progression or arrest.

In favorable environments, sensing neurons ASI and ASJ express ligands DAF-7/TGF-beta ligand and DAF-28/insulin ligand to activate TGF-beta and insulin cascades. These cascades, through their downstream effectors, regulate expression of DAF-9 in the endocrine XXX cells. The XXX cells themselves regulate dafachronic acid production in the hypodermis via daf-12, which modulates hypodermal DAF-9 expression in a feed forward circuit. This hypodermal DAF-9 expression generates the dafachronic acid dose required to overcome dauer formation induced by unliganded DAF-12 throughout each cell of the whole organism. In unfavorable environments, decreased DAF-7/TGF-beta ligand and DAF-28/insulin ligand expression in the sensory neurons decreases the activity of the involved cascades, ultimately leading to DAF-12 mediated repression of its transcriptional targets to cause dauer formation (Figure 3).

**Figure 3: Daf-9, daf-12 and din-1 downstream of TGF-beta / insulin signaling**

Sensing of favorable environmental condition increases TGF-beta and insulin signaling, which then activate DAF-9/CYP p450 and dafachronic acid synthesis. Daf-12 binds Dafachronic acid and activates target genes to promote developmental progression. Sensing of an unfavorable environment leads to loss of TGF-beta and insulin signaling, resulting in repression of DAF-9/CYPp450 activity and dafachronic acid production. Unliganded DAF-12 represses its transcriptional targets to cause dauer formation.
1.3.9 Conservation of the *C. elegans* dauer signaling cascade

Evidently, this model of a steroid ligand operated switch that executes the decision between developmental progression and arrest is conserved in other nematodes as well, including free-living *Pristionchus pacificus* and parasitic *Strongyloides papillosus* (Ogawa et al., 2009). In these species, *C. elegans* dafachronic acid hormones are functional to inhibit formation of the arrested and infective larval stage, respectively. However, this conservation seems to be less true for the upstream signaling cascades, such as TGF-beta and insulin signaling.

1.3.10 Heterochronic regulation

In the sections above I described how signaling cascades connect environmental conditions to endocrine regulation of developmental progression and fate choice on a global, whole-animal scale. In the sections below, I will introduce findings on genes that regulate the timing of developmental progression at the cellular level. When these genes are mutated they affect the temporal regulation of development in certain tissues. This class of genes is called “heterochronic genes”. Heterochrony was first used to describe differences in developmental timing between two species. An example for this is brain development in humans and chimpanzees that starts at similar developmental timing, but ends years earlier in chimpanzees compared to human brain development (Mitteroecker et al., 2004). In *C elegans*, mutations of heterochronic genes affect the timing of developmental programs compared to wild type background. Many mutations are known to affect the development of different tissues but for simplification the focus will be on two specific tissues: the gonad and the hypodermis. Many aspects of temporal control over developmental progression became obvious by looking at the mutations of different genes, the consequences of these mutations for developmental timing, and the epistasis of these genes to each other.

1.3.10.1 Tissues with heterochronic phenotypes

*C. elegans* became an important model organism for research on heterochronic regulation due to its invariable patterns of cell divisions during development. Each larval stage is defined by stage specific developmental programs and each of these programs is reflected in its own cell specific divisions and differentiations, all of which can easily be observed by light microscopy (Sulston and Horvitz, 1977; Sulston et al., 1983). Genes that regulate the time point in development at which a specific program is executed are called "heterochronic" genes. Mutations of these genes cause stage specific programs to be expressed either earlier or later than normal
In particular, precocious mutants cause a program of an early stage to be skipped and the programs of the succeeding stage to be executed instead. Conversely, retarded mutants repeat stage-specific programs one or more times, and delay the execution of the succeeding program. The main characteristic of these mutations is that they do not change the nature of a stage specific program but only the timing of its occurrence.

A tissue with obvious heterochronic phenotypes is the hypodermis. Hypodermal seam cells follow an invariant and asymmetric stem cell like division pattern with stage specific variations that have been used to track developmental progression (Figure 4). During this stem cell like division pattern, one daughter cell maintains stem cell character and continues to divide, while the other daughter cell exits the cell cycle and fuses to the hypodermal syncytium. Specifically during the L2 program, the seam cells first undergo a proliferative division directly followed by the stem cell like division pattern – acting as an easily identifiable developmental program executed during this stage only. Repetition or loss of this hypodermal L2 program leads to changes in overall seam cell numbers during later stages. The L4 to adult transition also has unique character, leading to exit from the cell division cycle, fusion to the hypodermis, and terminal differentiation (Figure 4).
form cuticle structures called "alae", symbolized as green lines. Images taken from (Sulston and Horvitz, 1977)

A second tissue with heterochronic phenotypes is the gonad. During L2 and early L3 stages, the two arms of the gonad grow out from the mid body towards head and tail along the ventral side of the body, led by migratory distal tip cells (Hedgecock et al., 1987; Kimble and Hirsh, 1979). During mid L3, the distal tip cells change direction and migrate from ventral to dorsal sides, where they then turn back towards mid body, and stop by L4.

![Figure 5: Distal tip cell migration in hermaphrodites](image)

Cylindrical projection of distal tip cell migration on the body wall of the animal, opened at the the dorsal midline. Dorsal is up and down, ventral is central. "S3" refers to L3 stage programs, "S4" to L4 stage programs, and "SA" to adult stage programs. The arrow symbolizes the migration path of the gonad, away from mid body along the ventral side during S3, turning dorsal during S4 program and reflexing back towards midbody along the dorsal side. Figure taken from (Antebi et al., 1998).

Heterochronic genes belong to diverse functional categories and include transcription factors, translation factors, chromatin factors, posttranslational regulators and microRNAs (Moss 2007). MicroRNAs represent a recently discovered class of regulatory genes, that is estimated to regulate 30% of the vertebrate genome (Lewis et al., 2005).

1.4 MicroRNAs

microRNAs were first discovered for their involvement in the heterochronic regulation of hypodermal development in C. elegans. Several different microRNAs and their target protein coding genes act at several different stage transitions during C. elegans development. In the following section, microRNAs involved in heterochronic regulation and what genes they have to interact with in order to achieve their function are described. These interactions will highlight mechanisms of heterochronic regulation in general.
1.4.1 MicroRNAs and the L1 to L2 stage transition

The first microRNA described was lin-4/microRNA (Chalfie et al., 1981) through its interaction with lin-14/nuclear protein (Liu and Ambros, 1989). LIN-14/nuclear protein is translated during L1, but translation is repressed during L2 stage in wild type, despite the mRNA still being present. Loss of the coding region of lin-14/nuclear protein causes a loss of L1 programs and hypodermal seam cells precociously execute the L2 stage programs during L1 stage (Arasu et al., 1991). The opposite phenotype results from loss or reduced function mutations of lin-4/miRNA, a consequence of lin-14/nuclear protein translation persisting during the L2 stage. The hypodermis of these mutants fails to timely progress from L1 to L2 stage program. Loss of LIN-14/nuclear protein is epistatic to loss of lin-4/microRNA, while loss of the 3’UTR of lin-14/nuclear protein acts as a lin-14-gain-of-function mutation exhibiting the same phenotypes as lin-4/microRNA. This indicates that lin-4/microRNA suppresses translation of LIN-14/nuclear protein mRNA (Figure 6 A).

These observations can be interpreted as LIN-14/nuclear protein driving L1 stage programs. Termination of L1, and progression to L2 stage programs requires lin-4/microRNA mediated repression of lin-14/nuclear protein translation. This lin-4 microRNA mediated translational repression is mediated by lin-4/microRNA interacting with the 3’UTR of lin-14/nuclear protein. Two publications (Lee et al., 1993; Wightman et al., 1993) were critical to the discovery and mechanism of microRNAs. In a groundbreaking work, Lee et al. 1993 showed that lin-4/microRNA gene is not a protein coding mRNA, but a non-translated RNA that is made as a larger precursor that is processed into 21-23 nucleotide long microRNA. Wightman et al. observed that lin-4/miRNA was partly complementary to the lin-14 3’UTR, and demonstrated that lin-4 post-transcriptionally inhibits lin-14/nuclear protein through its 3’ end, providing the fundamental mechanism of microRNA target regulation (Wightman et al., 1993). Later work showed that the mature microRNA is incorporated into a RNA-induced Silencing Complex (RISC) (Hutvagner et al., 2001), where it represses translation of genes that contain antisense elements in their 3’UTR (Olsen and Ambros, 1999). As a summary, lin-4/microRNA is required for a transition from L1 to L2 programs.

1.4.2 MicroRNAs and the L2 to L3 stage transition

L2-L3 transitions are primarily regulated by the C. elegans hunchback, hbl-1. Hunchback proteins are transcription factors containing cys2-his2 zinc finger motifs, and are important for
early embryonic pattern formation, as first described in Drosophila (Lehmann and Nusslein-Volhard, 1987; Tautz et al., 1987). In C. elegans, the hunchback homolog hbl-1 also plays a later role in larval developmental timing, promoting the hypodermal L2 program (Abrahante et al., 2003; Lin et al., 2003) hbl-1/hunchback directed RNAi leads to a loss of hypodermal L2 programs and precocious execution of L3 programs directly following the L1 stage (Table 1). Transition from the hypodermal L2 stage to the L3 stage is dependent on the disappearance of hbl-1/hunchback protein mediated by three let-7 family microRNAs: mir-48, -84, -241 (collectively referred to as "let-7s") (Abrahante et al., 2003; Lin et al., 2003). Loss of let-7s/microRNAs leads to persistent HBL-1/hunchback expression during L3 and failure of the L2 to L3 transition (Abbott et al., 2005). Thus, let-7s microRNAs are normally required for a transition from L2 to L3 programs (Figure 6 B).

1.4.3 MicroRNAs and the L4 to adult stage transition

The L4-to-adult transition is marked by the end of molting, with the seam cells exiting the cell cycle, terminally differentiating and fusing into a seam syncitium, which excretes a ridged cuticular structure called “alae” – a convenient marker for the adult fate (Page and Johnstone, 2007). Loss or reduction of function of several heterochronic genes cause failure of the L4-adult switch, including let-7/microRNA (Reinhart et al., 2000) and zinc-finger protein LIN-29/ZnFi (Figure 6 C) (Ambros and Horvitz, 1984).

Lin-29 regulation is complex. mRNA transcription already starts during the L2-L3 stage, but translation is suppressed by the lin-41/RBCC ring finger protein. Lin-29/ZnFi expression is derepressed when lin-41/RBCC itself is translationally suppressed by let-7/microRNA as it comes on during the L3-L4 stage. Thus, LIN-41/RBCC disappearance allows LIN-29/ZnFi translation during L4 stage and promotion of terminal differentiation in hypodermis, as well as in the vulva (Reinhart et al., 2000). However, more recent studies show that varying the expression level of mir-84 can modulate the phenotypes of let-7 and lin-29 mutants (Hayes et al., 2006), placing the microRNAs further downstream in the heterochronic pathway. RNAi knock down of several heterochronic genes - including those acting earlier in the pathway - can suppress the microRNA deficiency phenotypes of inhibited terminal differentiation. This highlights how complicated heterochronic regulation networks can be, consisting of multiple parallel branches of regulation.
1.4.4 Heterochronic regulation by DAF-12 signaling

Another regulator of the L2 to L3 transition is the nuclear hormone receptor *daf-12*. Loss of function alleles of *daf-12* lead to retarded developmental progression, reflected in a repetition of L2 stage specific programs in the hypodermal seam cells. Alleles like *daf-12(rh61)*, which affects the ligand binding domain, cause both stronger hypodermal phenotypes and gonadal migration phenotypes when the distal tip cell continues outwards migration during L3 stage and fail to turn back towards midbody (Antebi et al., 1998). These phenotypes are associated with weak repressing activities of DAF-12 and its co-repressor DIN-1 (Ludewig et al., 2004), when dafachronic acid synthesis or binding to the receptor is diminished. Indeed, gonadal heterochronic phenotypes are also caused by mutations affecting dafachronic acid synthesis such as *daf-9(rh50)* (Gerisch et al., 2001; Jia et al., 2002). Loss of dafachronic acid synthesis of the *daf-9(dh6)* null allele or alleles that severely decrease DAF-12 ligand binding domain affinity for dafachronic acid, like *daf-12(rh273)* or *daf-12(rh274)* lead to either constitutive dauer formation (Daf-c) or severe retarded phenotypes in the gonad (Antebi et al., 2000). All phenotypes of reduced dafachronic acid production or LBD affinity can be suppressed by knock-out of DIN-1, indicating that they are caused by various degrees of *daf-12* target repression. The function of *daf-12* in these tissue specific heterochronic phenotypes is not only to globally arrest development as described earlier, but also to actively promote tissue specific developmental progression.

From the discussion above, it is known that a loss of the cell specifically acting *let-7s* microRNAs causes the same phenotype as a loss of the global acting nuclear hormone receptor *daf-12*—an increase in the number of seam cells. By contrast, a loss of microRNA repression target *hbl-1* causes the opposite phenotype, a decrease in the number of seam cells.
Figure 6: V1-4 and V6 seam cell lineages in wild type and mutant backgrounds

*lin-14(n536n540)* loss-of-function (lf) mutants fail to execute hypodermal L1 programs while *lin-14(n536)* gain-of-function (gf) and *lin-4(e913)* lf fail to progress timely to L2 stage (A). RNAi against *hbl-1* results in a loss of L2 programs, while *daf-12(rh61rh411)* lf and *mir-48* -84, -241 triple deletion mutants fail to timely progress to L3 stage (B). *lin-29(n333)* lf and *let-7(n2853)* temperature sensitive (ts) lf mutant fail to terminally differentiate and timely progress to adult stage (C).

The above mentioned microRNAs are so far known as cell specific regulators of heterochronic development, which due to their tight spatial control of expression only affect a subset of tissues. Other heterochronic factors are listed in Appendix 6 or reviewed elsewhere (Moss, 2007; Rougvie, 2001).

1.5 Questions addressed in this dissertation

MicroRNAs were discovered only recently, but it is clear that they are involved in many important biological processes such as stem cell maintenance, development and cancer. Despite their importance, many aspects of their regulation and function remain unknown so far. Only few examples are described for *trans-* and *cis-* acting regulatory factors that coordinate their transcriptional regulation. A connection of microRNA transcriptional regulation to upstream signaling allows an understanding of a broad regulatory cascade in a physiologic context.

In *C. elegans*, the loss-of-function phenotype of *let-7s/microRNAs* resembles a loss-of-function of the nuclear hormone receptor DAF-12. In Chapter 2 of this dissertation, I
investigated the possibility that this phenotypic similarity is a result of the let-7s/microRNAs being direct transcriptional targets of DAF-12. This connection would link DAF-12, an important regulator of global developmental progression, as a trans-regulatory factor to the let-7s/microRNAs, which act principally as cell type specific heterochronic genes.

To understand the hormone dependence of microRNA regulation, it is important to understand the extent of nuclear hormone receptor regulation of microRNAs and how much other transcription factors contribute. Because hormone receptor/microRNA promoter interactions have not been described in detail so far, I isolated predicted daf-12 regulated cis-regulatory elements from microRNA promoters and tested the dependence of their activation on daf-12 and other transcription factors, as described in Chapter 3.

The activity of nuclear receptors depends on their interaction with co-regulating factors, and in Chapter 4, I test a candidate DAF-12 co-regulator, LIN-29/ZnFi for its in vitro and in vivo influence on DAF-12 dependent transcriptional regulation.

Altogether these studies shed light on fundamental aspects of microRNA regulation during metazoan developmental progression.
2. Nuclear hormone receptor regulation of microRNAs controls developmental progression

This work was published as:

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Adam Antebi contributed data to Figure 17, Figure 18 and Table 1
Introduction

Many examples are known of how environmental input can influence the progression of development of an organism, e.g. the delayed sexual maturation of humans caused by starvation, or the delay of metamorphosis of starving tadpoles (Gilbert, 2003).

The environment achieves this by influencing the endocrine regulation that is driving development. The endocrine signal originates from endocrine tissues that excrete signaling compounds like steroid or thyroid hormones that can diffuse throughout the whole body to orchestrate changes across different tissues. Environmental conditions influence these endocrine organs and hormone syntheses and influence organism wide progression or fate choices. In target tissues, nuclear hormone receptors convert this signal into local changes of gene expression patterns that define the morphology changes and developmental programs executed within each tissue. How exactly the hormone signals are converted into cell specific developmental programs, and how those are responsive to environmental changes remains mostly unclear and a lot of research on both mammalians as well as model organisms has been done to identify these hormones, their downstream targets and how they regulate them. In the model organism *C. elegans*, this research lead to the identification of steroid like hormones called dafachronic acids (Motola et al., 2006). Dafachronic acids are synthesized by a cascade of hormone synthetic genes only in presence of food and favorable environmental conditions. Hormone synthesis is regulated by insulin and TGF-beta signaling cascades that convey environmental sensing information and repress it e.g. in absence of food (Gerisch and Antebi, 2004). Dafachronic acids signal throughout the whole worm body to activate the nuclear hormone receptor DAF-12 and its target genes. In absence of dafachronic acids, DAF-12 tightly represses its transcriptional targets and causes developmental arrest and dauer larva formation. This repression seems to be the main function of DAF-12 for developmental regulation, since null mutants of *daf-12* fail to arrest, and show overall normal morphology. DAF-12 transcriptional activation seems to be required for developmental progression at least in one tissue, the hypodermal seam cells, that fail to properly transit from larval stage L2 to L3 absence of DAF-12 transcriptional activation (Antebi et al., 1998).

It is not well understood what target genes DAF-12 regulates to transduce the presence or absence of the global dafachronic acid signal into cell intrinsic gene expression changes.
However, knowing the identity of these genes and the dynamic of their regulation is fundamental to understanding this model system for not only how hormone signaling drives development, but also for how it connects to upstream signaling cascades that link development to environmental conditions.

The main purpose of this study was to gain insight into these processes by identification of direct DAF-12 target genes and their regulation. The primary aim was to find transcriptional activation targets of DAF-12 and connect them to upstream hormone signaling and downstream tissue specific effects. A starting point for this aim was to identify genes that cause a similar loss-of-function phenotype as \textit{daf-12}, hinting a functional connection. Candidates are \textit{mir-48, -84,} and \textit{-241}, members of the \textit{let-7} family of microRNAs, since their deletion leads to a loss of developmental progression from L2 to L3 stage programs in the seam cells (Abbott et al., 2005) as seen in \textit{daf-12} null mutant background. MicroRNAs are a recently discovered class of cell autonomously acting translational regulators coding for small hairpin RNA molecules that get processed and incorporated into a protein complex whose sequence specifically interferes with RNA translation of its target genes. Several repression targets for the \textit{let-7} family microRNAs have already been described, one of which is hunchback homolog \textit{hbl-1}, which is required for L2 specific seam cell program execution (Abrahante et al., 2003). This functional connection of translational regulator genes and their repression targets to DAF-12 and hormone signaling dependent functions is the reason why I tested these genes for DAF-12 dependent regulation. A direct connection would integrate cell specific factors like the microRNAs and \textit{hbl-1} into a \textit{daf-12} and hormone dependent signaling cascade. This cascade would not only span the gap between tissue specific development and organism wide hormonal control, but it would also connect it to already established cascades for environmental sensing. The final cascade would span from environmental sensing, through the insulin and TGF-beta cascade, onto the steroid synthesis pathway and \textit{daf-12} dependent regulation of tissue specific regulators that are part of cell specific developmental programs. In addition to this, it would be the first time that microRNAs are found to be integral parts of a signaling cascade.

In this chapter, I will present experiments that I undertook to test the promoters of the \textit{let-7} family microRNAs for direct DAF-12 regulation, and how this regulation affects microRNA downstream targets. I will test if the microRNAs are part of a cascade connecting global \textit{daf-12} hormone signaling to seam cell specific program execution, and if this cascade reacts
accordingly to signaling mutant backgrounds. Finally, I will look for literature examples to test if this regulation motif is conserved in other organisms.

**Results**

2.1 NHR activates microRNAs

2.1.1 *In vitro* promoter interaction

2.1.1.1 Direct transcriptional activation of microRNA-promoters by DAF-12 and ligand

To test the above stated hypothesis, I cloned promoter regions of several microRNA genes into a luciferase reporter construct and performed luciferase assays in Human Embryonic Kidney cells (also known as 293T cells). I tested promoters of *lin-4* and *lin-4* homolog *mir-237*, as well as *let-7* and its homologs *mir-48, -84, -241* (referred to as *let-7s*) promoters. Co-transfected was either DAF-12 or GFP expression vector, and activation was assayed in presence of either 400nM Δ^4^-dafachronic acid or EtOH as vehicle control. I found that only *mir-241p* and *mir-84p* reporters gave strong luciferase activation (Figure 7).

![Figure 7: Luciferase assay of microRNA promoters](image)

*Figure 7: Luciferase assay of microRNA promoters*

Expression vectors for DAF-12 or GFP as control were transfected into Human Embryonic Kidney cells, along with vectors containing luciferase cDNA downstream of a microRNA promoter followed by a minimal promoter. Dafachronic acid (Δ^4^-DA) strongly activates *mir-84* and *mir-241* promoters in the presence of DAF-12 *in vitro*, whereas other promoters were not visibly activated. Assay was repeated twice with similar results.

Activation was dependent on the presence of both DAF-12 and ligand, suggesting that DAF-12 can physically interact with both promoters, as well as with the basic mammalian transcriptional machinery.
To find the location of the DAF-12 response elements (RE) in the promoters, I generated a deletion series of the *mir-241* promoter to map the DAF-12 response elements. I found that a 500bp long truncation #4 retains highest DAF-12 and ligand dependent activation making it likely for DAF-12 response elements to be present in these 500bp.

To define a canonical RE that DAF-12 can bind to, Shostak (Shostak et al., 2004) used an approach testing *in vitro* DNA binding of genomic fragments to immobilized DAF-12 DBD. I generated a consensus DAF-12 response element by using the "Weblogo.com" tool of the UC Berkeley to combine various REs defined by Shostak. I will refer to this element as "Shostak-RE" (Figure 8).

![Figure 8: Shostak-response element for DAF-12](image)

DAF-12 response elements identified by Shostak were combined independent of their fold-induction potential to generate a canonical response element using the “Weblogo.com” tool.

EMSA experiments by Daniel Motola also showed an increased DAF-12 affinity to the RE when the Shostak-RE was preceded by a stretch of several adenosine nucleotides (Motola, personal communication). The #4 truncation fragment of the *mir-241* promoter contains four potential DAF-12-response-elements (REs) that fulfilled the above mentioned criteria. The two separately located sites were called 241a and 241b, and an inverted repeat with 9 nucleotides
spacing between response elements (IR9) was called 241cd. Two potential response elements were identified for the mir-84 promoter and called 84d and 84e.

In the full-length promoter context, deletion or mutation of the 241a and 241b elements of the mir-241 promoter and 84d and 84e elements of the mir-84 promoter led to a ~7 and ~3 fold decrease in activation, respectively (Figure 9). This was interpreted as DAF-12 and ligand dependent activation to depend on the presence of the identified response elements in the promoter in vitro.

Figure 9: Deletion analysis of mir-241 and mir-84 promoters
Deletion fragments 1 to 4 of the mir-241 promoter retained DAF-12 and dafachronic acid (400nM) dependent partial activation. The strongest activating fragment #4 harbored four Shostak-response elements, called 241a, 241b, 241c, 241d. Deletion or point mutation of 241a and 241b abolished most activation (A). Mir-84 promoter lost most activation after deletion of 84a and 84b sites. Due to different promoters used, the nomenclature of the 84a and 84b site changes in Chapter 3 to “84c” and “84e”. Assay was repeated twice with similar results.

2.1.1.2 Direct physical DAF-12 interaction with response elements in the promoter fragments
To test if DAF-12 can physically interact with those candidate DAF-12 response elements, I performed electrophoretic mobility shift assay (EMSA). Human Embryonic Kidney cells were transfected with expression vectors for either GFP or Flag-tagged DAF-12. Nuclear extract of transfected cell cultures were incubated with radio labeled, 30 nucleotides long oligo nucleotides spanning candidate DAF-12 response elements. I found that among unspecific background shift signals, specific shift signals were induced only by nuclear extract of daf-12 transfected cultures.
These specific shifts were super shifted by incubation with anti-Flag antibody as proof of DAF-12 being the shift causing protein. The shift was sequence specific, since unlabeled wild type sequence oligo competed away the shift signal, while oligos with a mutated daf-12 response element failed to do so (Figure 10 A).

The 241b element caused a clear shift signal, while the 241a element produced in addition to a specific signal an unspecific background band. The specific signal gained in intensity when incubated with daf-12 transfected nuclear extracts, and was super shifted after anti-Flag antibody incubation (Figure 10 B).

Figure 10: EMSA of 241a and 241b
Nuclear extract of cells expressing Flag-tagged DAF-12 induced a shift (s) of 241a element containing radio-labeled oligo that was partially overlapped by a non-DAF-12 dependent shift. The DAF-12 specific signal was super shifted (ss) after incubation with anti-Flag antibody, competed away by non-labeled wild type sequence oligo, but not competed away by oligos with point-mutated 241a (A) while shift and super shift of 241b are not overlapped (B). Green letters indicate DAF-12-REs while red letters represent mutated nucleotides. EMSA was repeated once with similar results.
The oligo containing the 242cd sites produced a more complex shift pattern, since either one (causing the “monomer s”) shift or both (causing the “dimer s”) shift response elements can be occupied to induce a shift by DAF-12 protein. Double occupancy resulted in a higher shift and was the predominant condition as seen by the more intense shift band. Anti-Flag antibody also generated a super shift even though more diffuse than the super shift signal observed for the single-response element containing oligos. Competition with cold wild type oligo competed DAF-12 away from the labeled oligos, while single or double mutated competitor oligo partially competed with the result of a more equal distribution between single and double DAF-12 occupancy of the oligo that is reflected in the more equal intensity between the two different shift signals (Figure 11).

![Figure 11: EMSA of 241cd](image)

Nuclear extract of cells expressing Flag-tagged DAF-12 induced two shift signals (s) of radio labeled oligo containing 241cd element. “monomer” shift (s) signal is caused by oligos bound to a single DAF-12 protein, while “dimer” shift (dimer s) is caused by oligos bound to two DAF-12 proteins. The DAF-12 specific signal was super shifted (ss) after incubation with anti-Flag antibody. Incubation with non-labeled wild type (wt) competitor oligo competed away the shift. Addition of oligo with one (mut 241xd or mut 241cx) or both (mut 241xx) daf-12 response elements mutated changed the ratio of “monomer” and “dimer” shift towards monomer bound, labeled oligo. Green letters indicate DAF-12-REs while red letters represent mutated nucleotides. EMSA was repeated once with similar results.
The 241c and 241d elements are spaced 9 nucleotides apart, which reflects nearly one complete turn of the DNA helix, positioning the two DNA bound DAF-12 proteins in direct proximity possibly reflecting direct DAF-12-DAF-12 homo-dimerization or hetero-dimerization with one alternative nuclear hormone receptor. Dimerization is common for NHRs and modifies transcriptional output (Forman & Evans 1995). The context of possible dimerization and in vivo transcriptional activation generated by these two sites are investigated in more detail in Appendix 5.

2.1.2 In vivo promoter activation

2.1.2.1 Mir-241 promoter regulation and daf-12 signaling mutations

To examine microRNA promoter activation in vivo, I generated transgenic worm lines containing the microRNA promoters fused to a gfp reporter gene (termed mir-241p::gfp or mir-84p::gfp). I found that wild type worms transgenic with the mir-241p::gfp reporter gave a broad expression pattern as described (Esquela-Kerscher et al., 2005).

![Figure 12: DAF-12 and dafachronic acid regulate mir-241 promoter in vivo](image)

(A to H) mir-241p::gfp. Images show representative L3 animals, with indicated cell types (white arrowheads and exc, excretory cell; outlined arrowheads, neu, neuron; mus, muscle; int, intestine; ph, pharynx). Bar graphs alongside the images quantify the percentage of worms with excretory cell GFP expression as either strong (green), weak (yellow), or off (red) (two independent experiments, left and right, n = 10 animals each, and representative images are shown). Wild type (N2, A and B) grown without ethanol (EtOH) or with dafachronic acid (250nM Δ5-DA) showed equal expression levels. Expression was decreased in daf-
null (C), strongly repressed in daf-9(dh6) null (E), or not activated in daf-9(dh6);din-1(dh149) double null (G), but was rescued nearly to wild type level by growth on 250 nM Δ⁴-DA (F and H). In wild type (B) or daf-12 null (D) animals, dafachronic acid supplementation had no effect. Point mutation of all four DAF-12 REs abolished differences of tested genetic backgrounds or dafachronic acid (I to N).

daf-12(rh61rh411) null mutants showed decreased expression most noticeably in the excretory cells (exc), as well as muscles (mus), pharynx (ph) and intestine (int) whereas neuronal expression (neu) seemed less affected, revealing tissue selectivity of daf-12 regulation (Figure 12 C-D). Previous research revealed that null mutants for cytochrome P450 daf-9(dh6) fail to produce dafachronic acid, and the unliganded DAF-12 interacts with its co-repressor DIN-1/SHARP to repress its transcriptional targets. According to this, mir-241p::gfp expression was tightly repressed in most tissues in daf-9 null background (Figure 12 E). Supplementation with dafachronic acid rescued this arrest and increased mir-241p::gfp expression back to wild type levels (Figure 12 F). Tight repression was also relieved in daf-9(dh6);din-1(dh149) double null mutants lacking both, the ligand and the co-repressor. Mir-241p::gfp expression levels in this double mutant strain were similar to daf-12 null mutants (Figure 12 G, H). Unlike daf-12 null mutants, dafachronic acid supplementation of daf-9;din-1 worms restored mir-241p::gfp expression back to wild type levels. Point mutation of all four daf-12-REs in mir-241p (termed mir-241p(mut abcd)::gfp) resulted in the same weak expression level in wild type, daf-12 null, daf-9 null, and daf-9;din-1 doubles with or without ligand, as previously seen of the mir-241 wild type promoter gfp construct in daf-12 null background (Figure 12 I-K).

This was interpreted as liganded DAF-12 acting through its REs to mildly activate mir-241p in some issues, whereas unliganded DAF-12 together with DIN-1 tightly represses expression in nearly all tissues. The loss of any influence of daf-12 signaling onto the point mutated reporter constructs further shows that DAF-12 does not interact with any other response elements in the promoter than the mutated four to mediate activation and repression.

2.1.2.2 Mir-84 promoter regulation in daf-12 signaling mutant backgrounds

As reported (Esquela-Kerscher et al., 2005; Hayes et al., 2006), mir-84p::gfp was expressed in pharynx, somatic gonad, seam cells, vulva cells, and occasionally in the intestine. The phenotype of daf-12 null or the let-7s deletion overlaps in the seam cells, and direct
transcriptional activation of the microRNAs would show as de-regulated mir-84p::gfp seam cell expression.

**Figure 13: DAF-12 and dafachronic acid regulate mir-84 promoter in vivo**
Images show representative L3 animals. Bar graphs alongside the images quantify the percentage of worms with excretory cell GFP expression as either strong (green), weak (yellow), or off (red) (two independent experiments, left and right, n = 10 animals each, and representative images are shown). Epidermal seam cells (arrowheads) expressed mir-84p::gfp in wild type (N2, A), but not in daf-12 null mutants (B). Seam cell expression was absent in hormone-deficient daf-9;din-1 animals (C), but restored to nearly wild type levels by dafachronic acid (Δ4-DA) supplementation (D).

Indeed, seam cell expression was absent in daf-12 null and daf-9;din-1 double null mutants, with expression increased to almost wild type levels in daf-9;din-1 animals grown on dafachronic acid supplemented plates (Figure 13 A-D). This finding possibly explains daf-12 heterochronic phenotypes as a failure to activate the microRNAs in tissues subject to temporal control of development. In contrast to seam cell expression, expression in other tissues such as the pharynx was less affected (Figure 13 E, F), again showing tissue-specificity of daf-12 regulation.

### 2.1.2.3 Mature microRNA dose measured by Q-PCR
Promoter fusions to gfp reporter genes reflect the transcriptional activation of the promoter, but do not account for regulation during the microRNA maturation process, which was also found to be highly regulated (Newman et al., 2008). To quantify mature microRNAs, I used Taqman Quantitative PCR (Q-PCR) and found that the transcriptional regulation of the let-7s is also reflected in the abundance of total mature microRNAs detected. daf-12 mutants and daf-9;din-1 animals without ligand showed decreased levels compared to wild type, whereas daf-9
null mutants showed tight repression of let-7s microRNAs (Figure 14 A-D). As expected, expression in daf-9, daf-9;din-1, but not daf-12 mutants was rescued by dafachronic acid. mir-1 was used as an endogenous control as it was reported to be not regulated during development of C. elegans (Lau et al., 2001).

**Figure 14: Relative quantification of let-7 family microRNAs by Q-PCR**

MicroRNA expression was decreased in daf-12(rh61) (B), daf-12(rh61rh411) null (C), daf-12(rh62rh157) null (D), daf-9;din-1 double null mutants (E) and repressed in daf-9 mutants (F) relative to wild type expression (A). In daf-9 genotypes, expression was dependent on growth in presence of 250nM Δ⁴-dafachronic acid. Q-PCR was carried out using the TaqMan system, using mir-1 quantification as endogenous control.
Figure 15: Quantitative PCR of mir-1

The amplification plot for miR-1 detector shows overlapping CT values for mir-1 in all genotypes tested i.e. daf-9(dh6) in presence versus absence of 250nM Δ^4-dafachronic acid. Therefore mir-1 expression does not change substantially in the genetic backgrounds used in this publication. In contrast to this, N2 dauers on starved plates express ~ 16x reduced amount of mir-1, which after 2h of re-feeding already increases 4x (A).

The only RNA purifications showing drastic changes of mir-1 content judged by the Q-PCR amplification plot were derived from starvation dauers. However, the absolute RNA amount used as template was below minimum concentration necessary for accurate quantification and therefore was not quantified prior to use, instead equal number of worms were extracted (100 animals per condition) (Figure 15). Variations in RNA input concentrations might be responsible for a general over- or underrepresentation of all four measured let-7s, but this specific misrepresentation is identical for all four microRNAs quantified for a specific condition.
However, relative quantification of the four *let-7* family microRNAs indicates a specific regulation of *mir-84* relative to the other three quantified miRs (Figure 16).

**Figure 16: mir-84 regulation during dauer recovery**
Quantification of *let-7* homologs in N2 starvation dauers (A), 2h after re-feeding (B) and 24h after re-feeding (C) on a single biological replicate consisting of 100 hand-picked worms. Fold change of *let-7* homologs relative to endogenous control miR-1 and calibrated to 24h of re-feeding. Note that miR-1 RNA is less abundant in RNA preparations of starvation dauers (Figure 15). One biological replicate of n=100 animals each condition.

2.1.2.4 MicroRNA promoter activation in *daf-12* upstream signaling mutants
In the introduction, I describe evidence for upstream neuronal input through TGF-beta and insulin signaling onto the global decision between developmental progression or arrest. This influence converges onto *daf-12* ligand production. Direct DAF-12 dependent *mir-84* and *mir-241* activation connects these upstream signaling cascades through *daf-12* and its ligand to cell intrinsic program execution in the hypodermis. To further investigate this dependence onto upstream cascades, and to show how the downstream, cell intrinsic *let-7s* expression is affected by global acting TGF-beta and insulin signaling, the regulation dynamics of *mir-84p::gfp* and *mir241p::gfp* reporter construct were analyzed in dauer constitutive mutant backgrounds of *daf-7/TGF-beta*, *daf-2/Insulin/IGF-I Receptor*, and *daf-9*. *Daf-2(e1368)* and *daf-7(e1372)* alleles are temperature sensitive and cause dauer formation only when cultivated at non-permissive temperature of 25°C, while *daf-9* is a null allele that does not produce dafachronic acid under any condition and constitutively causes dauer formation.

In *daf-2* and *daf-7* dauer larvae grown at non-permissive temperatures, *mir-241p::gfp* expression was nearly completely repressed, whereas *mir-84p::gfp* was down regulated in some
tissues such as the pharynx (Figure 17 A-D) but consistently up-regulated and expressed more penetrantly in others tissues such as the seam cells (Figure 17 E-G).

Figure 17: microRNA promoter regulation in different dauer signaling mutant backgrounds

Mir-241p::gfp showed high expression in continuously growing wild type (A), but low expression in daf-7(e1372) dauer larvae (B). mir-84p::gfp showed high expression in the pharynx of continuously growing wild type (C) but low expression in daf-2(e1368) dauers (D). mir-84p::gfp seam cell expression (E) was elevated in daf-2 and daf-9 mutants during dauer stage (F and H) and was even higher in daf-7 mutants during reproductive growth at 20°C (G). Penetrant seam expression was reversed by 500 nM dafachronic acid in daf-7, but not daf-2, during reproductive growth (I to M). Animals were assayed during L3 and/or L3d stages, n > 20 animals, and representative images are shown. Red bars represent percentage of worms showing expression when grown on ethanol vehicle, while green bars represent the same during growth on Δ³-dafachronic acid (SEM).

Mir-84 seam cell expression is increased even in the total absence of ligand synthesis in daf-9 null mutants (Figure 17 H). These findings are in contrast with the existing model of daf-12 signaling where the absence of ligand causes DAF-12 to tightly repress transcription of its target genes and to induce dauer formation.

It was found that in daf-2(e1368) and daf-7(e1372) mutant L3 worms grown under permissive temperature, seam cell expression was more penetrant than in wild type background, arguing for a ligand independent transcriptional activation of mir-84p in this tissue (Figure 17 G).

To test ligand influence on the expression in this tissue, worms grown under permissive temperature that were either ligand supplemented or non-supplemented were compared.
Dafachronic acid supplementation largely suppressed the increased expression in seam cells in *daf-7*, but not in *daf-2* background (Figure 17 I-M). These findings suggest that insulin/IGF and TGF-beta signaling distinctly regulate *mir-84p* expression in a dafachronic acid dependent and independent manner.

2.1.3 MicroRNAs and their involvement in signaling cascades

2.1.3.1 HBL-1 drives developmental progression in seam cells and is microRNA regulation target

The zinc finger protein *hbl-1*/hunchback is responsible for L2 proliferative programs of seam cells (Fay and Wood, 1999), and the protein is present during L2 stage but not during L3. *Hbl-1* loss during L2 stage leads to loss of proliferative L2 seam cell program, and a decreased seam cell number in later stages. *Let-7s* mutants result in both increased seam number and *hbl-1::gfp* expression during L3 stage, interpreted as *let-7s* repressing *hbl-1* through binding and repressing its 3’UTR (Abbott et al., 2005; Li et al., 2005). To examine if *daf-12, let-7s* and *hbl-1* form a continuous regulatory cascade, changes in L2 program repression during L3 stage in *daf-12* signaling mutant backgrounds were looked at by monitoring seam cell numbers in later stages as an indicator for L2 stage program repetition. Expected was an increased HBL-1 expression during decreased *let-7* transcription, with a resulting increase in L2 program repetition and seam cell numbers. Consistent with this idea of NHR signaling promoting *hbl-1* inhibition, an increase in seam cell number was observed in *daf-12(rh61)* and *rh61rh411* mutants and in *daf-9;din-1* double mutants. *Daf-12(rh61)* exhibits a penetrant extra seam phenotype interpreted as a more severe failure to inhibit L2 programs during L3 stage than seen in *daf-12* null mutant background (Table 1). The seam cell phenotype was dependent on functional *hbl-1(+),* an observation that by genetic epistasis places *daf-12* upstream of *hbl-1*. Restoring *daf-12* transcriptional activation back to wild type level by ligand supplementation of *daf-9;din-1* double null mutants also reversed seam cell number back to wild type level.
Table 1: Seam cell counts
Seam cells between posterior pharyngeal bulb and the anus were counted by DIC microscopy during the L3 stage. n≥20 animals, mean and SD are shown, P-values were calculated by T-test relative to wild type. (na, not applicable)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seam Number</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type N2</td>
<td>12±0</td>
<td>na</td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td>13±1.4</td>
<td>.00022</td>
</tr>
<tr>
<td>daf-9(dh6); din-1(dh149)</td>
<td>13±1.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>daf-9; din-1 + DA</td>
<td>12±0</td>
<td>na</td>
</tr>
<tr>
<td>let-7s: mir-48 mir-241(nDf51); mir-84(n4037)</td>
<td>16±1.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>daf-12(rh61)</td>
<td>22±1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>hbl-1RNAi</td>
<td>9±.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>daf-12(rh61) hbl-1RNAi</td>
<td>10±1.3</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

GFP-reporter fusions to hbl-1-UTR in daf-12 signaling mutant background that was shown to decrease let-7s microRNA transcription (Esquela-Kerscher et al., 2005) were analyzed to show that these phenotypes are caused by hbl-1-UTR mediated repression. In this background, expression level of two different gfp reporter constructs were analyzed, that both contained the hbl-1-promoter: the hbl-1p::gfp::unc-54-UTR reporter contained the unrepressed unc-54-UTR and is insensitive towards hbl-1-UTR mediated repression so that it only reflects transcriptional activation. The hbl-1p::gfp::hbl-1-UTR construct contained the hbl-1-UTR and is subject to hbl-1-UTR mediated repression. This reporter therefore reflects transcriptional activation minus hbl-1-UTR mediated translational repression.
Comparison of these reporters shows changes in \textit{hbl-1}-UTR mediated repression. When compared to wild type and \textit{daf-12} signaling mutant background, expression of \textit{hbl-1p::gfp::hbl-1}-UTR was up-regulated during L3 only in \textit{daf-12(rh61)} background with decreased \textit{let-7s} transcription, but not in wild type (Figure 18). Interestingly, the \textit{hbl-1}-UTR reporter containing strain showed up-regulation in the ventral nerve cord in the \textit{daf-12(rh61)} background, while the \textit{unc-54}-UTR reporter containing strain showed strong body muscle up-regulation – pointing towards direct or indirect \textit{daf-12} regulation of \textit{hbl-1} in this tissue.

\textbf{Figure 18: \textit{Hbl-1} UTR reporter is regulated by \textit{daf-12} signaling}

A \textit{gfp}-fusion to \textit{hbl-1} promoter and UTR was repressed in the hypodermis at mid L3 (28 hours) in wild type (A). In the \textit{daf-12(rh61)} mutants, reporter signal was up-regulated in the hypodermis (hyp) and other tissues (B) (exposure 250 ms). A \textit{gfp}-fusion to the \textit{hbl-1} promoter, containing the \textit{unc-54}–UTR lacked substantial up-regulation in the hypodermis (C and D), although body muscles (bm) showed modest reporter up-regulation (D) (exposure time 50 ms). \textit{n>10} animals were observed and representative images are shown.

The observed up-regulation in the seam cells was interpreted as caused by reduced \textit{hbl-1}-UTR-mediated repression due to decreased \textit{let-7s} transcription. The observation of equal hypodermal expression levels of the construct without \textit{hbl-1}-UTR in both wild type and \textit{daf-12(rh61)} background verifies that the difference is caused by \textit{hbl-1}-UTR mediated repression.

\section*{Discussion}

\subsection*{2.2 Overview}

In this work, I show that the nuclear hormone receptor DAF-12 directly regulates the \textit{let-7} family members \textit{mir-84} and \textit{mir-241}. Direct regulation of a microRNA has not been shown so far for NHRs, only few TFs are known, e.g. \textit{lin-12} and \textit{lag-1} in \textit{C. elegans} and \textit{p53} in mammals, to directly regulate microRNA transcription (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Yoo and Greenwald, 2005).
*Let-7s* microRNAs mediate tissue specific programs of developmental progression and their absence leads to arrest or failure to progress in development. By showing direct DAF-12 and dafachronic acid mediated activation of these microRNAs, animal wide endocrine regulation of development was connected to the cell autonomous activity of microRNAs. These findings were interpret as a “molecular switch” explained further down and it was proposed that this “molecular switch” is a model for the regulation of stage transitions and how the combined function of DAF-12/NHR and its directly activated *let-7s* microRNAs is required (Figure 19).

But before talking about the molecular switch idea, I explain in more detail the integration of these hormone regulated microRNAs into signaling cascades. I begin this explanation by looking at the most upstream components of TGF-beta and insulin signaling that are expressed in sensory neurons. These components are regulated according to environmental input, and favorable environment increase, while unfavorable environment reduce signaling of both cascades that then activate the biosynthesis of dafachronic acids through transcriptional control over DAF-9 in the endocrine tissues XXX neurons and hypodermis (Gerisch and Antebi, 2004). Dafachronic acids activate the type II clear receptor DAF-12 that acts as a switch between two states: liganded DAF-12 activates transcription of its targets to cause developmental progression while unliganded DAF-12 represses them and causes developmental arrest/dauer formation. The mechanism behind this process was analyzed in hypodermal seam cells, where a loss of *daf-12* activation during L3 stage leads to a failure to progress and a repetition of L2 programs. The same phenotype is observed in absence of the *let-7s* (Abbott et al., 2005), while the opposite phenotype is observed in absence of the *let-7s* repression target *hbl-1* (Abrahante et al., 2003). It was found that direct DAF-12 mediated transcriptional activation of the *let-7s* is necessary to suppress *hbl-1* during L3 to prevent L2 fates from reoccurring, and that loss of *daf-12* transcriptional activation leads to a failure to suppress L3 expression of *hbl-1* and a failure to progress (Figure 19). This connection integrates the cell autonomous acting *let-7s* into global insulin/TGF-beta signaling cascades, and spans regulation from animal wide to cell type specific program execution.
Figure 19: Model for NHR-microRNA signaling cascades
In response to favorable environmental signals, activated insulin/IGF and TGF-b pathways induce Δ^4-DA biosynthesis through DAF-9/CYP450. (Right) DAF-12 with the ligand activates L3 programs and expression of let-7s and thereby inhibits HBL-1 and genes of L2 programs, which result in developmental progression. (Left) During unfavorable conditions, DAF-12 without the ligand, together with DIN-1, repress L3 programs and let-7s, which allows de-repression of L2 programs or developmental arrest. Dauer signaling also has Δ^4-dafachronic acid–independent outputs onto microRNAs.

2.3 Transcription factor and microRNA act as a “molecular switch”
Within the regulatory context that was analyzed, NHR transcriptional activation prevents arrest, while the direct NHR mediated activation of microRNAs as "activated repression" of "old programs" drives developmental progression. These two functions could be interpreted as a combined "molecular switch" that mediates stage transitions or the discrimination between two stages in a single tissue. To see if this hormone regulated "molecular switch" function is involved in other important processes in other systems, the "molecular switch" hypothesis was tested by reviewing several similar cases from the literature. However, to date there are no NHRs described to directly activate microRNAs.

2.3.1 Mammalian p53 and the "molecular switch"
An important TF mediating the decision between continued development or cell cycle arrest, DNA repair, senescence or apoptosis is p53 (Zhao et al., 2000). After cell stresses like UV or ionizing irradiation, p53 is activated and induces cell cycle arrest, DNA repair, senescence or apoptosis. This is a vital protection mechanism, and absence or decrease of p53 function is
associated with 50% of all known cancers (Harris, 1996). p21 is an important transcriptional activation target of p53, and p21 deletion represses major aspects of p53 mediated cell stress responses (Waldman et al., 1995) but does not completely suppress p53 mediated growth arrest (Brugarolas et al., 1995), indicating that p21 redundant functions also are important for the p53 cell stress response. Recently, He (He et al., 2007) and others (Chang et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007) showed that p53 directly activates transcription of mir-34a-c. mir-34 microRNAs repress genes that promote cell cycle progression - the opposing function to p53 - and mir-34 transfection into cells leads to cell cycle inhibition and senescence. These observations can be interpreted as miR-34s silencing genes that oppose the effects of p21 by supporting the transition from cell cycle progression to arrest. In other words, mir-34s and p21 mediate similar functions to achieve p53 downstream effects. When looking at p53 and the mir-34s with the "molecular switch" hypothesis in mind, the above mentioned findings can be interpreted as p53 and mir-34 forming a "molecular switch": p53 directly activates transcription of mRNAs for genes like p21 that promote a.o. cell cycle arrest, while it simultaneously activates mir-34 microRNAs that repress translation of genes with opposite function, e.g. cell cycle progression. The conclude is that the "molecular switch" can be considered as being conserved in this system and that it indeed might be a paradigm for understanding not only hormone dependent developmental progression as in this case, but also the function of other TFs involved in fundamental processes such as tumor formation, stem cell differentiation or maturation in metazoans.

2.3.2 Consequences of the "molecular switch" idea

A general goal of the "molecular switch" idea is to understand the function of microRNAs that are directly activated by certain TF as the "activated repression" function of this TF, as p53 in the above example. As a consequence, the investigation of the function of a TF would at an early time point already involve experiments to identify directly activated microRNAs. This would facilitate the understanding of more integrated functions of the TFs in question, as it did in example shown in this chapter, where it was essential to identify the let-7s microRNAs as direct transcriptional targets and as an "activated repression" function of daf-12 in order to make sense of the hypodermal heterochronic phenotypes of daf-12.

The application of the "molecular switch" idea may be especially important in understanding how progression of development is controlled. Progression from one developmental stage to the
next also involves actively terminating the previous stage to prevent overlap. When this switch involves a nuclear hormone receptor, this transition can be achieved by a single signal in the form of a hormone, acting on a type II nuclear receptor that changes from active repression in absence, to activating transcription in presence of the ligand. But also non-hormonally induced changes of the functional status of a TF can achieve the goal to mediate a transition from one program to different one, as the p53 example shows. The invariant cell lineage of *C. elegans* facilitated the discovery of this regulation principle, and it is likely to be less apparent in more complex animals whose cell lineages are more dynamically regulated and less well known.

### 2.4 NHR-signaling paradigms

DAF-12 signaling dependent regulation of the *mir-84* and *mir-241* promoters also validates nuclear hormone receptor signaling paradigms set up by several key publications over the last three decades (Mangelsdorf et al., 1995).

Abundance of the dafachronic acid ligands is ultimately regulated by a single, non-redundant gene, cytochrome p450 *daf-9* (Gerisch et al., 2001; Jia et al., 2002). The ligand products of this enzyme bind to the NHR DAF-12, a type II nuclear receptor that in the presence of its ligand activates transcription of its target genes (Motola et al., 2006) to allow progression through development (Antebi et al., 1998). *C. elegans* co-activators for DAF-12 have not been identified, but ligand bound DAF-12 can interact with mammalian co-activator SRC-1 (Motola et al., 2006). In the absence of ligand, DAF-12 interacts with co-repressor DIN-1 (Ludewig et al., 2004; Motola et al., 2006), an homolog of human co-repressor SHARP, to suppress transcriptional activation of its target genes. The importance of active activation and active repression is reflected in the severity of phenotypes of mutations affecting different components of this cascade: a null allele of *daf-12* leads to less severe phenotypes like increased seam cell divisions or loss of dauer formation, while an increase of DAF-12 interaction with DIN-1 causes constitutive target gene repression that leads to migration defects of the gonad, reduced bruit size or increased dauer formation. These phenotypes result from two different types of mutations, one type effecting the DAF-12 ligand binding domain e.g. *daf-12(rh61)* or *daf-12(rh273)* (Antebi et al., 2000), and the other limiting or abolishing the synthesis rate of DAF-12 ligand e.g. *daf-9(rh50)* or *daf-9(dh6)* (Gerisch and Antebi, 2004). Constitutive DAF-12 repression, in e.g. *daf-9(dh6)* null background, involves constitutive dauer formation, and can be completely suppressed by either additional loss of *din-1* function or ectopic hormone supplementation. The
daf-9;din-1 double null mutant exhibits in most parts phenotypes resembling daf-12 null alleles in absence, and wild type phenotypes in presence of supplemented daf-12 hormone. These hypotheses based on genetic epistasis experiments were validated by quantifying gfp reporter fusions to the mir-241 and mir-84 promoters in various trans-mutant backgrounds in Figure 12 and Figure 13, discussed in further detail below.

2.4.1 Mir-241 promoter regulation

mir-241p::gfp reporter showed moderately reduced activation in several tissues in a daf-12 null background, but no tissue lost all activation, indicating that daf-12 is only one TF among others contributing to the overall transcriptional activation of this promoter. Loss of ligand production in the daf-9(dh6) null allele causes DAF-12 to interact with co-repressor DIN-1 to mediate unconditional, tight transcriptional repression of the mir-241 promoter in nearly all tissues, with the exception of a few head neurons. This demonstrates that unliganded DAF-12 actively represses its target promoters throughout most of the worm body. The strong daf-9 null phenotype is changed to the weak daf-12 null phenotype in daf-9(dh6);din-1(dh149) double null mutant background since this genetic background is deficient for both, daf-12 activation as well as repression. Activation was restored back to wild type levels by ligand supplementation of daf-9(dh6) and daf-9(dh6);din-1(dh149) mutant backgrounds, validating that daf-9 is a non-redundant step in the dafachronic acid synthesis pathway.

DAF-12 regulates the mir-241 promoter by interacting with a defined number of cis-regulatory elements present in the DNA sequence of the promoter, called response elements (Mangelsdorf and Evans, 1995). All DAF-12 mediated transcriptional control of the mir-241 promoter passes through four response elements, and their deletion renders the promoter completely insensitive to mutations affecting daf-12 signaling, like daf-9 null and daf-9;din-1 double null background with or without ligand supplementation. The epistatic relation of these factors is shown in the model in Figure 19.

2.4.2 Mir-84 regulation and dafachronic acid independent activation

Pharyngeal expression of the mir-84p::gfp reporter was regulated in a similar way as the mir-241 promoter, but seam cell expression was completely lost in daf-12 null. Seam cell expression was also lost in daf-9;din-1 double nulls, but restored to wild type levels by ligand addition, and surprisingly increased in daf-9(dh6), daf-2(e1368) and daf-7(e1372) constitutive dauer mutants or daf-2(e1368) and daf-7(e1372) mutants growing at permissive temperature as non-arrested
worms. Ligand supplementation was able to suppress up-regulation of seam cell expression in daf-7, but not daf-2 background. This indicates a daf-12 ligand independent promoter activation following distinct mechanisms for TGF-beta or insulin cascades.

One possible explanation is DAF-12 hetero dimerization with alternative NHRs as (Forman et al., 1995) described for mammalian RXR with VDT, TR, PPAR delta, that - when hetero dimerized - render the receptor dimer insensitive to the RXR ligand, but responsive to the ligand of the hetero dimerization partner. This effect relied on interaction between ligand binding domains, independent of the DNA binding capacity of the second dimerization partner. Given that the C. elegans genome contains 284 genes coding for NHRs (Antebi, 2006), the possibilities are vast and might not even rely on actual presence of a second ligand, since many NHRs are suspected to be orphan receptors. This observation poses an interesting opportunity to further study general NHR signaling functions and their intercalation with insulin and TGF-beta cascades. To our knowledge, no other experimental system integrates all these functions into a single, in vivo model with as drastic outcomes as the ones observed here.

An alternative explanation is that other transcription factors act in these genetic backgrounds. Dependence on DAF-12 trans-regulatory action is supported if the increased seam cell GFP expression in these backgrounds is suppressed by loss of DAF-12. This trans-regulatory influence of DAF-12 or other factors binding to DAF-12-REs can be tested by using a GFP reporter fused to a mir-84 promoter that has both DAF-12-REs point mutated, as seen in Figure 9B. One candidate for an alternative TF activating promoter of the let-7s family is notch-receptor lin-12, described in the following paragraph.

2.4.3 Lin-12/notch receptor in hypodermal differentiation

Despite its best described function during vulva development (see below), a recent publication connects lin-12 also to seam cell differentiation (Solomon et al., 2008). Lin-12 gain-of-function mutants exhibit molting phenotypes during all molts, and seam cells precociously underwent terminal differentiation not after the L4 stage but already after the L3 stage. Lin-12 gain-of-function mutations also show enhanced let-7 transcription which might explain the precocious phenotype, since let-7 is required for terminal differentiation in the hypodermis and loss of let-7 results in delayed phenotypes in vulva and hypodermis. How the increased let-7 levels affect daf-12 signaling requires further investigation since daf-12 and let-7 interaction is complex, as discussed in the following section.
2.5 Feedforward and feedback loops

While DAF-12 transcriptionally activates let-7 family members (Bethke et al., 2009) and temporally regulates let-7 itself (Johnson et al., 2003), several lines of experiments also showed that DAF-12 itself is a repression target of let-7 during later stages. Current bioinformatic prediction algorithms indicate the presence of let-7 seed-regions inside the daf-12-UTR (e.g. http://pictar.mdc-berlin.de/ or http://microrna.sanger.ac.uk). Experimental investigation showed that phenotypes caused by loss of let-7 activity are suppressed in daf-12 null mutant background, placing daf-12 downstream of let-7 (Grosshans et al., 2005). These observations suggest that daf-12 and the let-7 family microRNAs are involved in both, feedback- and forward loops, to drive stage transitions.

To date, many microRNAs are described to be involved in feedback inhibition, and to better understand the daf-12 involvement into these regulatory loops, I will shortly discuss several cases of microRNAs feedback and – forward loops that mostly involve either hormone signaling or heterochronic regulation.

**Figure 20: Models of regulatory loops**

In this work it was shown that DAF-12 directly activates the let-7s, while Johnson et al (2003) showed let-7 transcriptional regulation by DAF-12, and Grosshans et al (2005) showed experiments that suggest DAF-12 to be downstream of let-7. The dashed lines symbolize possible let-7 suppression of DAF-12 through let-7 binding sites in the DAF-12 UTR.
2.5.1 *Lin-12 activates mir-61 to form a feedback loop*

During vulva formation, *lin-12/notch* is directly activating *mir-61/microRNA* in vulva precursor cells (VPCs) while they are induced to follow three different cell fates during vulva formation (Yoo and Greenwald, 2005). One central cell, the p6 cell, follows primary vulva cell fates, while the two adjacent p5 and p7 cells follow secondary vulva cell fates, all three cells giving rise to cells forming the vulva, while p3, p4 and p8 cells follow tertiary cell fate to fuse into the hypodermal syncytium. EGFR-Ras-MAPK signals onto vulva precursor cells p5, p6, p7 with highest intensities in p6 promoting primary vulva cell fates, while *lin-12/notch* is up-regulated in p5 and p7 to promote secondary cell fates. One direct target of *lin-12/notch* is *mir-61*, a microRNA that represses the negative regulator of *lin-12/notch*, *vav-1*. This cascade acts as a feedback loop to enhance *lin-12* signaling once it reaches a certain threshold. However, this feedback loop has only a dispensable function during vulva formation, since for example loss of negative regulator *vav-1* does not prevent proper vulva formation.

Applying the "molecular switch" idea, *lin-12* can be interpreted as the TF that initiates the transition from "precursor" to "differentiation" programs in the VPCs, namely secondary vulva precursor cell fates. *Vav-1* belongs to the program that suppresses secondary vulva precursor cell fate formation and therefore is target of the "activated repression" mediated by *lin-12* activated *mir-61*. At the same time as repressing *vav-1*, *lin-12* mediates direct activation of protein coding genes that are already part of the vulva differentiation programs, as LIN-11/homeodomain transcription factor (Marri and Gupta, 2009). As a summary, *lin-12* activates the repression of *vav-1* that opposes differentiation, while directly activation *lin-11* that contributes to differentiation.

2.5.2 Neuronal differentiation in *C. elegans* and feedback

A second example of a non-hormonal loop involving TFs and microRNAs is the double negative feedback loop described by (Johnston et al., 2005) that regulates bilateral cell fate decision of two gustatory neurons, ASE left and ASE right, during *C. elegans* development. This feedback loop involves two pairs of TF/microRNA combinations, with the microRNA being a direct activation target of the TF. Each pair defines one of two possible cell fates in a pair of gustatory neurons. The downstream microRNA of one pair suppresses the upstream TF of the other pair. To define a choice between the two possible states, outside influence enhances one TF to unbalance the loop and to increase expression of the regulated microRNA that in turn
represses the other TF of the alternative fate choice. This repression leads to a loss of repressing microRNA counteracting the primarily enhanced TF, and the system reaches a stable output.

2.5.3 EcR and feedback
An example for a feedback loop involving hormone signaling involves the Ecdysone receptor (EcR) that is feedback inhibited by mir-14. Deletion of mir-14 leads to several phenotypes, a.o. increased adult survival and an increase of EcR expression levels. A genetic decrease of EcR dose in an EcR +/- background suppresses the adult survival of a mir-14 null background (Varghese and Cohen, 2007). The authors interpreted this as a lack of mir-14 mediated EcR repression causing an over expression that ultimately leads to the observed phenotype. However, the majority of mir-14 deletion phenotypes remained unaffected by decreasing EcR levels. Unfortunately, it was not investigated if 20-OH-Ec mediated repression of mir-14 activation is a direct effect of EcR and its ligand ecdyson, or if other intermediate regulators are necessary.

2.5.4 C/EBP alpha and NFI-A and feedback
Another example for hormonal induction of a microRNAs involving a feedback loop has been described in mammals. It involves the interplay of retinoic acid, C/EBP alpha, NIF-A and mir-223 in "acute promyelocytic leukemia" (APL) (Fazi et al., 2005). In APL cell lines, blood cells are arrested during their development into different blood cell lineages, but a high dose of retinoic acid overcomes this block and induces developmental progression and terminal differentiation, involving expression of several microRNAs, among others mir-223. The factor NFI-A binds to a response element in the mir-223 promoter and transcriptionally activates it to generate a constant baseline expression level, while at the same time, NFI-A also is a repression target of mir-223, and these two components form a negative feedback loop. Retinoic acid application up-regulates C/EBP-alpha which then binds to response elements in the mir-223 promoter that overlap the REs of NFI-A, which is displaced from the promoter, while increasing mir-223 expression at the same time increases repression of NFI-A. This is an example of an indirect hormone induction of microRNA up-regulation that drives development and differentiation, including a feedback inhibition loop involving TFs driving transcription of microRNAs that at the same time repress those TFs.

The literature shows examples of feedback loops that, involving TFs and miRs, regulate temporal coordination of stage transitions in one cell type (e.g. mir-223 in mammalian blood cell lineage), or amplify regulatory input that drives the decision towards one of two possible fates.
(e.g. neuronal or vulva cell fates in *C. elegans*). Direct hormonal involvement into microRNA feedback loop regulated differentiation processes has been shown in this work for *C. elegans* hypodermal cells, but not yet for the mammalian blood cell lineage differentiation. The results from the *C. elegans* model in this Chapter 2 might point the way for mammalian research on this area.

**Outlook**

### 2.6 Cell non-autonomy of microRNAs

The observed spatial separation of *mir-84* regulation and HBL-1 translational repression is most likely caused by either of three possibilities:

a) cell non-autonomous microRNA function

b) low level HBL-1 expression and repression in the seam cells

c) low level HBL-1 regulation in the hypodermal syncytium and signaling into seam cells

In order to test hypothesis a) of cell non-autonomous microRNAs activity, it is necessary to identify the mode of microRNA transportation between cells. The hypothesis is supported if inactivation of this transport mechanism leads to *let-7s* loss-of-function phenotypes in the seam cells while maintaining wild type like microRNA expression levels. In recent literature, two different transport mechanisms for small RNAs have been described, assuming that microRNA procession takes place inside the transcribing cell and not the target cell.

One cell-to-cell transport mechanism for small RNA molecules has been described in context of global RNAi effects in *C. elegans* that require the function of *sid-1* and *sid-2* proteins involved in uptake of small double stranded RNA molecules into cells (Winston et al., 2002; Winston et al., 2007). For these genes to be involved in the cell non-autonomous microRNA effect, the seam cell expressed microRNA would be required to get excreted and taken up by the hypodermis via a *sid*-protein based mechanism. However, *sid*-protein independent effects were also observed, and no genes have been assigned to these alternative functions.

A second cell-to-cell transport mechanism for small RNA molecules is hinted by the observation that endosomes or microvesicles described for mammalian cell culture contained not only protein coding mRNA, but also microRNAs (Valadi et al., 2007; Yuan et al., 2009).
Differences in RNA content between the vesicles and the cell they originate from suggests that regulated deposition of RNAs into the budding vesicles occurs.

An alternative approach for testing hypothesis a) is to do in situ hybridization for mature and precursor “pri-miRNAs” in seam cells and hypodermis tissue at the L3 stage. The hypothesis would be supported if mature microRNAs are detected in hypodermal syncytium and seam cells, while premature microRNAs are only detected in the seam cells. This can be further tested by knocking out the transport mediating genes and detecting mature microRNAs only in the same tissue as the pri-miRNAs. Those findings would be the first proof of microRNA function requiring directed microRNA transportation across cell membranes.

2.7 Ligand independent activation of DAF-12 / mir-84 promoter

The observed mir-84 reporter activation in a daf-12 ligand less background might be caused by ligand independent activation of DAF-12, or by alternative transcription factors like LIN-12/LAG-1.

DAF-12 trans-dependence can be tested by observing mir-84 seam cell expression of daf-7, daf-2 or daf-9(dh6) alleles in daf-12(rh61rh411) background: loss of ligand less activation in daf-12 null background strongly indicates daf-12 dependence. Cis-dependence on daf-12 response elements in the mir-84 promoter can be tested by observing a mir-84p::gfp reporter with all daf-12 REs point mutated and crossed into the above mentioned genetic backgrounds. Remaining seam cell GFP expression in daf-12 null background of wild type promoter reporter, but loss in mutant promoter reporter would argue for non-DAF-12 TF mediating the initially observed, ligand independent activation.

2.7.1 Alternative transcription factors: lin-12

Independent of DAF-12 dependence for the ligand-independent activation, mir-84 reporter activation in lin-12 gain- and loss-of-function mutant background needs to be established.

lin-12 gf activates let-7 precociously (Solomon et al., 2008), but mir-84 has partly redundant function with let-7 for terminal differentiation in the hypodermis (Hayes et al., 2006), and mir-84 might also be subject to lin-12 dependent activation. The influence of lin-12 onto mir-84 promoter regulation will be investigated in further detail in Chapter 3.12, while a possible connection of mir-84 and let-7 mediated terminal differentiation will be investigated in Chapter 4.
2.8 Feedback regulation by let-7

Epistasis places daf-12 upstream and downstream of let-7 family microRNAs, of which some are transcriptionally regulated by daf-12, making daf-12 in combination with its target microRNAs a candidate for feedback repression.

Feedback repression of daf-12 directly involving microRNA activity will modify daf-12 translation independently of transcriptional activation of the daf-12 promoter. Analysis of these parameters would require measurement of translation independent of transcription. Due to the tight control of spatial expression of let-7 family microRNAs, tissue specific measurement would help identify involved, partially redundant microRNAs. A technique to achieve this is tested in Appendix 3. The described reporter for translational repression needs to lose feedback-repression in deletion background of either the involved microRNAs, or daf-12 null background.

The biological function of the daf-12 feedback control can be revealed by looking at what phenotypes loss-of-feedback regulation has. This can be done by successively mutating let-7 binding sites in the daf-12-UTR of a gfp reporter construct, and testing it for an increase in expression in response to let-7 site mutations. Additionally, a construct containing the daf-12 genomic region instead of a gfp can be tested for in vivo phenotypes caused by let-7 site mutations and the resulting DAF-12 upregulation. Expected phenotypes include abnormal seam cell division, dauer formation or precocious terminal differentiation of the hypodermis – see Chapter 4.
3. Daf-12 activation of *cis*-regulatory elements in microRNA promoters
Chapter 2 showed how the nuclear hormone receptor DAF-12 directly activates microRNAs of the *let-7* family. Through this connection, global nuclear hormone receptor signaling exerts temporal and cell type specific control of microRNA gene activation. However, the observed dependence of microRNA promoter activation upon *daf-12* signaling was limited in many tissues, with most of the activation during developmental progression depending on non-DAF-12 TFs. In addition to that, some of the observed regulation dynamics contradicts the current model of dafachronic acid dependent activation of DAF-12 target genes. These observations prompted a more detailed investigation of the mechanisms of microRNA promoter activation by *daf-12* signaling and by alternative cascades or transcription factors.

Previous research has shown how the transcriptional activation of a gene is regulated by *trans*-regulatory modules ("TRM") i.e. the transcription factors (TF) and co-regulators that bind to *cis*-regulatory modules ("CRM") i.e. TF binding sites within the promoter region of the regulated gene (Davidson, 2006). In this chapter, specific CRMs were tested for activation by DAF-12 or non-DAF-12 TFs. This was done by using a new technique to isolate single CRMs out of the full length promoter, while enhancing their individual transcriptional activation of *in vivo* reporter assays. Each of the isolated CRMs was tested for transcriptional activation in genetic backgrounds of mutants either for *daf-12* signaling or alternative TFs candidate for *daf-12* signaling interaction. The aim was to identify tissues with high and low dependence on *daf-12* and hormone dependent regulation. A tissue with high *daf-12* signaling dependence is a candidate for showing phenotypes that connect *daf-12* signaling and the microRNAs to biological functions. Identification of non-DAF-12 TFs regulating microRNA transcription was expected to link their upstream cascade to the downstream acting microRNA promoters and functions and was hoped to reveal how these cascades intersect with *daf-12* mediated hormonal regulation.

The following section of the introduction reviews several key publications for the theory of promoter regulation in *C. elegans*, with several examples of *trans*- and *cis*- regulatory modules and their function as well as recently described factors involved in *let-7* family promoter regulation. This is done in order to build the background for a detailed analysis of DAF-12 and
hormone dependent and independent regulation of cis-regulatory modules present in promoters of let-7 family microRNAs.

The result section discusses possible new sequences for DAF-12 CRMs and presents experimental data of reporter activation by the CRMs from Chapter 2 after they were isolated out of full length promoter context. A candidate DAF-12 CRM from the let-7 promoter was investigated in the same way, and reporters for all isolated CRMs were tested in various genetic backgrounds, including new candidate daf-12 interacting.

3.1 Promoter regulation studied in C. elegans

C. elegans as a model organism offers several advantages for the study of transcriptional regulation. One important advantage is that the natural expression pattern of most C. elegans genes is sufficiently recapitulated by small promoter regions: Null mutants of most genes are rescued by re-introduction of a wild type copy that requires only 2kb of upstream promoter sequence to rescue the null allele (Okkema and Krause, 2005). Further more, stable transformation of reporter constructs into C. elegans by germline DNA injection is a fast and reliable process that requires less than two weeks to obtain strains that stably transmit the injected DNA as an extra chromosomal array (Fire, 1986). In addition to this, the C. elegans genome is simple and most functions are executed by non-redundant genes (Wood, 1988), so that the activation of a promoter in a specific tissue often relies on a single TF, and null mutant background for this TF causes a complete loss of activation (Flames and Hobert, 2009). Below I review some of the literature on transcriptional regulation in C. elegans.

3.2 Initial studies of the myo-2 promoter

Initial research on the mechanisms of promoter activation was done on the myosin heavy chain gene myo-2 that has yielded many important insights of which several will be mentioned here. The myo-2 promoter studies revealed that transcriptional activation in a specific tissue nis caused by defined, few hundred base pair long sequence elements in a promoter, and that several genes expressed in the same tissue share these elements in their promoters (Okkema et al., 1993). A combination of multiple, different CRMs, each bound and activated by a different TF, defines the "full length" expression profile of a promoter, and isolated fragments containing only a subset of CRMs activate expression only in a subset of tissues. In particular, enhancer elements smaller than 200bp were discovered that were able to direct transcription activation to the
downstream transcription start site on their own. By various in vitro binding experiments, ceh-22/homeobox domain protein (Okkema and Fire, 1994), TGF-beta signaling component daf-3/co-smad (Patterson et al., 1997; Thatcher et al., 1999) and pha-4/forkhead factor (Kalb et al., 1998) were identified as proteins binding and contributing to regulation of defined enhancer elements present within a 127bp stretch of the myo-2 promoter, called C183. It is important to notice that each factor is bound to its own individual CRM, the short sequence of DNA its DNA binding domain recognizes, and that the C183 contained multiple, seperated CRMs, each being bound by a different factors. In vivo regulatory interaction of those factors with the C183 element were validated for all three factors. A bioinformatic approach further revealed DAF-12 to bind and regulate elements in the myo-2 as well as the ceh-22 promoter (Ao et al., 2004). Interestingly, the identified daf-12 dependent cis-regulatory element, (CACACA) is different from the DAF-12-RE defined by Shostak (Shostak et al., 2004), (AGTGCA), the one used in this work. Deletion of this binding site decreased both, pharyngeal activation during normal growth conditions, as well as repression during dauer stage or in mutant backgrounds that set daf-12 signaling to repression rather then activation. Research like this revealed the combinatorial nature of specific promoter regions or CRMs and gave rise to the idea of the "gene battery".

3.3 The “gene battery” define the tissue characteristics

The character and properties of a terminally differentiated tissue are defined by the genes expressed in it, collectively described as a "gene battery" (Davidson, 2006). Promoters of genes belonging to this gene battery can share the same CRMs. Application of current laboratory techniques like microarrays and cell sorting techniques have helped to first identify the set of genes expressed in a specific tissue. Analysis of the promoter regions of these genes led to the identification of cis-regulatory promoter elements that are required for this tissue specific expression. These CRMs are not only conserved between genes of the same gene battery, but also between species as different as yeast and mammals. Of importance for this study is that the presence and function of a CRM contributes the gene to a gene battery, and subsequently to a biological function. The importance of this is reflected in two key publications involving daf-19/x-box binding factor and pha-4/forkhead factor.
3.3.1 DAF-19/x-box binding factor defines ciliated neurons

RFX-type transcription factors are highly conserved and involved in processes as distinct as DNA damage checkpoint regulation in yeast or MHC class II gene regulation in humans. They share a characteristic DNA binding domain distinct from other described DBDs that recognizes sequences called “x-box”, which are as well highly conserved. The *C. elegans* genome codes for one x-box binding factor, called “*daf-19*” due to its constitutive dauer forming phenotype. However, *daf-19* was initially discovered during a screen for mutations that affect dye-filling, a characteristic of ciliated sensory neurons in *C. elegans* (Perkins et al., 1986) that make up 60 out of a total of 302 hermaphrodite neurons. Loss of function mutants of *daf-19* cause a failure to form these cilia while other aspects of neuron morphology seem little unaffected, making *daf-19* the key factor for the induction of cilia formation (Swoboda et al., 2000). The sequence of the DNA recognition motif of human RFX factors was described (Emery et al., 1996; Reith et al., 1994), with very similar motifs present in promoters of known ciliated neuron expressed factors of *C. elegans*, leading to the identification of a consensus DAF-19-CRM. Reporter constructs containing a DAF-19-CRM were expressed in ciliated neurons, and expression was lost either by point mutating the DAF-19-CRM in the reporter or by crossing the wild type sequence reporter into *daf-19* null allele background. In an attempt to define a ciliary transcriptome, genome wide searches for genes bearing this consensus CRM in their promoter led to the discovery of many more candidate cilia related genes with a high percentage of experimentally validated positives (Blacque et al., 2005).

These observations underline the potency of a single factor that binds to well defined consensus DNA motifs spanning only few base pairs. These consensus CRMs then activate their target genes that define a precise morphological feature of their cell, in this case cilia formation. However, individual characteristics of variations of a consensus CRM can also have functional consequences, as was shown by the differences in function of variant forms of the CRM of the forkhead factor pha-4.

3.3.2 PHA-4/forkhead factor defines a specific organ

The *C. elegans* forkhead transcription factor PHA-4 is a homolog of human FoxA and acts as an organ identity gene defining the pharynx and the foregut. The precursor cells that during embryogenesis in wild type give raise to pharynx/forget cell lines fail to do so in loss of function alleles, while over expression of PHA-4 leads to an excess of pharyngeal/foregut cells (Horner et
The PHA-4 response element was defined with help of the homology to human FoxA response elements and was identified in nearly all members of the pharynx and foregut defining gene battery (Gaudet et al., 2004; Kalb et al., 1998). Pharyngeal expression of PHA-4 begins during early embryogenesis and lasts until late larval stages, a time span reaching from initial cell fate decisions to post embryogenic development. This long lasting presence of PHA-4 raises the question if all target genes of pha-4 are activated evenly during this time span or if gene battery members are regulated differentially and if this is still achieved through the PHA-4-CRM. The answer to this question came from analysis of promoters of the pharynx/foregut gene battery that revealed the presence of different variations of PHA-4-CRMs. These variations differed in PHA-4 binding affinity, with high affinity sites driving early expression and low affinity sites driving late activation (Gaudet et al., 2004). This way it is possible that different CRMs are activated by the same upstream factor and connect their promoter to the same upstream cascade, but can still maintain individual features that distinguishes their function from other CRMs for the same trans-acting factor, in this case differing in temporal onset of expression.

### 3.3.3 TTX-3 and CEH-10 homeodomain factors define gustatory neurons

Fragmentation of a single promoter can be reveal a CRM that activates expression in a specific tissue of interest. A bioinformatic search looking for genes that harbor this specific CRM in their promoter reveal other members of the gene battery that contribute to the characteristics of this tissue of interest, an approach more sensitive then forward genetics. Wenick and Hobert (Wenick and Hobert, 2004) used this approach in their work on the gene battery defining the gustatory AIY neuron in *C. elegans*, and the corresponding CRMs that were activated by the homedomain proteins *ttx-3* and *ceh-10*. During this study, the authors again showed that the architecture of promoters is highly modular, and that separate CRMs each activate transcription in a single type of cell or tissue. They also validated that within a promoter, separate CRMs behave in an additive manner to yield the spatial and temporal expression profile seen for the full length promoter. General observations were that the identified CRMs usually did not have repressive function. Nor did promoter truncations lead to expression in other tissue types than the full length promoter. A third observation was that most CRMs behaved in an enhancer like manner in that the orientation or distance to the transcription start site varied.
3.4 Repeat generation of defined promoter regions

This introduction chapter explained so far that CRMs connect promoters to upstream signaling cascades (3.2) and that genes required to define the characteristics of a tissue or an organs are grouped into a “gene battery” with all members of this gene battery having the same CRMs (3.3) in their promoters. One factor can be sufficient to drive a specific gene battery to define morphological features of a tissue or a whole organ (3.3.1). But despite the sequence similarities of a consensus CRM for a specific trans-factor, individual CRMs still have individual function (3.3.2). Single CRMs only partially recapitulate the expression pattern of a full length promoter, while each additional CRM for the same trans-factor increases the intensity of the promoter activation (3.3.3). A consensus CRM for DAF-12 was discussed in Chapter 2, but daf-12 signaling alone does not define a specific tissue or organ, but rather influences activation of many gene batteries according to animal wide dafachronic acid signaling activity and developmental fate choices.

This DAF-12 and dafachronic acid dependence of DAF-12-CRMs is one of the two main topics of this chapter. The other point of interest is to find other TFs and signaling cascades that connect to either DAF-12-CRMs or CRMs in close proximity.

To address the first topic, genomic promoter regions were isolated to in vivo investigate the transcriptional activation DAF-12-CRMs in context of their direct adjacent sequences and CRMs.

The second topic is investigated by crossing reporters for these isolated CRMs into mutant backgrounds of signaling cascades that are candidate to functionally interact with DAF-12 signaling. The hope is to connect the microRNAs to new cascades via those new trans- or cis-connections.

Since Chapter 2 shows that DAF-12 influence on the investigated promoters is limited, a low transcriptional activation of an isolated DAF-12-CRM alone is expected that requires an enhancement of this transcriptional activation. Previous observations (Wenick and Hobert, 2004) showed an additive effect of CRMs onto overall promoter activation, indicating that generation of multiple tandem repeats of short promoter fragment covering the CRM of interest could already lead to an increase in signal output of a one specific CRM in its genomic sequence environment.
In order to generate two-, four- or six-fold repeats of any genomic sequence in a time efficient way, a PCR technique was established that rapidly generates repeats from genomic DNA templates arranged in identical orientation. Those PCR-generated repeats were placed upstream of a pes-10 minimal promoter (Fire et al., 1990) that provides a transcription start site able to recruit the basal transcription apparatus, followed by a yfp coding region.

3.5 Transcription factors influencing expression of let-7 family microRNAs
In the this part of the introduction I will introduce the promoter characteristics to be investigated in this chapter, and TFs that are candidates for daf-12 signaling interaction on the let-7 microRNA family promoters.

With the importance and function of CRMs in mind, it becomes clear that the interaction of trans-acting factors (TFs) with CRMs of the let-7s microRNA promoters will show how daf-12 dependent and independent upstream signaling converges onto the let-7s microRNAs. Several of these DAF-12-independent trans-acting factors and CRMs for the let-7 family promoters have already been described and will be discussed further below.

3.5.1 Tissue specificity of mir-84 promoter regulation
In Chapter 2.4.2, mir-84 promoter reporter regulation in seam cells and pharynx was discussed and how the regulation in the seam cells, but not the pharynx, contradicts the current idea of ligand dependent DAF-12 activation. This chapter describes experiments that try to find if the unexpected seam cell regulation originates from a distinct cis-regulatory module than the one responsible for the pharyngeal regulation, and if DAF-12 or non-DAF-12 TFs act through those CRMs or CRMs in their direct proximity.

3.5.2 Daf-12 independent activation of the mir-241 promoter
Chapter 2.1.2.1 showed that a reporter with the mir-241 promoter fused to gfp continues to be expressed in many tissues like neurons, body muscle, excretory cells even in absence of daf-12 signaling. The remaining "background" activation was interpreted as not DAF-12 dependent, indicating that non-DAF-12-TFs can activate CRMs in the mir-241 promoter region. To test if these CRMs are the same or different from DAF-12-like CRMs, DAF-12-like CRMs were isolated from the remaining promoter sequence to either separate background and daf-12 dependent activation or to reavel if these isolated CRMs respond to both daf-12 dependent and independent activation.
3.5.3 **DAF-12 influence on the let-7 promoter**

Several experiments discussed in Chapter 2.5 indicate that DAF-12 acts upstream as well as downstream of let-7, and might be part of a feedback regulation loop involving let-7. This ambiguity could be solved by showing that DAF-12 is capable of directly regulating CRMs of the let-7 promoter.

3.5.4 **FLYWCH transcription factors regulate microRNA promoters**

Ow and Ambros (Ow et al., 2008) identified by yeast-one-hybrid assays three FLYWCH zinc finger protein like TFs that bind promoters of microRNAs *lin-4*, *mir-48* and *mir-241*. Simultaneous knock-out of multiple FLYWCH TFs lead to precocious expression of microRNA repression targets, e.g. *lin-14*, but also embryonic lethality. To find heterochronic phenotypes further downstream in the pathway, the authors monitored seam cell proliferation and expression level of specific collagen genes that mark the adult stage. However, they were unable to record FLYWCH gene dependent heterochronic phenotypes. This indicates that the FLYWCH TF regulation of the *let-7s* microRNAs does not play an essential role in miR target regulation.

The identified FLYWCH CRMs are located in close proximity to DAF-12 regulated CRMs described in Chapter 2, indicating a possible functional interaction with daf-12 signaling. This was the reason to test RNAi knock-out of *flywch* genes in daf-12 signaling background for synthetic phenotypes.

3.5.5 **Daf-12/nhr regulates microRNA promoters**

Regulatory influence of DAF-12 on promoters of the *let-7* microRNA family was already discussed in Chapter 2.5.

3.5.6 **Lin-12/notch regulates microRNA promoters**

Regulatory influence of *lin-12* on promoters of the *let-7* microRNA family was already discussed in Chapter 2.5.1.

### Results

3.6 **DAF-12 dependent cis-regulatory elements**

The first canonical DAF-12 RE (referred to as “Shostak-RE”) sequence was defined by Shostak et al through *in vitro* DNA affinity assays of an immobilized DAF-12 DNA binding domain (Shostak et al., 2004). The authors showed *in vivo* DAF-12 dependent activation of promoters containing this RE and I was able to verify the function of these Shostak-REs in
Chapter 2. However, the REs present in the tested microRNA promoters differed from the conserved core of the Shostak-RE. This resulted in a changed canonical RE that I from now on refer to as “DAF-12-like CRM” (Figure 21), which I try to detect and analyze in the promoters of members of the let-7 family of microRNAs.

After discussing the results of previously analysed protein coding genes, and before presenting the results from my work on microRNA promoters, I will shortly discuss the reason for choosing specific promoters to analyse, namely promoters of mir-84, mir-241 and let-7.

Figure 21: DAF-12-like CRM
Validated DAF-12 response elements from Chapter 2 were combined using the “Weblogo” tool to give the canonical sequence of the DAF-12-like cis-regulatory module. The previously published Shostak-sequence (Shostak et al., 2004) is overlayed in red, while the DAF-12-like CRM resulting from this study (Bethke et al., 2009) is overlayed in green.

3.7 Repeat generating PCR strategy
Promoter stretches between 100 and 150bp length surrounding DAF-12-like CRMs were isolated out of the promoters of mir-84, mir-241 and let-7 and converted to multiple repeats to enhance signal intensity. For this repeat generation via PCR, a novel method was used based on primers that contain the 5' and 3'tag DNA sequence juxtaposed. Inclusion of this tag renders the PCR product capable of self-priming after completion of the first round for amplification of the genomic DNA template to generate repeats of the desired length (Figure 22).
Figure 22: Repeat-generation PCR scheme
Colorcoded regions show homolog sequences independent of their orientation. Primers bind to the template DNA and define the content and the length of the repeated promotor fragment. 5’ tags of the primers were chosen to bind to the sequence at the opposite end of the targeted promotor fragment, allowing the initial PCR product to self-prime and generate repeats with a defined orientation (A). PCR product was separated by agarose gel electrophoreses, DNA with the length of the desired repeat length was gel extracted (B) and sub-clonated into TOPO vector,
where it was excised with restriction enzymes cutting on both sides of the insert (C). The result
of the sequencing reaction of the vector containing a three fold repeat of the 84e element shows
the orientation of the color coded primer regions within the generated repeat (D). Red highlights
the Shostak-REs for DAF-12.

3.8 Repeats of isolated mir-84 promoter regions

The PCR repeat generation was used on two 120bp long stretches of the mir-84 promoter
surrounding the 84d or the 84e elements (following page: Figure 23) to generate repeats of
different repeat lengths.

3.8.1 The nomenclature of CRMs within the mir-84 promoter

The nomenclature of CRMs within the mir-84 promoter differs between Chapter 2 and 3
because the promoter region analyzed changed during the course of this work (following page:
Figure 23). The initial luciferase assay was performed on a 2.8kb promoter element, as described
by Esquela-Kerscher (Esquela-Kerscher et al., 2005). The worm line transgenic with the 2.8kb
promoter gfp reporter lacked seam cell expression, while a 1kb and a >8kb mir-84 promoter
reporter showed similar seam cell expression (Hayes et al., 2006). Therefore, worm lines were
chosen that are transgenic with the described 1kb promoter reporter. Two Shostak-REs were
identified within the initially used 2.8kb promoter fragment, and site directed mutagenesis of
these two sites showed 3x reduced DAF-12 and ligand dependent activation in luciferase assay
(see Chapter 2, Figure 7). The 1kb promoter used for the in vivo gfp reporter construct contained
one of those two Shostak-REs, but site directed mutagenesis did not severely affect in vivo
reporter activation according to observation by eye in n>10 worms. Reanalysis of the 2.8kb
promoter identified five Shostak-REs, of which two - named 84d and 84e (following page:
Figure 23 and Figure 24) - fall into the 1kb promoter fragment present in the in vivo reporter
construct. Worm strains transgenic with a reporter that has these two Shostak-REs point mutated
are pending detailed analysis if they indeed loose DAF-12 dependent transcriptional activation.

following page: Figure 23: CRMs of the mir-84 promoter
A 2.8kb and a 1kb promoter region of the mir-84 microRNA were used in this study. DAF-12-like
and other CRMs are marked in red, with an arrow indicating the CRM orientation. Note that
the “84a” from Chapter 2 is now called “84e” , and the “84b” element of Chapter 2 is now called
“84e”.
3.8.2 Increased repeat number enhances activation

To test the principle that increased repeat number increases promoter activation, worm lines were generated that are transgenic with constructs containing one-, two- or a four-fold repeat of the 84e element (Figure 24 A). The 1x84e gave very low GFP intensity in the pharynx, that was increased by 2x84e, and even further increased by 4x84e. The 4x84e is an extremely potent transcriptional activator, of which germline injection of only 2.5ng/ul DNA concentration resulted in transgenic lines with high GFP intensity. Injection of higher concentrations led to no transgenic offspring - this was probably caused by a lethal phenotype due to sequestration of vital TFs away from their endogenous target genes to the 4x84e DNA present in high dose. Conclusion was that increased number of repeats of a CRM strongly increases expression level, facilitating the analysis of tissue specific expression and regulation.

3.8.3 84d and 84e repeat reporters

The 4x84e reporter is expressed only in the pharynx (Figure 24 A). In comparison to this, a 4x84e and a 6x84 reporter were analysed, of which the 6x84e reporter was oriented in reverse orientation to the 4x84e reporter. The 6x84e reporter showed no obviously different tissue specificity than the 4x84e reporter (n>100 animals, observation by eye). In contrast to the 4x84e reporter, the 4x/6x84d reporters lack expression in the pharynx, and instead shows weak expression in excretory cell, and rectal gland under favorable growth conditions (Figure 24 B-F). However, under dauer inducing conditions, the 4x/6x84d reporter showed strong seam cell expression in preliminary experiments (Figure 24 G), and starved worms show a strong vulva expression after refeeding (Figure 24 H).

Consistent with the full length reporter construct, the 4x84e reporter does not show strong DAF-12 dependence, pointing to non-DAF-12 TRMs being responsible for expression in this specific tissue. Candidate TFs for this function will be discussed later in this chapter.
Figure 24: CRM repeats of the *mir-84* promoter

Sequence of the 84d and 84e elements and their location within the 1kb full length promoter of the *mir-84p::gfp* reporter used in Chapter 2. Red highlights the Shostak-REs for DAF-12. Repeated promoter elements are fused to a *pes-10* minimal promoter (Fire et al., 1990) and a *yfp* reporter gene. Expression of the 84e reporters *in vivo* increases drastically with increasing numbers repeated 84e elements. 4x/6x84e show strong activation in the pharynx only (= ph, A) while 6x84d was expressed weaker and in more cell types: neurons (= neu, B), excretory cell (= exc duct, B), hypodermis (= hyp, C), vulva precursor cells (= vulva prec, D), gonad (E), rectal gland and tail (F), but not in the pharynx (= ph, B). Dauer worms showed strong expression in seam cells (G) and stared worms show strong expression in vulva cells (H) after re-feeding. Panels D, E and H show 4x84e, while panels B, C, F, G show a 6x84e reporter that is oriented in opposite orientation. Change of orientation did not have any obvious effect on expression.
pattern. Expression in each shown tissue was observed in n>10 animals and representative images are shown.

3.9 Repeats of isolated mir-241 promoter regions

Two repeats were generated of a 150bp region surrounding 241ab and a 100bp region surrounding 241cd.

The 4x241ab reporter shows expression only in the excretory cell and a few head neurons (following page: Figure 25 A). The excretory duct expression is completely abolished in *daf-12* null mutant background (following page: Figure 25 B) and is therefore 100% *daf-12* signaling dependent, while in *daf-9;din-1* double null mutant background it is low. Ligand supplementation to the latter genotype induced a strictly ligand dose dependent increase of expression level, while very high ligand dose led to a total loss of expression (following page: Figure 25 C). The 2x241cd reporter showed expression only in the pharynx and the gut cells (following page: Figure 25 D), with an incomplete DAF-12 dependence in *daf-12* null mutant background (following page: Figure 25 E), and a moderate ligand dependent rescue of the decreased expression in a *daf-9;din-1* double null mutant background.

following page: Figure 25: CRM repeats of the mir-241 promoter
Sequence of the 241ab and 241cd elements and their location within the 2kb full length promoter of the *mir-241p::gfp* reporter used in Chapter 2. Red highlights the Shostak-REs for DAF-12. Repeated promoter elements are fused to a *pes-10* minimal promoter (Fire et al., 1990) and a *yfp* reporter gene. The 4x241ab reporter is expressed in the excretory cell (exc, white arrows) and several head neurons (A), while excretory cell expression is completely lost in *daf-12(rh61rh411)* null mutants (B). In a *daf-9;din-1* double null background, expression level is dependent on ligand dose (C). The 2x241cd reporter is expressed in the pharynx and the gut (C), and shows moderate decrease of expression level in *daf-12* null background (D) compared to wild type. Binding site of the *flh-1* between the 241a and 241b elements discussed below is shown in blue. Expression in each shown tissue was observed in n>20 animals with one replicate and representative images are shown.
3.10 Let-7 TRE reporter

Luciferase assays discussed in Chapter 2 show a very low absolute activation of the let-7 promoter for all four conditions tested (Figure 26 A). However, plotting the fold induction of ligand supplemented condition versus control indicated a 10 fold DAF-12 and ligand dependent transcriptional activation of the tested let-7 promoter fragment (Figure 26 B).

![Figure 26: Re-analysed luciferase assay of the let-7 promoter](image)

Plotting the data presented in Figure 7 with decreased unit size of the y-axis (A) reveals DAF-12 and ligand dependent activation of the let-7 promoter construct in terms of absolute activation and ligand dependent fold induction (B).

The TRE element was identified as an important CRM for let-7 transcriptional regulation (Johnson et al., 2003) (Figure 27 A). Closer examination reveals similarities of the TRE to the Shostak daf-12 RE (Figure 27 B).

![Figure 27: Let-7 "TRE" element](image)

A let-7 promoter element that is important for let-7 reporter expression was called “temporal regulatory element” or “TRE” (Johnson et al., 2003) (A), and can also be interpreted as a direct repeat of two DAF-12-like CRMs with 5 nucleotides pacing (DR5) (B).
EMSA with oligos containing wild type TRE sequence showed weak DAF-12 specific shift signals that were supershifted by antibody incubation and competed away by incubation with cold competitor oligo. Oligos with a TRE mutated to the same sequence shown to abolish DAF-12 DNA interaction in Chapter 1 failed to show any shift signal, while oligos with a TRE sequence mutated into a perfect Shostak-RE revealed strongly enhanced DAF-12 specific shift signals (Figure 28).

**Figure 28: EMSA of TRE-element**
Nuclear extract of cells expressing Flag-tagged DAF-12 induced a shift (s) of TRE element containing, radio labeled oligos. Weak shift (s) and super shift (ss) signals are obtained with the wild type TRE sequence, that are lost completely when the TRE site is mutated to a validated non-DAF-12-interacting sequence ("mut TRE -"), while they are strongly enhanced by mutating the TRE into a canonical DAF-12-like CRM sequence ("mut TRE +"). Grey bars represent a dose increase of the non-labeled competitor oligos. Green letters indicate DAF-12-REs while red letters represent mutated nucleotides. EMSA was done once.
To test for \textit{in vivo} tissue specificity of the TRE-CRM, a 4xTRE reporter strain was generated and found expression in head neurons, weak expression in seam cells, and very strong expression in the rectal gland (Figure 27).

\textbf{Figure 29: CRM repeat of the TRE}
Sequence of the TRE element and their location within 2kb of promoter. Red highlights the Shostak-REs for DAF-12. Repeated promoter elements are fused to a \textit{pes-10} minimal promoter (Fire et al., 1990) and a \textit{yfp} reporter gene. The 4xTRE reporter is expressed in excretory duct (= exc duct, A), neurons (=neu, A), seam cells (= seam, B), and strongest in the rectal gland and tail (= rect / tail, C). Expression in each shown tissue was observed in n>10 animals and representative images are shown.

Preliminary rectal gland expression analysis of this reporter construct in DAF-12 null background (observation by eye, n>10 animals) did not reveal strong expression level changes, pointing again towards non-DAF-12 TRMs as the main source of activation in rectal gland expression. A \textit{daf-12} dependent regulation of the 4xTRE would link the regulation to heterochronic phenotypes of \textit{let-7} and the effects of \textit{daf-12} null mutant background on reporter expression in this tissue remain to be tested.

\subsection{3.11 Conserved elements in analyzed promoters}
The above described DNA elements were chosen with the aim of isolating DAF-12 dependent \textit{cis}-regulatory elements, and therefore are located around the identified DAF-12 binding sites. By extending the searching to binding motives that were assigned previously to TFs other then
DAF-12, several genes were identified as candidates for interaction with CRMs present in the 84e element.

3.11.1 Daf-3/co-smad
A DAF-3-like CRM present in the 84e element is similar to a CRM described for the myo-2 promoter (Horner et al., 1998; Thatcher et al., 1999) (following page: Figure 23). Pharyngeal expression of the repeat reporter overlaps with the expression pattern of daf-3 regulation target myo-2 and also connects the repeat reporter expression pattern to a tissue with daf-3 dauer regulation function (Patterson et al., 1997). Based on those findings, daf-3(e1376) mutant allele background was expected to cause a loss or gain of 4x84e reporter expression level during developmental progression or arrest. However, preliminary analysis (observation by eye, n>10 animals) indicated no change of expression level in mutant background compared to wild type.

3.11.2 Forkhead transcription factors pha-4 and daf-16
A potential forkhead transcription factor binding site was also identified (following page: Figure 23). Loss-of-function of forkhead factor pha-4 leads to a loss of pharynx formation (Horner et al., 1998), and pharyngeal expression of several reporter constructs was shown to be PHA-4 mediated (Gaudet et al., 2004). Forkhead factor daf-16 is an important dauer regulator with expression and function in the pharynx (Ogg et al., 1997). Previous studies, using in vitro binding experiments with degenerated oligos found a 8nt long sequence of the elements that DAF-16 and three mammalian forkhead factors can physically bind. However, in 25 of 25 cases, the identified sequence differed in the most 3' nucleotide from the one present in the 84e element (Furuyama et al., 2000). To still test influence of daf-16 null mutant background, the 4x84e reporter was rossed into daf-16(mu86) background and compared reporter expression level to that observed in wild type. Preliminary analysis (observation by eye, n>10 animals) indicated no clear loss or gain of reporter expression level in daf-16(mu6) mutant background during normal growth. Promoter activation in pha-4 knock-out background still remains to be tested.

3.11.3 Ceh-22/homeobox domain
The homeobox domain protein CEH-22 regulates promoter activity in the pharynx. It binds to DNA sequences very similar to the canonical DAF-12 REs (Okkema and Fire, 1994) (Figure 30). CEH-22 function as well as activation of DAF-12-like CRMs 241cd and 84e all locate to the
pharynx, which raise the possibility of regulatory CEH-22 and DAF-12 interaction on DAF-12-like CRMs in this tissue.

**Figure 30: CEH-22 and DAF-12 response elements**
A sequence protected from DNaseI degradation by CEH-22 protein incubation (Okkema and Fire, 1994) (below) shows high similarity to the consensus DAF-12-like CRM when compared to the Shostak-RE sequence and the sequence defined in Figure 21 (above).

### 3.12 Lin-12/notch receptor
Other TFs are interaction candidates not based on CRM sequence but on functional connection to microRNA regulation. Solomon et al describe heterochronic seam cell phenotypes of the lin-12/notch gain-of-function (gf) allele n137n460 discussed in Chapter 2.7.1 (Solomon et al., 2008). The authors observed increased let-7 promoter reporter expression in the seam cells, accompanied by a precocious terminal differentiation phenotype in the hypodermis. This precocious hypodermal differentiation is also known to involve mir-84 function (Hayes et al., 2006). A second link of lin-12 to mir-84 is the overlapping expression of mir-84 CRM reporter 4x84d in vulva precursor cells (Figure 24 D) and reported lin-12 dependent activation of mir-61 (Solomon et al., 2008). For those reasons, the reporter 4x84d and 4xTRE were crossed into the lin-12(n137n460) gf mutation background and tested for lin-12gf regulatory effects on hypodermal expression. Both reporters and genotypes were assayed at the non-permissive temperature of 15°C during food availability. Preliminary observations indicate that daf-12 null
(n=15) and lin-12gf (n=5) background decreased expression of the 4x84d CRM reporter during L3 stage in seam cells, vulva precursor cells and cells of the excretory system when compared to wild type background (n=33). The 4xTRE CRM reporter seemed to be unaffected in daf-12 null background (n=8), while lin-12gf background during L4 stage (n=13) decreased the number of animals expressing the reporter in the seam cells compared to wild type. This decrease of the 4xTRE is unexpected, since published phenotype and full length promoter reporter regulation in line-12gf allele suggested to expect an increase. However, these are only preliminary observations and the experiments need to be repeated to give conclusive results. Other growth conditions need to be assayed in addition to this, since strong seam cell expression was only observed in dauer larvae and strong vulva expression was only observed in L4 worms recovering from starvation. The whole regulation dynamics of dauer / starvation recovery has not been investigated, conditions under which the mir-84 full length promoter displays very interesting regulatory dynamics (see Chapter 2.4.2. and 2.7).

3.13 FLYWCH transcription factors flh-1, flh-2, flh-3

Previous publications describe that flh-1(bc374) loss-of-function leads to precocious expression of mir-241 (Ow et al., 2008). The identified FLYWICH-CRM is located between the 241a and 241b sites of the 150bp long promoter sequence of the 241ab repeat (following page: Figure 25), and it resembles closely a CRM identified to be present in all C. elegans microRNA promoters (Ohler et al., 2004). The co-localization of the CRMs makes it appear likely that this is the place where FLYWCH and DAF-12 signaling converge. For those reasons it was decided to test the influence of flh-gene knock-down onto reporter constructs of DAF-12 dependent and independent 241ab, 241cd, 84d and TRE CRM repeats. Knock-down of either one or both TFs regulating the CRMs was expected to cause a change in expression intensity or pattern. However, worms transgenic with either of those reporter constructs grown on flh-1, flh-2, flh-3, or flh-1/flh-2 double RNAi (observation by eye, n>20 per condition) did not show striking changes in expression pattern when compared to the same worm lines grown on L4440 empty RNAi vector control. To observe a reporter regulation effect, a more precise measurement of GFP expression level might be required. Another possibility is that RNAi might decrease flh-1 dose insufficiently to generate a reporter phenotype.
Discussion

3.14 Regulation of CRMS present in the mir-84 and the mir-241 promoters

Daf-12 signaling is not the only signaling cascade regulating the let-7 family microRNA promoters. A DAF-12-like CRMs of 100 to 150bp length was isolated to test for tissue specificity and dependency of transcriptional activation onto DAF-12 or non-DAF-12 TFs. Increasing repeat length clearly enhanced the reporter signal of the DAF-12-like CRM constructs and facilitated analysis. With the help of these constructs I found that isolated DAF-12-like CRMs activate transcription only in a subset of tissues compared to the full length promoter. DAF-12 and ligand dependence of transcriptional activation of those isolated DAF-12-like CRMs greatly varied. Complete dependence was observed for the 241ab-CRM expressed in the excretory cell, while pharyngeal and gut expression of the 241cd-CRM and pharynx expression of the 84e-CRM show only partial dependence, but reflects tissue specific recapitulation of full length promoter regulation. The same seems to be true for the 84d-CRM, that appears recapitulate some of the mir-84 regulation aspects described in 2.1.2.4 and Figure 17. No obvious daf-12 dependence was found for the TRE-CRM expressed a.o. in seam cells and rectal gland. This led to the assumption that other TFs regulated by other cascades activate those CRMs, and two ways of determining the importance of the observed activation were tested: knock-down of candidate TFs discussed below and analysis of promoter sequence conservation presented in Appendix 5.

The chosen strategy of CRM-isolation and amplification appears to be successful for the mir-84 promoter in separating two very distinct regulatory pattern, namely pharyngeal and seam cell regulation, as well for the mir-241 promoter, where it successfully isolated DAF-12 and dafachronic acid dependent regulation, namely regulation in the excretory cell, away from other, hormone independently activating factors. These regulatory functions of the full length promoters can now be assigned to specific CRMs, given that the preliminary results can be validated by repeated experiments.

3.15 Candidate TFs for let-7 family microRNA regulation

Several TFs were tested that are, based on prior publications, candidates to regulate let-7 family promoters. Several TFs are candidates to directly bind to DAF-12- and non-DAF-12-like CRMs present in the repeated promoter sequences, while others were found to have a strong functional connection to let-7 microRNA promoter regulation. Candidates for non-DAF-12-like CRM binding are DAF-3/co-smad, DAF-16/foxo, PHA-4/foxo and FLYWCH TFs. Candidate
for interaction with DAF-12-like CRMs is CEH-22/homodomain protein, and TF connected via function to let-7 family promoter regulation is LIN-12/notch. However, daf-3/co-smad, daf-16/foxo and flywch mutant alleles as well as RNAi knock-down did not noticeably affect reporter activation and might need to be re-examined in more stringent ways, while candidates pha-4/foxo, ceh-22/homeodomain protein remain to be tested. Preliminary observations for lin-12/notch dependent regulation will be discussed below.

3.15.1 Lin-12/NOTCH receptor is a candidate for daf-12 interaction

The notch receptor lin-12 has important functions in vulva differentiation and uterus-seam connection (Greenwald et al., 1983), dauer recovery (Ouellet et al., 2008) and terminal differentiation of the hypodermis (see Chapter 2.4.3 and 2.7.1), the latter by activating expression of the let-7 microRNA (Solomon et al., 2008). Several observations made during this work suggest potential interaction of mir-84 and lin-12. Q-PCR data indicates that mir-84 is the only let-7 family member to be up-regulated during and directly after induction of dauer recovery (Figure 16). Based on the observations of strong dauer-related up-regulation in the seam cells of reporters for both, mir-84 full length promoter (Figure 17 G-M) as well as the 4x/6x84d CRM (Figure 24 G), the expectation is that the up-regulation observed by Q-PCR to take place in the hypodermis - suggesting a function for mir-84 during dauer recovery in the seam cells. Mir-84 together with let-7 drive terminal differentiation in the hypodermis (Hayes et al., 2006), further connecting mir-84 to this process. Activation of the mir-84 promoter during these lin-12 influenced processes in lin-12 target tissues makes functional interaction possible, and suggests that lin-12 might act through not only regulating let-7, but also mir-84 to drive developmental progression and terminal differentiation of the hypodermis. Preliminary observations presented in Chapter 3.12 underline these expectations but need to be repeated.

LIN-12 drives transcriptional activation by interaction with the DNA binding LAG-1. LAG-1 binds to DNA sequence RTGGGA (Christensen et al., 1996), but these sites are absent from the repeated promoter fragments. However, presence of several LAG-1 binding sites in the ins-18 promoter that were conserved in C. briggsae did not result in lag-1 dependent reporter construct regulation (Ouellet et al., 2008), raising the possibility of a TRTGGGA-CRM independent regulation function of lag-1. This still leaves room for a direct or indirect lin-12 regulation of these CRMs that require further investigation.
3.15.2 Direct let-7 promoter regulation through DAF-12

EMSA of the DR5 containing TRE CRM shows a much weaker DAF-12 binding to DNA than observed for tested CRMs of the mir-241 promoter. However, the full length let-7 does induce a DAF-12 and ligand dependent transcriptional activation in luciferase assays. These two findings support a direct interaction of DAF-12 with the let-7 promoter. However, preliminary data presented in Chapter 3.12 rather disproof a daf-12, and suggest a lin-12 involvement, pending further analysis.

Also interesting is the low over-all luciferase activity specifically observed for the let-7 promoter. This could be interpreted as endogenous mammalian factors present in the Human Embryonic Kidney Cells binding and suppressing the promoter activation, possibly through a high degree of conservation of CRM / TRF couples, as observed by Flames (Flames and Hobert, 2009). Loss of this activation after point mutation of the TRE CRM would clarify if DAF-12 activates the let-7 promoter by interacting with the TRE.

Outlook

The reporter constructs described in this chapter still need to be accurately analyzed for loss of expression intensity relative to wild type background when loss-of-function is induced for the following genes:

3.16 Connecting CRMs to signaling cascades

Let-7 microRNA family regulation has so far not been connected to TGF-beta signaling directly, only through TGF-beta influence on dafachronic acid synthesis and DAF-12 activity. Identifying the potential daf-3/co-smad binding site adjacent to the 84e CRM (following page: Figure 23 and Figure 45) as an actually functional one of would show this direct connection of TGF-beta downstream signaling and let-7 family microRNA regulation.

Forkhead transcription factors play an important role in developmental regulation, insulin signaling, stress resistance and longevity. However, no daf-16/foxo or pha-4/foxo forkhead factor dependence has been shown to date. Identifying the potential forkhead binding site adjacent to the 84e CRM (following page: Figure 23 and Figure 45) as actually functional would directly connect forkhead transcription factors to let-7 microRNA regulation, a direct connection that has not yet been described, either.

A functional, linear interaction of ceh-22/homoedomain protein and DAF-12 has already been described in the pharynx (Ao et al., 2004). Finding that a canonical DAF-12-CRM (Figure 30)
has a very similar sequence to a CEH-22-CRMas regulation would indicate that these two factors also can act in parallel to regulate the same gene battery, connecting homeo domain protein function to DAF-12 hormonal control. This possible connection needs to be established by showing direct CEH-22 can interact with the DAF-12-like CRMs by EMSA.

A flh-1, -2, -3 flywch gene regulation would validate already published gene functions (Ow et al., 2008).

3.17 Lin-12 in terminal differentiation
The let-7 regulation through lin-12 described previously (Solomon et al., 2008) together with the gfp reporter regulation of the 84e CRM repeat shown in Figure 24 indicate a possible functional interaction of lin-12 with mir-84 as well. This interaction needs to be analyzed in a lin-12(n137n460) gain-of-function mutant background, with an increase in mir-84 promoter activation as expected outcome. This lin-12 dependent activation would connect lin-12 to mir-84 regulation and complement the let-7/mir-84 mediated suppression of larval programs described earlier (Frand et al., 2005; Hayes et al., 2006; Ouellet et al., 2008). Testing a lin-12(n137n460);daf-12(rh61rh411) for suppression of precocious alae formation of lin-12 alone would directly establish epistasis between the these two factors.

Positive results of any of the suggested experiments would also make lin-12 a candidate for influencing the unexpected ligand independent DAF-12 activation of the mir-84 promoter in the seam cells observed in Chapter 2.1.2.4.

3.18 Daf-12 in terminal differentiation
I showed luciferase assay (Figure 26) and EMSA (Figure 28) experiments that indicate a direct DAF-12 interaction with the let-7 promoter. If daf-12 influence onto the let-7 promoter is mediated by direct interaction with the TRE (Johnson et al., 2003), DAF-12 and hormone dependent luciferase activation shown in Figure 26 should be abolished by point-mutating the TRE sites from the full length promoter. This would connect daf-12 directly to the let-7 mediated terminal differentiation of the hypodermis, in addition to the already established role of daf-12 in hypodermal seam cell differentiation during L2 to L3 stage transition.
4. DAF-12 and its co-regulator LIN-29 form a “molecular switch” driving terminal differentiation of the hypodermis.

Linyan Meng contributed data to Figure 31, Figure 33 and Figure 34.
Introduction

DAF-12 is ubiquitously expressed, but transcriptional activation of DAF-12 target genes described in Chapter 2 and 3 shows extensive tissue specificity that is brought about by the promoter sequences. Trans-regulatory modules (TRMs = TFs) that act on different cis-regulatory modules (TF binding elements in promoters of regulated proteins, called CRMs) present in the promoter have been connected to tissue specific promoter activation. However, tissue specific differences in the cellular co-regulator pool can also modulate the transcriptional output of a TF (O'Malley, 2006) in a tissue specific way. For this reason, it was investigated to what extend tissue specific and temporally coordinated changes in transcriptional output of DAF-12 are caused by co-regulators influencing DAF-12, perhaps even independent of dafachronic acid.

The only known DAF-12 co-regulator is co-repressor DIN-1 (Ludewig et al., 2004), but DAF-12 also interacts in vitro with human co-activator SRC-1 (Motola et al., 2006). However, no C. elegans co-activator for DAF-12 has been described so far. DAF-12 and its ligand dependent activation is implicated in heterochronic regulation during L2 to L3 stage transitions (Antebi et al., 1998), when let-7 family microRNAs, as its direct targets, also play a role during larva-to-adult transition (Hayes et al., 2006). For these functional roles of DAF-12, potential DAF-12 co-regulators were expected to also be involved in heterochronic regulation and to exhibit heterochronic phenotypes.

The chosen candidate, LIN-29, is a zinc finger protein with homology to mammalian EGR2. Lin-29 null alleles continue to execute larval programs during the adult stage, for example continued seam cell division and molting, interpreted as failure of terminal differentiation in hypodermis and vulva (Liu et al., 1995; Rougvie and Ambros, 1995). Loss-of-function of let-7 leads to similar phenotypes as lin-29 null, a constellation similar to the seam cell division pattern of daf-12 null alleles and the let-7s (mir-48, -84, -241) microRNA deletions discussed in Chapter 2 that were explained by DAF-12 directly activating the let-7s. One of the let-7s microRNAs, mir-84, was recently connected to hypodermal terminal differentiation as well. Hayes et al. found that molting phenotypes of hypomorphic let-7 alleles are enhanced by further loss-of-function of mir-84, while over-expression of mir-84 not only rescues let-7 hypomorph phenotype, but also the molting phenotype of lin-29 null alleles (Hayes et al., 2006). This already connects one direct
target of *daf-12* during L3 to the L4 to adult transition. However, the *mir-84* phenotypes are much weaker than those of *let-7* or *lin-29*, suggesting a smaller role of *mir-84* in this process.

Current epistasis models place *lin-29* at the most downstream position of the heterochronic pathway and suggesting it to be the ultimate factor driving terminal differentiation (Hayes et al., 2006). However, finding that *lin-29* acts upstream of *mir-84* and *let-7* microRNAs would change this model and place the microRNAs rather then *lin-29* to the very end of the heterochronic. According to this new model, the two microRNAs would act downstream of *lin-29* as the “activated suppression function” to repress larval genes while *lin-29* directly activates protein coding genes for the adult stage to mediated terminal differentiation. Loss-of-function of either the microRNAs or the TF activating the microRNA transcription would both result in failure to suppress larval programs and failure to drive adult programs, which could explain the continued molting during the adult stage. The observation that over-expression of *mir-84* rescues the continued molting of *let-7* and *lin-29* null mutants partially verifies this model (Hayes et al., 2006). However, it is still not clear if this happens by direct microRNA activation through LIN-29, or LIN-29 acting as a co-factor that can interact with the promoters of both genes. A candidate for the latter option is DAF-12, since the direct activation of *mir-84* thorough DAF-12 is validated in Chapter 2, and Chapter 3 suggests a potential direct *let-7* activation by DAF-12, despite the fact that loss of DAF-12 alone does not cause defects in terminal differentiation.

This led to the question of whether the microRNA promoters are directly activated by LIN-29, or co-activated by LIN-29 acting through DAF-12. To address this question, the *mir-84* promoter was *in vitro* tested in luciferase assays for direct LIN-29 dependent activation, or LIN-29 co-activation of DAF-12. To complement the *in vitro* assays, *in vivo* assays were performed for transcriptional activation of a *mir-84* promoter fusion to *gfp* in wild type, *daf-12*, *lin-29*, and *daf-12;lin-29* double mutant background.

### Results

#### 4.1 Co-activation assay of LIN-29 and DAF-12 on the *mir-84* promoter

It was found that LIN-29 co-transfection leads to a two fold increased DAF-12 and ligand dependent activation of the *mir-84* promoter during *in vitro* luciferase assays, while *lin-29* transfection in absence of DAF-12 does not cause promoter activation (Figure 31). This was interpreted as LIN-29 co-activating DAF-12. However, it cannot be ruled out that the failure of
LIN-29 alone to activate the *mir-84* promoter is due to the lack of an activation domain compatible with the mammalian transcriptional machinery.

Point mutation of the DAF-12 CRMs leads to a strong loss of DAF-12 and ligand dependent activation, but *lin-29* co-transfection still enhances the remaining activation. This observation rules out the possibility that LIN-29 binds to a CRMs other than the DAF-12 CRMs to indirectly recruit liganded DAF-12 to result in activation. However, it does not distinguish whether LIN-29 binds to the DAF-12 CRM or directly to DAF-12 to enhance activation.

The *lit-1* promoter is DAF-12 and ligand activated, but co-transfection of *lin-29* decreases transcriptional activation instead of increasing it as it did with the *mir-84* promoter. This points towards a sequence specificity of the LIN-29 and DAF-12 co-activation that is only achieved by the *mir-84* promoter.

![Figure 31: Luciferase assay of LIN-29/DAF-12 co-activation](image)

Expression vectors for DAF-12, LIN-29 or GFP as control were transfected into Human Embryonic Kidney cells, along with luciferase reporter vectors containing the *mir-84* promoter and the *lit-1* promoter as used in Chapter 2. Positive control *lit-1* promoter and the wild type *mir-84* promoter were used in Figure 7, while the *mir-84* promoter with point mutated DAF-12-CRMs was used in Figure 9 B. LIN-29 co-transfection increased DAF-12 and ligand dependent activation of both *mir-84* promoters, but decreased *lit-1* promoter activity. Assay was repeated once with similar results.
4.2 EMSA with LIN-29 and DAF-12 on DAF-12 regulated CRMs

The question was addressed if LIN-29 can *in vitro* interact either with the DAF-12 CRM directly or with DAF-12 protein and EMSA was performed with oligos containing the 241cd CRM validated in Figure 11 to interact with DAF-12. The expectation was a shift signal in LIN-29 presence if LIN-29 can bind the oligo directly. If LIN-29 binds to DAF-12, a further increase of the DAF-12 induced shift in presence of both, LIN-29 and DAF-12 was expected. To test if this interaction is hormone dependent, shift experiments were performed in presence of both, DAF-12 and LIN-29, and in absence and presence of ligand (Figure 32). No LIN-29 mediated changes in shift pattern were detected under any of the experimental conditions. However, presence or absence of the ligand during EMSA changed the ratio of the DAF-12 induced monomer and dimer shift bands towards single occupancy in this experiment. Whether this was LIN-29 dependent or independent remains to be tested with EMSA in the presence of only DAF-12 and presence or absence of ligand.

4.3 Mammalian two hybrid assay for Lin-29 and DAF-12LBD interaction

Mammalian two hybrid assay were performed to test if the co-activation is caused by physical interaction of LIN-29 with DAF-12 (Figure 33). The *daf-12* ligand binding domain was fused to the *vp16* activation domain, and *lin-29* was fused to *gal-4dbd*. When only the *lin-29* construct is transfected, LIN-29::GAL-4DBD binds to the promoter of the luciferase reporter, but fails to activate transcription due to lack of an activation domain. This can change when the *daf-12lbd* construct is co-transfected. Under these conditions, physical interaction of LIN-29 with the DAF-12LBD locates the fused VP16 activation domain to the LIN-29::GAL-4DBD bound to the luciferase reporter. Once the VP16 domain is located to the luciferase promoter, it will induce activation of the luciferase gene. This activation is only achieved by interaction of the proteins that were fused to the GAL-4DBD and the VP16 domain, in this case interaction between LIN-29 and the DAF-12LBD. Negative controls were transfected with the empty vectors either expressing GAL-4::DBD or VP16 tag only. DNA was transfected into Human Embryonic Kidney Cells, as done in Chapter 2.
Figure 32: EMSA with DAF-12 and LIN-29
Nuclear extract of Human Embryonic Kidney cells expressing Flag-tagged DAF-12 induced two shift signals (s) of radio labeled oligo containing 241cd element as discussed in Figure 11. LIN-29 expression did not cause a shift alone or in presence of DAF-12, while ligand presence enhanced “monomer” binding over “dimer” binding. Green letters indicate DAF-12-REs. EMSA was done once.
Lin-29::gal-4dbd and daf-12lbd::vp16 co-transfected cultures showed strong ligand dependent luciferase activation, indicating the possibility of ligand dependent, direct protein-protein interaction of LIN-29 and DAF-12LBD. However, DAF-12LBD::VP16 alone also caused noticeable ligand dependent luciferase activation, raising the possibility of unspecific interaction of the DAF-12LBD with the GAL-4::DBD.

Figure 33: Mammalian Two Hybrid Assay with LIN-29 and DAF-12
Expression of GFP or LIN-29::GAL-4DBD very weakly activated a GAL-4 response element regulated luciferase reporter while luciferase in DAF-12-LBD::VP16 expressing cultures was activated weakly in absence, and strongly in presence of LIN-29::GAL-4DBD. DAF-12::FLAG expressing cultures showed tweak activity in absence and presence of LIN-29::GAL-4DBD. Assay was repeated once with similar results.

4.4 GFP reporter analysis for LIN-29 and DAF-12 co-activation in vivo
After testing for co-activation and physical interaction in vitro, in vivo co-activation was investigated next. The mir-84p::gfp promoter reporter was crossed into daf-12(rh61rh411) or lin-29(n546), or the double mutant daf-12(rh61rh411);lin-29(n546), and GFP expression in the pharynx was measured during the L3 stage. Daf-12 null mutants were found to express less reporter than wild type, as reported in Chapter 2 (Figure 13). Lin-29 null mutants expressed less
GFP than *daf-12* null mutants, and double mutants hardly expressed any reporter. This experiment shows that depletion of *daf-12* has a weak, and depletion of *lin-29* has a strong effect on *mir-84p::gfp* reporter expression level. The double mutant shows a further decrease of reporter expression. This experiment indicates that DAF-12 and LIN-29 both individually activate the *mir-84* promoter, and that LIN-29 dependent *in vivo* reporter activation is partially or totally independent of DAF-12.

**Figure 34: In vivo gfp reporter analysis in *daf-12* and *lin-29* mutant backgrounds**

Expression of a *mir-84* promoter activated *gfp* reporter in the pharynx at L3 stage was weaker in *daf-12(rh61rh411)* null mutant background than in wild type. Expression in *lin-29(n5463)* null mutant background was further decreased and *daf-12;lin-29* double null mutant background nearly abolished all detectable GFP expression. n=20, GFP intensities were quantified once and relative GFP intensities observed multiple times.

**Discussion**

The *in vitro* co-activation assays show that LIN-29 presence enhances DAF-12 and ligand dependent transcriptional activation of *mir-84* promoter luciferase reporter, while LIN-29 alone does not have any *in vitro* effect. Daf-12 CRM deletion decreases activation but retains LIN-29 co-activation capacity. The *lit-1* promoter reporter shows a reduction of ligand dependent luciferase activation in the presence of LIN-29, indicating that co-activation is promoter sequence specific. These results were interpreted as LIN-29 acting as a co-activator for DAF-12 *in vitro* for the *mir-84* promoter, by either acting through DAF-12 CRMs or through DAF-12. However, these experiments do not clarify if LIN-29 is able to bind directly to the DNA, or if it binds to DAF-12 to achieve its co-activator function. And even if LIN-29 can bind to the
promoter tested by luciferase assay, it might not be able to recruit mammalian co-activators and the basic transcriptional machinery as DAF-12 is able to. At least for a human LIN-29 homolog, EGR2, it was shown to activate luciferase reporters containing two EGR2 response elements (Crosby et al., 1991), proving that this human LIN-29 homolog is able to bind its CRM and cause transcriptional activation in luciferase assays. It remains unclear whether the conservation between EGR2 and LIN-29 is high enough that LIN-29 maintains this capacity.

![Figure 35: EGR2-like CRM in the mir-84 promoter](image)

**Figure 35: EGR2-like CRM in the mir-84 promoter**

Human ZincFinger protein and closest *C. elegans* LIN-29 homolog EGR2 binds to sequences (Crosby et al., 1991) similar to those found in *mir-84* promoter regions 89bp downstream of the 84e CRM.

241cd oligos only interacted with DAF-12, but not with LIN-29, and LIN-29 was not able to influence the DAF-12 dependent shift pattern, indicating that under the chosen conditions, LIN-29 cannot interact with either DAF-12 regulated 241cd, nor with DAF-12 protein itself. However, it was the *mir-84* promoter that showed co-activation, and the EMSA needs to be repeated on oligos containing CRMs present in this promoter to rule out sequence-specific effects on the LIN-29 mediated co-activation.

While the *in vitro* experiments show that LIN-29 acts through DAF-12, *in vivo* analysis of *mir-84* promoter expression indicates at least a partially DAF-12 independent activation of the *mir-84* promoter by LIN-29 in the pharynx. Further analysis of reporter expression in phenotype related tissues like seam cells is necessary to establish epistasis of *lin-29* and *daf-12* in context of terminal differentiation of the hypodermis.

The results together place *mir-84* activation downstream of *lin-29*, in contrast to previously published models (Hayes et al., 2006). The new interaction of *lin-29* and *mir-84* allow
modifications to the published model of late stage heterochronic regulation pathway. The presented data can be interpreted as a re-occurrence of "molecular switch" with a combination of TFs and microRNAs driving developmental progression. However, further experiments described in the outlook below are necessary to solidify this hypothesis.

According to this hypothesis, the molecular switch would use the activation of microRNAs mir-84 and let-7 to suppress molt-activating genes, most likely nhr-23/-25, belonging to the old stage. At the same time it would activate programs of the new, adult stage. As the molecular switch described in Chapter 2, the transcriptional activation of let-7 family microRNAs would lead to terminal differentiation and a loss of stem cell character of the target tissue, while loss of microRNAs or the TFs activating them causes a failure of both developmental progression and terminal differentiation. This simplified model does not account for all regulatory functions of the larval-to-adult transition in the hypodermis, which becomes clear by looking at the regulation of adult specific genes: Larval and adult cuticles contain different collagens. The collagen gene col-19 is specific for dauer larvae and adult stage and appears to be downstream of mir-84, let-7 and lin-29 since COL-19 expression is decreased in let-7(mg279) and further decreased in let-7(mg279);mir-84 mutants (Hayes et al., 2006) as well as in lin-29 mutant background (Liu et al., 1995). This can be interpreted as the loss of repressing microRNAs leading to the loss of a hypothetical TF activating col-19, rather then col-19 being directly activated by lin-29 as the "molecular switch" idea would suggest, shown in Figure 36. Also DAF-12 may be repressed by feedback loops involving mir-84 and let-7 (Grosshans et al., 2005) as discussed in Chapter 2.5.

If the modified model holds true, it would connect daf-12 signaling to terminal differentiation of the hypodermis. If the LIN-29 co-activation of DAF-12 is ligand dependent, these experiments would identify, for the first time, a steroid hormone that is directly regulating aspects of nematode molting. All DAF-12 transcriptional activation described so far is ligand dependent, but the presented data does not support a ligand dependence of hypodermal terminal differentiation so far.

Our results imply that the larval to adult transition of the hypodermis in C. elegans requires the ligand dependent or independent DAF-12 mediated activation of mir-84 and let-7 microRNAs to suppress larval program promoting genes like nhr-23, nhr-25, hbl-1 etc to prevent re-occurrence of molting during adult stage (Frand et al., 2005).
Figure 36: Revised regulation model for larvae-to-adult transition
Upper box is a current regulation model (Hayes et al., 2006), while the lower box is a new model based on the results of this work that suggest that the mir-84 / let-7 microRNAs act downstream of lin-29 / daf-12 that together might form a “molecular switch” discussed in Chapter 2. This “molecular switch” would activate microRNAs to suppress the old programs – molting in this case – while it directly activates adult programs.

The homology between nematode LIN-29 to mammalian EGR2, and nematode LIN-29 co-regulator MAB-10 (Harris and Horvitz, 2007) to mammalian EGR2 co-regulators NAB1/-2 (Le et al., 2005) and their role in terminal differentiation of stem cell like cells allows to hypothesize that EGR2 could be part of a molecular switch in mammals mediating transitions between successive developmental programs. Experiments testing this possibility are explained in detail in the outlook to this chapter below. If this connection can be shown experimentally, it would link the C. elegans observations to mammalian stem cell differentiation processes and underline the conservation of the “molecular switch” idea. This conservation would also implicate that this mechanism is present in various vital processes of mammalian biology already mentioned in the discussion of Chapter 2.
**Outlook**

4.5 Epistasis experiments of daf-12 signaling, microRNA expression and molting phenotype

Epistasis experiments need to be performed to reveal the in vivo connection of daf-12 signaling to the suppression of larval stage programs during adult stage. A hypomorph let-7(mg279) allele shows 30% supernumerary molts (Hayes et al., 2006). And additional loss of mir-84 increases the molting phenotype to 100% penetrance, while over expression of mir-84 reverses this and rescues let-7(mg279) to 0% penetrance. It needs to be shown how modulating DAF-12 transcriptional activation modulates mir-84 levels at the L4 stage and the penetrance of supernumerary molting of let-7(mg279) at the same time. The ideal mutant background for this would be a let-7(mg279);daf-9(dh6);din-1(dh149) triple mutant. This background lacks endogenous dafachronic acid production as well as DAF-12 repressor function. As a result, transcriptional activation of DAF-12 is dependent on the ligand dose supplemented in the growth medium, as shown in Chapter 2. Loss of DAF-12 transcriptional activation of mir-84 should increase penetrance of the molting phenotype of let-7(mg279), as mir-84 loss did in let-7(mg279);mir-84 double mutants. Supplementation of DAF-12 ligand to this mutant background will restore DAF-12 transcriptional activation and mir-84 transcription to wild type levels and should rescues the increased supernumerary molting phenotype, at least back to let-7(mg279) penetrance.

Since clues for DAF-12 activation of let-7 are discussed in Chapter 3, a control is necessary to show that this is due to DAF-12 activating mir-84, or activating mir-84 and let-7. This control would be the daf-9(dh6);din-1(dh149);mir-84(tm1304) triple mutant, tested for molting phenotype increase on non-ligand supplemented plates. An increase in penetrance in this case is likely due to decreased DAF-12 activation of let-7.

Contradicting with this model is a publication that describes rescue of let-7 null allele phenotypes in a daf-12 null allele background (Grosshans et al., 2005), placing daf-12 downstream of let-7, while results presented in Chapter 2 place daf-12 upstream of let-7 family members (Bethke et al., 2009). These contradicting phenotypes might be due to modifications of the feedback inhibition involving mir-84, let-7 and daf-12. To circumvent the feedback loop, an alternative approach might be necessary that involves lin-29 hypomorphic alleles to modulate the activity of the molecular switch. A pilot experiment would monitor expression level of a let-7
promoter fusion to *gfp* in *lin-29* null background. Regulation according to the model would result in a decrease in expression, while the epistasis suggested by Grosshans places the *daf-12*, now including its co-activator *lin-29*, downstream of *let-7* regulation, in which case *let-7* reporter activation would remain unaffected. Not taken into account however is the regulatory loop which connects *daf-12* and *let-7* discussed in Chapter 2.5.

4.6 LIN-29 activation of the *mir-84* promoter independent of DAF-12

EMSA of oligos containing the so far identified DAF-12-CRMs present in the co-activated *mir-84* promoters would rule out sequence specific effects onto LIN-29 co-activation.

A VP16 domain fused to LIN-29 would force transcriptional activation, as long as the LIN-29DBD can bind the luciferase promoter, making transcriptional activation independent of the ability to recruit co-activators through LIN-29 itself. Luciferase assays with *mir-84* promoter reporter and LIN-29::VP16 would reveal the presence of any LIN-29 regulated CRM.

The DBD of LIN-29 shows high homology to mammalian EGR2, and a DNA motif resembling a validated EGR2 CRM is located in direct proximity of the 84e CRM (Figure 35). This potential CMR is as a candidate for direct regulation by LIN-29. EMSA of this putative LIN-29 CRM will establish if LIN-29 physically interacts with the CRM. As a second step, luciferase assays with LIN-29::VP16 and the *mir-84* promoter luciferase reporter will show if LIN-29 also activates this CRM in full length promoter context. Sequence specificity of LIN-29 for this CRM can be shown by luciferase assay with a *mir-84* promoter that has the putative LIN-29 CRMs point mutated – a loss of luciferase activation would support sequence specificity.

Promoters of *col-7*, *col-17* and *col-19* were published as *lin-29* targets (Liu et al., 1995; Rougvie and Ambros, 1995), but the presented experiments did not completely rule out the possibility of an indirect activation. If these promoters are in deed direct targets, they can act as a positive control for LIN-29 activation in luciferase assays.

4.7 Molecular switch homolog functions in mammalian tissue differentiations

To test the model of molecular switch driven terminal differentiation in mammals, a system is needed where activation of TFs homolog to *lin-29* is required for terminal differentiation. A well studied and potentially homologous differentiation process is the myelination of neurons of the mammalian periferal nervous system that occurs after neurons transit from premyelination program to myelination program. EGR2 and NAB1, -2 regulated transcription is required for
differentiation of neuronal Schwann cells past the premyelination state, as well as keratinocytes and chondrocyte differentiation (Le et al., 2005). The myelination program is suppressed by EGR2 mutations (Topilko et al., 1994), analog to LIN-29 loss-of-function that suppresses the terminal differentiation of the hypodermis in *C. elegans* (Liu et al., 1995; Rougvie and Ambros, 1995). The implication is that EGR2 directly activates mammalian microRNAs to suppress the neuronal outgrowth program in order to allow for myelination program execution and terminal differentiation. A test for this model would be the quantification of *let-7* family microRNAs in EGR2-/- animals to show that their transcription is indeed lost.

The next step would be to test if forced expression of the down-regulated *let-7* microRNAs can suppress the EGR2 -/- myelination defect. These experiments would validate or falsify the conservation of the molecular switch in mammals.
5. Materials & Methods

DNA manipulation
Standard techniques were used to manipulate DNA (J. Sambrook, 1989).

Plasmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen 27106) or GeneElute HP Plasmid Midiprep Kit (Sigma NA0200-1KT). Restriction enzymes were purchased from NewEnglandBiolabs, used according to manufacturer’s protocols. Shrimp alkaline phosphatase (Promega M820A), Taq plus Precision (Stratagene 600210-51), Pfu ultra HF (Stratagene 600380-51), Roche rapid ligation kit (11635379001), Primers were standard ordered oligo nucleotides” purchased from Integrated DNA Technology or Sigma Genosys.

5.1 PCR protocols
Primers used in this work were generated without software support. Most primers had 25-30 nucleotides homolog to the target sequence with a 5’ extension containing restriction enzyme recognition sequences. Primers or primer lengths were chosen that the four most 3’ nucleotides contained two guanidine or cytosine, with most 3’ nucleotide being of them. It also was a criteria that the homolog region contained no more than four identical nucleotides directly adjacent to each other, and that all nucleotides were present in equal ratio. For most PCR reactions, melting temperature was 92°C with one initial 2 minute melting step and a 30 seconds melting step during cycling, annealing temperature was 50°C for 1 minute, extension temperature was72°C with 1 minute for 1 kb product length.

DNA constructs

5.2 Expression vectors for mammalian cell culture
DAF-12 cDNA was amplified with primers 326166 (cgggatccatgggcacaaatggagtc) and 326164 (ccgctcggttggtaaaattctc) and cloned into pCMVTag2b (Stratagene #211172). Lin-29 cDNA was excised from vector Ba 282 using XmaI, Xhol and cloned into pCMVTag2b (Stratagene #211172). Lin-29 cDNA was sub-clonated into pCMX gal-4DBD to give pCMX lin-29::gal-4DBD. Vectors pCMX gal-4-dbD, pCMX vp16, pCMX vp16::daf-12-lbd and pCMX daf-9 were obtained from the Mangelsdorf lab (Motola et al., 2006).
5.2.1 Luciferase reporter vectors
microRNA promoters were PCR amplified using the following primers: mir-48 (JRP6, JRP3) and mir-241 (JRP7, JRP2, (Esquela-Kerscher et al., 2005)),
mir-84 with primers 27723-001 (GGATCC gatgccacaggcagctgtatgatgaatgt) and
27723-002 (gtcgacctcacaattatgagttgca),
let-7 with primers 33872767 (accggatcacagtgtagacgctccaccaatcg) and
33872768 (actgtgacaattgtgtaagggctcattgtgctgca),
lin-4 with F59G1.5658+.SalI.lin-4 (gtcgacagagcggctcctctctacg) and
F59G1.6169-.BamHI.lin-4 (ggatccagccggaacataactataaacc),
mir-237 with 44938-001 (ggatcctctgatcgtcctctgtgtc) and
44938-002 (gtcgagcttaattacactcctcactg) and inserted into ptk luc reporter vector (Motola et al., 2006) upstream of a thymidine kinase (tk) minimal promoter that was followed by the firefly luciferase cDNA. The mir-241 promoter was subcloned into Fire Lab vector L4053 (Fire Lab C. elegans Vector Kit, 1999 plate) using BamHI to generate mir-241p::4xNLS::GFP, called A215.

mir-241 truncations #1 (called A224) was generated with primers 44113-005
(cgggatcc_cgggtgctgtgctgtcataatgttcc) and 50390-002 (ggatcc_ggaagggatgtcgtcaccgcatatat),
#2 (called A225) was generated with primers 44113-007
(gttgac_acggttcggtggtccataatgttcc) and 46031-001 (cgggatcc_actgtcgtggtggccggttcc), #3
(called A226) was generated with PCR primers 50390-
001(gttgac_agctggtccggtggtggtgcagttgca) and 46031-001
(cgggatcc_actggtcgtggtggtggtgcagttgca), and truncation #4 (called A227) was generated with primers
44113-007 (gtcgac_actgtcgtggtggtgcagttgca) and 44116-006
(ggatcctctgatcgtcctctctctgatcctctgatcctct)

pG5 luc was obtained from the Mangelsdorf lab (Motola et al., 2006), ptk luc daf-9 (A194)
contains a PCR product amplified primers 6555-084 (gtcgacacagagcgtgcatactgacacacac),
and 6555-085 (ggatcctctgatcgtcctctctctctgatcctct), ptk luc him-4 contains a PCR product amplified
with primers 6555-082 (gtcgacacagagcgtgcatactgacacacac), and 6555-083
(ggatcctctgatcgtcctctctctctctctct)
with primers 6555-080 (gtcgacacagaatcatgtagctcactactgt), and 6555-081
(gtcgacacagaatcatgtagctcactactgt).

5.2.2 Site directed mutagenesis of daf-12-like CRMs

Point mutations were introduced using Quikchange Multi. Vector A215 was mutated with
primers 59450-001 (CTCAATGTACTAATATAAGGTACCATT TCTCTCTCTCTCTCACGC) and 59450-003
(GTCTGGCGTCCAAAGGTACCTT TCTCTCTCTCTTCTCCACCG) to give mir-241p(mut ab)::4xNLS::GFP (A275). The insert was subcloned into ptk luc to give ptk luc mir-241p(mut tab) (A279). A275 was template for site directed mutagenesis using Quikchange II and primers
30037678 (ccaggctcggtgccgGgtacCtttataaaGgtAcCtccctgacg), 30037679 (cgtcagggaGgTacCtttataaaGgtacCeggeacgagctgg) to give mir-241p(mut abcd)::4xNLS::GFP, called A455. Luciferase reporter ptk luc 241c with one of two DAF-12 sites of the 241cd CRM mutated (Figure 50) was generated using site directed mutagenesis as described above using
primers 46031-002 (gcgctgtacctttaaaaGGTACCtccctgaegcgeggagacgc) and primer 46031-003
(ggctcgcggcgcgtcagGGTACCtttataaaagtacacggc) to give vector A214. Luciferase reporter
ptk luc 84b with the 84b DAF-12-RE mutated (following page: Figure 23) was generated using
site directed mutagenesis (as described in Chapter 1) using primers 50622-001
(ccctgcctttccaaagaGgtAcCaCagaaatggaaggcaag) and primer 50622-
002(ccctgcctttcaagtgtgctGgTacCtttggggagaagcaag) described above.

5.3 CRM-repeat generation

Repeat-PCR-products described in Chapter 3 were generated by using 50μl PCR reaction
containing 50ng of plasmid DNA harboring any template containing sequence (e.g. the
corresponding ptk luc vectors used in Chapter 2 ) 2.5μ Taq Precision or 1.25u Pfu Ultra, 5μl
manufacturer supplied 10x PCR buffer, 1μl of each primer working solution (working solution is
10pmol/μl), 1μl of the 50 mM dNTPs solution. Cycling parameters were 2min initial
denaturation at 95°C, then 35 cycles of 30sec 95°C denaturation, 1min 50°C primer annealing,
30sec 72°C extension time. PCR-products were separated on a 0.5x TBE 0.8% agarose gel,
bands of different sizes corresponding to different repeat numbers were gel extracted and sub-
clonated into TOPO TA or TOPO BLUNT vector according to used polymerase and sequenced to
verify identity, repeat number and orientation. Inserts were then excised and sub-clonated into
the multiple cloning site of a modified \textit{yfp} or \textit{cfp} reporter plasmid upstream of a \textit{Apes-10} minimal promoter and the chromophore coding region.

The modified vectors containing a \textit{yfp} coding region were called A423 and A424 depending on the orientation of the multiple cloning site. They were generated as the following: The original \textit{yfp} (L4817, internal name A246) or \textit{cfp} (L4816, internal name A245) reporter plasmids are taken from the 1999 Fire Lab Vector Kit. Vector backbone and chromophore cDNA were excised out of L4816 or L4817 by digestion with \textit{HindIII} and \textit{BamHI}, the \textit{Apes-10} minimal promoter sequence was excised out of Fire Lab vector L4053 (called A170) by digestion with \textit{NheI} and \textit{BamHI} and the 4x241ab repeat was excised out of \textit{Topo TA} vector (called A384) by digestion with \textit{HindIII} and \textit{XbaI}. All fragments were gel purified and ligated in a single step with equimolar amount of DNA fragments. The \textit{yfp} containing plasmid was called A391 (Figure 37), the \textit{cfp} containing A388. Oligo nucleotides for forward and reverse strand of a multiple cloning site containing in 5' to 3' order restriction sites for \textit{SalI}, \textit{Nhel}, \textit{XbaI}, \textit{ApaI}, \textit{SpeI}, \textit{HindIII} (forward oligo 28227516: agctgtgcagatgctgtaatgctgactagcatctagagggcccactagtttaagcttattgca, and reverse 28227517: ataagcttaaactagtgggccctctagatgctagcatgtcgactatgcag) were annealed as described for EMSA oligo annealing. The resulting double stranded oligo contained sticky end overhangs for 5' \textit{HindIII} and a 3' \textit{PstI} ligation. The oligo overhangs also had a mismatch in order to destroy the restriction sites they were annealed to. The double stranded oligo was ligated into A391/A388 backbone digested with \textit{PstI} and \textit{HindIII} and gel extracted to eliminate the insert. The \textit{yfp} containing product was called A424 (Figure 37). A423 vector was generated using oligos 28227518 (agcttttactagtgggccattctagaagctagcatgtcgactactgca) and 28227519 (gtagtcgacatgctagcttctagaatgggcccactagtaaa) with restriction sites in opposite orientation as A424 (\textit{HindIII}, \textit{SpeI}, \textit{ApaI}, \textit{XbaI}, \textit{Nhel}, \textit{SalI} in 5’ to 3’ order, forming a 5’ \textit{HindIII} and a 3’ \textit{PstI} overhang that was not destroyed after ligation).
Primers 42692097 (agccatcacc_tctctcctagtcgctggtcagtg, forward) and 42692097 (ctaggagagag_gtgatggctgtaagagagaag, reverse) were used to PCR-generate reporter repeats “4x84d” and “6x84d”. The PCR product was separated on a 1.2% agarose gel and DNA fragments between 500 and 1000bp length were gel-extracted, sub-clonated into TopoTA (A632 for 4x84d and A628 for 6x84d) and sequenced to verify identity, repeat number and orientation. 4x84d::Δpes-10::yfp was generated by excising the inserts out of A632 and A628 with XbaI, SpeI and ligation into A424 digested with XbaI, followed by shrimp alkaline phosphatase treatment (SAP). 4x84d::Δpes-10::yfp was called A634. The 6x84d::Δpes-10::yfp vector was called A631, containing the 6x84d repeat in opposite orientation to the yfp.

Primers used to generate repeats “1x84e” to “4x84e” were 35910036 (gacacccataggctcgctggtcagtg, forward) and 35910037 (ggaacgaacgcaagcagcagctggtggtcagtggtcagtg, reverse). For the generation of 2x84eMut with a mutated DAF-12-like CRM were generated by using A479 as template. The PCR product was separated on a 1.2% agarose gel and DNA were gel-extracted corresponding to various fragment lengths (Figure 22), sub-clonated into TopoTA (A632 for 4x84d and A628 for 6x84d) and sequenced to verify identity, repeat number and orientation. Promoter repeats were sub-clonated into TopoTA (A520 for 1x84e, A521 for 2x84e, A523 for 4x84e). A424 vector and insert vectors were
digested with HindIII and XbaI and ligated. 1x84e::Apes-10::yfp vector was A529, 2x84e::Apes-10::yfp vector was A530 and 4x84e::Apes-10::yfp vector was called A532).

4x241ab repeat was generated with primers 87436-003 (ccaaaccatgcgtcacaacctactactactacaataagg, forward) and 87436-004 (agtgtgagagc_gaatcggattttgtgaatgggttggtggattgatg, reverse), sub-clonated into Topo TA and called A384 and further processed as described above.

2x241cd was generated with Pfu Ultra polymerase and primers 87436-001 (gagcagaagta_actagtctcaatgtactaatataaagtacc, forward) and 87436-002 (gtacattgagactagt_tacctctgctctgaaactcggtggagaaacagagag, reverse), sub-clonated into Topo BLUNT and called A401. Backbone of A391 and insert of A401 were gel-purified and ligated after vector digestion with HindIII and NotI and the 2x241cd::Apes-10::yfp product was called A406.

4xTRE repeat was generated with primers 31355948 (ggtgtagtcgcccaggtcaccgcaataaaatacc) and 31355949 (ggtgagctggccgacacccgattaaattcg), and sub-clonate into TOPO TA to give vector A457. Backbone of A391 and insert of A457 were gel-purified and ligated after vector digestion with HindIII and NotI and the 4xTRE::Apes-10::yfp product was called A459.

5.4 Double-tagging constructs

The constructs listed in Table 2 were generated for the CFP/YFP double tagging-project described in Appendix 3. However, not all constructs were used during this project. The data is shown here to give a complete list of reagents created.
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<th>UTR</th>
<th>construct</th>
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</table>

Table 2: List of cfp / yfp tagging vector constructs

myo-3p::cfp/yfp::unc-54-UTR: Constructs myo-3p::cfp-unc-54-UTR (A245) myo-3p::yfp-unc-54-UTR (AA246) were taken out of the 1999 Fire Lab Vector Kit where they are called L4816 and L4817, respectively.

daf-12p::cfp/yfp::unc-54-UTR: A 5kb fragment upstream of the daf-12 start codon was PCR amplified with Taq Precision using primers 22507-003 (cta GCT AGC cttccgaaacatacatgtgtg) and 22507-004 (gg GGT ACC ttaattgcccgttgccgaaatgtg) and subclonated into TOPO TA vector to give vector A177.

The promoter was cut out of A177 with Sall and BamHI und sublonated into vector L4053 from the 1995 Fire Lab Vector Kit to give vector A249. The promoter was excised out of A249 with HindIII and AgeI and subclonated into vector A392 (cfp) and A393 (yfp) to give vector A433 (cfp) and A434 (yfp). A392 (cfp) and A393 (yfp) were derived previously from A312 (cfp) and A313 (yfp) by digestion with BamHI and relegation of the vector backbone. A312/A313
were derived from A245/A246 by digestion with *HindIII* and *XbaI* and ligation with *HindIII* and *XbaI* digested *TOPO TA* vector to exchange the *myo-3* promoter with the multiple cloning site of *TOPO TA*.

*daf-12p::cfp/yfp::daf-12-UTR*: Vectors A453 (*cfp*) and A454 (*yfp*) were derived by cutting the DAF-12 UTR out of vector Ba359 of the Antebi Lab Bacterial collection using *EcoRI* and *ApaI* and ligation into A304 (*cfp*) and A306 (*yfp*). A304 and A306 were obtained earlier by subclonation of the *daf-12* promoter out of A177 into A245 (*cfp*) and A246 (*yfp*) using *SalI* and *BamHI* to give vectors A251 and A252.

*daf-12p::cfp/yfp::hbl-1-UTR*: The hbl-1-UTR was PCR amplified using *Pfu* ultra polymerase and primers 29030250 (gggccctgatgatccgattaattgtactttg) and 29030249 (CGGCCGtgaggacgcctcgttaaggaacac) and subclonated into *TOPO BLUNT* to give vector A442. hbl-1-UTR was cut out of A442 with *ApaI* and *EcoRI* and ligated into A453 (*cfp*) and A454 (*yfp*) that were obtained earlier as described above to give A443 (*cfp*) and A444 (*yfp*).

*myo-3p::cfp/yfp::daf-16-UTR*: The *daf-16*-UTR was PCR amplified with *Taq* precision and primers 84160-001 (GAATTCGTCGAacgatcagccactgatggatactatggatgttg) and 84160-002 (GTCGACtggttaatttccgctccttg) and subclonated into *TOPO TA* to give vector A355. A355 was digested with *EcoRI* and *Sall* and ligated into A392 (*cfp*) and A393 (*yfp*) to give vector A414 (*cfp*) and A415 (*yfp*).

*daf-16p::cfp/yfp::unc-54-UTR*: A 5kb fragment upstream of the start codon of the *daf-16 B*-isoform was PCR amplified using primers 87436-005 (CCCCGGGcgtttgtcctgcgttcatctctttg) and 87436-004 (ACCGGTcgtttgctcactcactgatagtcccatgc) and subclonated into *TOPO XL* to give vector A432. The promoter was excised from A432 using *XbaI*, *AgeI* and ligated into only *AgeI* digested and Shrimp alkaline phosphatase treated A492 and A393 to give vectors A437 (*cfp*) and A438 (*yfp*).
**daf-16p::cfp/yfp::daf-16-UTR**: The promoter of *daf-16B* isoform was excised from A432 using *XbaI*, *AgeI* and ligated into only *AgeI* digested and Shrimp alkaline phosphatase treated A414 and A415 to give vectors A435 (*cfp*) and A436 (*yfp*)

**Nematodes**
Standard techniques were used to maintain nematodes (Brenner, 1974). For ligand supplemented plates, 10μl of 75μM Δ⁴-DA (Motola et al., 2006) in ethanol were mixed with 90μl of over night OP50 culture in LB medium and seeded onto 3cm plates containing 3ml NGM agar.

5.5 **RNAi knock-out**
RNAi mediated gene knock out was achived by feeding, using *E. coli* strain *HT115(DE3)* that was transgenic for a vector containing a short, 100 to 500bp long stretch of DNA sequence of the coding region of the targeted gene, flanked by two T7 polymerase binding sites facing into the insert. An *HT115* culture was grown over night at 37°C in LB medium containing 75-100μg Ampicillin and 12.5μg Tetracyclin. The culture was pelleted and resuspended in LB in 1/5 of the original volume and plated onto NG plates supplemented with 50μg/ml Ampicillin and 1mM IPTG to induce the T7 polymerase to generate the double stranded RNA. RNAi clones were taken out of the “Arringer library” (Kamath et al., 2003). As a negative control, one RNAi plate was plated out with *HT115* transformed with 4440, the empty RNAi vector. Positive control was RNAi aginst *unc-22* to cause the easily scoreable “uncoordinated” phenotype

5.6 **Analysis of GFP expression**
10 to 20 gravid adult worms were placed on a 6cm NG agar plate for 6h at 20°C to lay eggs and then removed. Plates were incubated at desired temperature and scored when the worms reached desired stage. Used as a negative control was a plate that was For the analysis of extra chormosomal arrays in different mutant backgrounds, N2 worms transgenic with the same extra chromosomal array were used. GFP intensity was observed using a Leica FL-MZIII binocular or the Zeiss Axioscope2 equipped with fluorescence filter sets described below.

5.7 **Worm transformation**
Worm lines transgenic with reporter constructs were generated using microinjection (Mello, 1991) of DNA mix with 100ng/μl total concentration into the germline. Germline injected animals gave offspring that at low frequency had plasmid DNA rearranged to form an extra
chromosomal array that shows relatively stable transmission. Each event of extra chromosomal array formation shows within limits an individual expression level and transmission rate, which is why each individual, array transmitting worm is considered an individual worm line that received a unique “dhEx” number after entering into the lab worm collection, identifying all offspring and crosses done with this line. For the individual variations between different dhEx lines, multiple dhEx lines of injections with the same construct were kept.

5.8 Worm lines transgenic with reporter used in Chapter 2
dhEx458 (= mir-241p::4xNLS::gfp), dhEx480 (= mir-241p(mut abcd) ::4xNLS::gfp) were generated by injection of 20ng/μl reporter construct and 80ng/μl coel::rfp (Miyabayashi et al., 1999). Worm strain GR1427 (=mir-84p::gfp) was obtained from Hayes (Hayes et al., 2006). Worm strains BW1932 (hbl-1p;;hbl-1::gfp) and BW1891 (hbl-1p;;unc-54::gfp) were obtained from Abrahante (Abrahante et al., 2003). Gene alleles /strains are listed in Table 4.

5.9 Worm lines transgenic with CRM-repeat reporter constructs
6x and 4x 84d repeats were injected with 10ng/μl A631 and A634 as well as 10ng/μl of A245 as marker. 1x84e, 2x84e, 2x84eMut and 4x84e were generated by injection of 2,5ng/μl of construct A529, A530, A532 and A532 with A245 as marker. 4x241ab, 2x241cd and 4xTRE repeats were injected with 20ng/μl of vector A391, A406 and A459, respectively, and 80ng/μl of coel::rfp.

Extra chromosomal arrays for 4x84d reporter are dhEx500 till dhEx504 and for the 6x84d reporter dhEx513 and dhEx514.

Extra chromosomal arrays for 1x84e reporter are dhEx521, dhEx522. Extra chromosomal arrays for 2x84e reporter are dhEx501, dhEx502 (this one was used for crosses with other genetic backgrounds), dhEx503 and dhEx504. Extra chromosomal arrays for 2x84eMUT reporter are dhEx509 till dhEx512. Extra chromosomal arrays for 4x84e reporter are dhEx500 till dhEx504.

4x241ab: Extra chromosomal array is dhEx464
2x241cd: Extra chromosomal array is dhEx519
4xTRE: Extra chromosomal arrays are dhEx493 till dhEx495, the latter used for crosses with other genetic backgrounds.
5.10 Worm lines transgenic with double tagging constructs

The following worm lines were generated or planned to be generated for the CFP/YFP double
tagging-project described in Appendix 3. Each chromophore vector is present in equimolar
amounts relative to 10ng/μl *daf-12p::yfp::unc-54-UTR*, and filled up with *coel::rfp* vector
construct to 100ng/μl. Not all constructs were used during this project. Worm lines were
generated by injecting by injecting different mixes of one or two *yfp / cfp* vectors, a marker
vector. *Coel::rfp* was used as a marker. The data is shown here to give a complete list of reagents
created. Table 3 shows of which reporter constructs the generated lines / *dhEx* lines consist, all
lines contain the *coel::rfp* marker.

<table>
<thead>
<tr>
<th>injection mix</th>
<th>reporter</th>
<th>dhEx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>daf-12p::yfp::unc-54-UTR</em> -</td>
<td>467</td>
</tr>
<tr>
<td>2</td>
<td><em>daf-12p::yfp::unc-54-UTR</em> <em>daf-12p::cfp::unc-54-UTR</em></td>
<td>471, 472, 473</td>
</tr>
<tr>
<td>3</td>
<td><em>daf-12p::yfp::unc-54-UTR</em> <em>daf-12p::cfp::unc-54-UTR</em></td>
<td>468, 474, 475</td>
</tr>
<tr>
<td>4</td>
<td><em>daf-12p::yfp::unc-54-UTR</em> <em>daf-12p::cfp::daf-12-UTR</em></td>
<td>469</td>
</tr>
<tr>
<td>5</td>
<td><em>daf-12p::yfp::unc-54-UTR</em> <em>daf-12p::cfp::hbl-1-UTR</em></td>
<td>470, 476</td>
</tr>
<tr>
<td>6</td>
<td><em>daf-12p::yfp::hbl-1-UTR</em> <em>daf-12p::cfp::unc-54-UTR</em></td>
<td>477, 478</td>
</tr>
</tbody>
</table>

Table 3: Double tagging injected extra chromosomal arrays

Worm strains shown in Error! Reference source not found. of Appendix 3 were generated
during the pilot experiment for the double tagging project by germline injection using 20ng/μl
for each chromophore vector, 10 ng/μl A245 construct as marker and *pCR 2.1* vector to reach
100 ng/μl total concentration of injected DNA: “Y9-1” is transgenic with *daf-12p::yfp::daf-12-
UTR*. Strains called “C1-4” and “C1-5” are individual lines transgenic with the reporter *daf-
12p::cfp::daf-12-UTR*. Strains called “CY1-2”, “CY1-3”, “CY5-1” and “CY5-9” are individually
formed lines transgenic with equimolar amounts of both \textit{daf-12p::yfp::unc-54-UTR} and \textit{daf-12p::ycfp::unc-54-UTR} constructs.

**Gene alleles / strains**

N2

daf-2(e1368)
daf-7(e1372)
daf-12(rh61rh411)
daf-12(rh61)
din-1(dh14)
daf-9(dh6), mir-48(n4097)
mir-48 mir-241(nDf51)
lin-29(n546)
daf-12rh61rh411; lin-29(n546)
lin-12(n137n460)
daf-16(mu86)
BW1932, ctIs39 hbl-1p;;hbl-1::gfp
BW1891, ctIs37 hbl-1p;;unc-54::gfp
ctIs39 daf-12(rh61)
ctIs37 daf-12(rh61)
dhEx458(mir-241(wt)::GFP), N2
dhEx458, daf-12(rh61rh411)
dhEx458, daf-9(dh6)
dhEx458, daf-9(dh6); din-1(dh149)
dhEx458, daf-2(e1368)
dhEx458, daf-7(e1372)
dhEx480(mir-241(mut-abcd)::GFP), N2
dhEx480, daf-12(rh61rh411)
dhEx480, daf-9(dh6)
dhEx480, daf-9(dh6); din1(dh149)
GR1427, mgEx674(mir-84::GFP), N2
mgEx674, daf-12(rh61rh411)
mgEx674, daf-9(dh6); din-1(dh149)
mgEx674, daf-7(e1372)
mgEx674, daf-2(e1368)
mgEx674, daf-9(dh6)

Table 4: Gene alleles / strains

Microscopy

5.11 Microscope
Microscopy was performed with a Zeiss Axioscope 2plus and an Axiocam MRm, using Axiovision 4.1 software. Excretory cell GFP expression (mir-241p::gfp) or seam cell expression (mir-84p::gfp) was scored by eye (n>20), as well as quantified by comparison of micrographs taken with 100X or 63X lens, usually 10 or more pictures per genotype/condition. Worm tissues scored as "off" had no detectable GFP, "low" as hardly detectable GFP, and “high” as everything else easily seen.

5.12 Fluorescence microscopy filter sets
The following filter sets were used together with the Zeiss Axioscope2 for fluorescence quantification by eye and by Axiovision software:
  - GFP – Chroma Technologies #41018 “Endow Longpass Emission”
  - YFP – Chroma Technologies #41028 “YFP/GFP BP (10C/TOPAZ)”
  - CFP – Chroma Technologies #31044v2 “Cya GFP”
  - dsRed – Chroma Technologies “Filter Set 15”

5.13 Bionocular
A Leica MZ FLIII was used for fluorescence observation and analysis of worms on plates. The binocular was equipped with the following filter sets: GFP, dsRed, CFP

Cell culture and luciferase assay
HEK293T cells were cultivated in DMEM with 25mM glucose (GibcoBRL 11995-065) supplemented with 10% FBS (HyClone SH30088.03) for normal cultivation and 10% charcoal/dextran-treated FBS ("ssFBS", HyCloneSH30068.03). Luciferase assay was performed in white, clear bottom 96 well plates (Corning Costar 3610) with 30,000 cells in 100μl ssFBS-DMEM per well. Cells were transfected 16h after seeding with 0.4μl of a 2M CaCl2 solution,
30ng transcription factor vector, 30ng luciferase reporter vector, 5ng beta-gal expression vector and 60ng *TOPO TA* vector (Invitrogen 45-0641) in 3.7μl total volume per well. This was combined while vortexing with an equal volume of 2xHBS buffer (280mM NaCl, 10mM KCl, 1.5mM Na₂HPO₄, 12mM dextrose, 42mM HEPES) to form calcium phosphate precipitate and directly applied to cells. 8h after transfection, a ssFBS-DMEM-ligand solution with 2.16x ligand concentration was prepared and 92.6μl were applied per well. Luciferase activity was assayed 16h after ligand addition: Medium was decanted and 50μl of assay buffer were added per well.

Assay buffer consisted of 10% "10x core buffer" (300mM Tricine pH7.8, 80mM Mg Acetate (Sigma M0631), 2mM EDTA), 1% Triton X-100, 0.5mM D-Luciferin (Invitrogen L2912), 1.5mM ATP (Sigma A8937), 0.5mM Coenzyme A Sodium salt (Sigma C3144), and 0.7% 2-Mercaptoethanol (Sigma M6250) and incubated for 1min at 24°C before reading in a Luminoskan Ascent (Lab Systems) luminometer. Subsequently, beta-galactosidase activity as transfection control was measured after the luciferase reading in the same well by adding 125μl beta-gal buffer consisting of 60mM Na₂HPO₄, 40mM NaH₂PO₄, 8mM KCl, 0.8mM MgCl₂, 0.04% 2-Nitrophenyl-β-D-galacto-pyranoside (Sigma N1127), 0.3% 2-Mercaptoethanol. After 10min incubation at 24°C, absorption at 405nm was measured in a Power-Wave XS (Bio-Tek) plate reader. Relative luciferase units were divided by the beta-galactosidase OD₄₀₅ to generate the luciferase activity corrected for variations in transcription efficiency.

5.14 Luciferase assay data

Four different combinations of transcription factor expression vector, luciferase reporter vector and ligand conditions were tested for each reporter including the empty *ptk-luc* luciferase vector:

a) DAF-12 expression vector, reporter vector, subsequent addition of Δ⁴-DA (shown as solid green in Fig. 1) to see transcriptional effects dependent on both DAF-12 and ligand

b) GFP expression vector, reporter vector, subsequent addition of Δ⁴-DA (shown as striped green in Fig. 1) to see transcriptional effects dependent only on the ligand and cell culture endogenous factors

c) DAF-12 expression vector, reporter vector, subsequent addition of ethanol as vehicle (shown as solid red in Fig. 1) to see transcriptional effects dependent on DAF-12 without ligand
d) DAF-12 expression vector, reporter vector, subsequent addition of ethanol as vehicle (shown as striped red in Fig. 1) to see transcriptional effects dependent only on ethanol and cell culture endogenous factors.

Each of these four conditions was tested in triplicate for each of the used promoter reporter constructs. Figure 7, Figure 26 A, Figure 31, Figure 33, Figure 41 and following page: Figure 42 show luciferase quantification values as “Relative Luciferase Units” to show transcriptional effect of any of the four tested conditions, while Figure 9 and Figure 50 show fold change of luciferase quantification values between DAF-12 expression vector transfected wells with ligand in presence of either wild type or mutant promoter to show the change caused by the promoter modifications. Figure 26 B shows fold induction of ligand supplemented versus ethanol control wells that were both transfected with DAF-12 expression vector and the same reporter.

Error bars of diagrams showing RLU are standard deviations, while error bars of diagrams showing fold-induction values reflect the compound error.

5.15 Gel Shift
Protocol for Electrophoretic Mobility Shift Assay was modified after Timchenko (Timchenko et al., 1999). A 10cm dish of HEK293T cells was transfected using Fugene6 reagent (Roche 11 814 443 001) according to manufacture’s protocols. 6μg DNA and 18μl Fugene6 were used for a 10cm culture. Cultures were placed on ice for nuclear extraction, washed with Buffer A (25mM Tris-HCl pH 7.5, 50mM KCl, 2mM MgCl2, 1mM EDTA, 5mM Dithiothreitol in water) and detached with a cell scraper into 1ml Buffer A that was transferred into a 1.5ml tube. Cells were pelleted and supernatant aspirated before the cell pellet was passed eight times through a syringe (BD 1cc Insulin Syringe” U-100 28G 1/2") for cell rupture. Released nuclei were pelleted by 10min centrifugation at 10,000g and 4°C. The cytosolic fraction in the supernatant was removed and the pellet re-dissolved in Buffer B (25mM Tris-HCl pH 7.5, 420mM NaCl, 1.5mM MgCl2, 0.5mM EDTA, 1mM Dithiothreitol, and 25% sucrose in water) and incubated for 30 min with occasional vortexing for nuclei rupture and release of nuclear proteins. The suspension was centrifuged for 20min at 20,000g to pellet cell debris, and supernatant was frozen at -20°C for later use.

Single stranded oligos were annealed by heating 10μg of each oligo in 100μl with 10% 20xSSC to 95°C for 1min and returning to 24°C over three hours. Half the annealing mix was separated on a wide pocket 15% acryl amid gel (stock 30% Acrylamid/Bis 19:1, Bio-Rad 191-107
0154) in 0.5x TBE and stained with ethidium bromide (Sigma E1385). The overloaded, unstained band of the ds oligo was cut out, transferred to a 0.5ml tube with the tip perforated and stuffed with glas wool (Supelco 2-0411) and placed inside a 1.5ml tube. Water / ssoligo was eluted by centrifugation at 20,000g for 10 min. 2µl of the eluted ds oligos were labeled by incubation with 2u Klenow(3’exo’) fragment (NEB M0212S), NEBuffer 2, 2µl of each 10mM ATP, GTP, TTP and radio labeled CTP (Amersham, AA0075) for 1h at 24°C. The labeled oligo was purified by spinning through a G50 Sephadex Column (Roche #11 273 965 001) with 1100g for 4min after a 2min and 1100g column pre-run.

Following oligos were used:

241a (wt sequence forward: agg_tactaataaaagtacctttct and reverse: agg_agaatgtgactttatattga, mutant forward: agg_tactaataaaGgtacctttct and reverse agg_agaatgtgactttatattga)

241b (wt sequence forward: agg_tccaaagtgcgcttctctctgt and reverse: agg_acagagagaagcacttttgtga, mutant forward: agg_tccaaagGgtAcCttttctctctgt and reverse agg_acagagagaagGgTacCttttgga)

241cd (wt sequence forward: agg_tgtacttttataaagtgcacctccct and reverse: agg_agggaatgcaccttttataaagtac, mutant for cd forward: agg_tgtacttttataaAGTACCtccct and reverse agg_aggggaGGTACCtttttataaagtaca, mutant for c only forward: agg_tactaataaaGgtaccctttct and reverse agg_agaaatgggtacCtttattagta, mutant for d only forward: agg_tccaaagGgtAcCttttctctctgt and reverse agg_acagagagaagGgTacCttttgga)

TRE-wt (wt sequence forward: AGG_atcaaaagtggaacaaagttccattttcctt and reverse: AGG_aagaaatgtgcctttcattttctt, negative mutant forward: AGG_atcaaaGgtGccttttataaagttccattttctt and reverse: AGG_agggaatgggtacCttttgtGgTacCttttgga, positive mutant forward: AGG_aagaaatGgtGCttttttcattttgga, and reverse AGG_agggaatgggtacCttttgtGgTacCttttgga).

5-10µg Protein was bound to 1µl of the oligo labeling reaction by 30min incubation at 24°C with 20mM Tris-HCl ph 7.5, 100mM KCl, 2mM MgCl2, 5mM DTT and 10% glycerol). The
whole annealing reaction was separated on a 18x20cm 5% acryl amide/TBE gel and stopped when bromophenol blue marker migrated 2/3 towards the gel bottom, transferred to 3MM chromatography paper (Whatman 3030347) and vacuum dried on a gel dryer (Bio-Rad Model 583). The dried gel was exposed for 10min to 1d onto 8x10in BioMax XAR film (Kodak F5513).

**RNA extraction**

For total RNA extraction, 20 young adult worms were picked to a new, seeded NGM plate and incubated at 20°C. After two and three days, 100µl of 10x over night OP50-LB culture was added to the plate and after four days now mostly adult staged worms were bleached and eggs were pipetted to plates with the final growth conditions, using glass Pasteur pipettes. When most worms reached the desired stage, worms were washed off the plate and sedimented for 15min in a vertical 5ml glass tube on ice to separate worms from OP50 bacteria. Sedimented worms were transferred with glass Pasteur pipettes into a new 1.5ml bullet tube and pelleted. M9 volume was aspirated until 100µl M9/worm pellet remained. This was mixed with 1ml TRIzol Reagent (Invitrogen 15596-018). Worms were freeze-thawed in liquid nitrogen and 37°C water bath six times, and RNA was extracted according to GibcoBRL Trizol Reagent manual #15596.

**Taqman Q-PCR**

Total RNA was DNase treated (Invitrogen 18068-015) and diluted to 2ng/µl as template for Q-PCR using Taqman MicroRNA Assay (Applied Biosystems) reagents and its protocols to quantify cel-mir-48 (#4373463), cel-mir-84 (#4373490), cel-mir-241 (#4373438), has-let-7a (#4373169), using Taqman microRNA RT Kit (4366597) and Taqman Universal PCR MasterMix (4324018). hsa-miR-1 (# 4373161) was used as endogenous control. PCR reactions were performed in a ABIprism 7000 machine controlled by ABI SDS software version 1.2.3 which was also used for data analysis. Each bar represents mean relative quantification value of a technical triplicate on a single biological experiment, with error bars indicating maximum and minimum RQ values of the triplicate. A test for cross-reactivity of microRNA specific primers and probes is shown in Figure 38.
Figure 38: Cross reactivity of TaqMan Q-PCR primers and probes
Primers and probes were tested for cross reactivity by performing Q-PCR for mir-48, -84, -241 on wild type and deletion strain with either one of the mir-48, -84, -241 microRNAs deleted. One biological replicate with n>4000 L3 stage animals per genotype.

Data analysis
EC50 values were calculated using GraphPad5 to fit a non-linear least squares regression curve to the data values. Error bars in luciferase assay data plots are explained in the “Luciferase assay data” paragraph.
6. Appendices

Appendix 1: Him4/hemicentin is transcriptionally regulated by daf-12

The cytoskeleton and the extracellular matrix interact to give shape and movement to cells during development, since they can generate force and displacement. HIM-4 is an extracellular matrix protein with a size of 5198aa (Vogel and Hedgecock, 2001). It contains conserved domains, among others N-terminal secretion signal, 48x repeat of Ig modules and a 3fold repeat of EGF. HIM-4 is expressed and secreted from a wide variety of cells, where it induces hemidesmosome formation, or multimerises to form fine, linear tracks to guide cell migrations, e.g. for linker cell of male gonad that leads gonad migration, or to lesser extend migration of the distal tip cell (DTC) of hermaphrodite gonad. Defects of \textit{him-4} lead to germline chromosome loss, tissue fragility and defective cell migration of the male linker cell. A GFP reporter consisting of a \textit{him-4} promoter with in-frame \textit{gfp} fused to the N-terminus lacking the excretion domain is expressed in hermaphrodite DTC during the migration turn towards dorsal and back to midbody. This expression coincides with the heterochronic “Mig” phenotype that certain \textit{daf-12} signaling mutants show. The ligand binding domain truncation \textit{daf-12}\textit{(rh61)} or point mutation alleles \textit{rh273} and \textit{rh274} show this phenotype, as well as a \textit{daf-9}\textit{(rh50)} dafachronic acid synthesis hypomorphic allele. The \textit{daf-12}(\textit{rh61}\textit{rh411}) null allele only shows a migration defect when in background of other heterochronic mutants, e.g. a null allele of the \textit{lin-29}/ZiFi protein or a hypomorph of the \textit{dre-1}/F-box protein.

When analyzing \textit{him-4} promoter activation in different backgrounds I found a partial \textit{daf-12} dependence. Wild type and \textit{daf-12} null mutants do not show a Mig phenotype and 100\% of all DTC express \textit{him-4p::gfp} (Figure 39). RNAi knock down of \textit{lin-29} and \textit{dre-1} caused migration defects in \textit{daf-12}(\textit{rh61}\textit{rh411}) null background, and loss of \textit{lin-29} does not affect \textit{him-4} DTC expression, while loss of \textit{dre-1} does.

Dafachronic acid synthesis hypomorphic \textit{daf-9}\textit{(rh50)} allele and the \textit{daf-12}(\textit{rh61}) allele both cause highly penetrant Mig phenotype, and both can be rescued by RNAi knock down of \textit{daf-12} co-repressor \textit{din-1}, but only \textit{daf-9}\textit{(rh50)} is rescued for both, migration defect and \textit{him-4} expression by ligand supplementation (Figure 39 E).
Figure 39: Him-4 regulation is *daf-12* dependent

*Him-4p:*gfp expression in distal tip cell at 30h-45h of age in wild type (A), *daf-12*(rh61rh411) (B), *daf-12*(rh61) (C) and *daf-9*(rh50) (D) background on RNAi against *din-1, dre-1* or *lin-29*. Shown are percent of distal tip cells expressing GFP (green) or no GFP (red), percent of distal tip cells that are reflexed (“R”) and non reflexed showing the Mig phenotype (“M”), n>20 for each bar. Only in mutations affecting upstream acting *daf-9*, but not in *daf-12lbd* mutants is *him-4p:*gfp reporter expression dafachronic acid dependent (E). A distal tip cell showing Mig phenotyp (F) or wild type reflexed migration (G) in *him-4p:*gfp reporter background.

However, DAF-12 and a *him-4* promoter luciferase reporter were co-transfected into human embryonic kidney cells, no luciferase activation was noticeable after ligand application, arguing for a either a non-direct *daf-12* regulation of the *him-4* promoter, or the requirement for *C. elegans* specific co-factors not available in human cell culture.
Appendix 2: Dafachronic acid derivatives and their effect on DAF-12

The luciferase assay used in Chapter 2 allows rapid in vitro testing if compounds can bind to DAF-12/NHR and influence its transcriptional activation. By using this assay, I tested several hypotheses.

6.1 Glycinoeclepin A derivatives as DAF-12 ligand

Δ⁴- and Δ⁷-dafachronic acid were suggested to be the natural ligands of DAF-12 (Motola et al., 2006). However, a structurally related compound, Glycinoeclepin A, was reported to act as a potent egg hatching factor in plant parasitic nematodes (Fukuzawa et al., 1985; Masamune et al., 1982). The main structural difference of this compound compared to the dafachronic acids is a further oxidation and ring-opening. The structure and function of the egg hatching factor might be conserved in other nematode species, and similar compounds might be present in C. elegans, acting as natural DAF-12 ligands of even higher activity. This led the lab of E.J. Corey to wonder if a ring-opening reaction performed on the dafachronic acids, that converts them into Glycinoeclepin-like compounds, would turn them into more potent ligands for DAF-12, noticeable in higher transcriptional activation of DAF-12/NHR.

Simon Giroux of the E.J. Corey lab synthezised the Glycinoecleptin A-like oxidation product of dafachchronic acids and I tested the product along with intermediates and other derivatives for their biological activity in vitro and in vivo. Luciferase assay was performed with cells co-transfected with DAF-12 expression vector and lit-1 promoter luciferase reporter (Shostak et al., 2004). Different concentrations of compounds were tested on these cells for their capacity to induce DAF-12 dependent transcriptional activation.
Figure 40: Dafachronic acid derivatives
Structure of the dafachronic acid derivate tested for the E.J. Corey lab. Panel A shows nomenclature of ring and carbon atom positions. Panel B: (25R)-Δ⁷-dafachronic acid; (25S)-Δ⁷-dafachronic acid; Δ¹-desmethyl dafachronic acid; (25S)-7,8-dihydro dafachronic acid; (25S)-diketodiacid-dafachronic acid. Drawings by E.J. Corey. Structures of (25S)-Δ⁴-dafachronic acid is shown in Figure 2.

I found that compounds with a double bound in Δ⁴-position are generally less active than those with a double bound in Δ⁷-position: EC₅₀ values of (25S)-Δ⁷- and (25R)-Δ⁷-dafachronic acid are 26 and 64 nM. (4S and 4R vs. 7S and 7R in Figure 41). Chirality at position 25 also influenced activity, with the R-configuration in all cases being more active than the S-configuration (4S and 7S vs. 4R and 7R in Figure 41). Removal of the methyl group at position 25 of Δ⁷-dafachronic acid strongly decreased the activity (7DM in Figure 41 A). Saturation at the Δ⁴- or Δ⁷-double bound only mildly decreased activity to an EC₅₀ value of 114nM (7,8DH in Figure 41 B), while ring opening at that position to a diKeto-diAcid derivative similar to Glycenoelepin A lead to a total loss of activity (diKdiA Figure 41).
Various dafachronic acid derivatives were tested for their transcriptional activation of DAF-12 in a luciferase assay with mir-241 promoter luciferase reporter. “4S” = (25S)-Δ⁴-dafachronic acid; “4R” = (25R)-Δ⁴-dafachronic acid; “7S” = (25S)-Δ⁷-dafachronic acid; “7R” = (25R)-Δ⁷-dafachronic acid; “7DM” = Δ⁷-desmethyl dafachronic acid; “7,8DH” = (25S)-7,8-dihydro dafachronic acid; “diKdiA” = (25S)-diketodiacid-dafachronic acid. EC₅₀ values shown in B in Mol. Assays were repeated once with similar results.

6.2 Ascr#3 interact and DAF-12/NHR or DAF-9/CYP450

*C. elegans* excretes compounds, called “dauer pheromones”, that are capable of inducing dauer formation even during good environmental conditions. Recently, the chemical structures was elucidated for dauer pheromone component ascr#3, member of a compound class called...
“ascarosides” (Butcher et al., 2007; Pungaliya et al., 2009; Srinivasan et al., 2008). I hypothesized that these compounds exert their effect by interacting with either one of the dauer preventing genes DAF-12/NHR or DAF-9/CYP450 to block dafachronic acid activation of DAF-12, or dafachronic acid production. I tested the ascarosides for interference with either DAF-12 transcriptional activation or with DAF-9/CYP450 mediated biosynthesis of the DAF-12-ligands in an in vitro luciferase assay. The co-repressor DIN-1/SHARP is the main co-regulator of DAF-12 during dauer formation (see Chapter 1.3.7), and candidate for ascaroside induced DAF-12 interaction. For that reason I co-expressed DIN-1/SHARP during several of the luciferase assays.

6.2.1 Ascr#3 and DAF-12/NHR

Dafachronic acid and DAF-12 activate transcription of the promoter of a luciferase reporter containing the promoter of mir-241, a direct transcriptional target of DAF-12 (Bethke et al., 2009). If ascarosides can interact with DAF-12, they are likely to suppress the dafachronic acid dependent activation. I found that increasing concentrations of ascr#3 do not strongly inhibit dafachronic acid dependent transcriptional activation by DAF-12, and conclude that ascr#3 does not interact directly with DAF-12 to prevent DAF-12 mediated promotion of developmental progression in vivo (following page: Figure 42 A). The ascr#3 concentrations used compare to up to 400nM found in a very dense C. elegans liquid culture under optimal cultivation conditions (Frank Schroeder, personal communication). Concentrations in nature are expected to be far lower.

6.2.2 Ascr#3 and DAF-9/CYP450

DAF-12 is unable to activate the mir-241 promoter reporter in presence of lathosterone instead of dafachronic acids (Motola et al., 2006). Lathoseterone is the direct precursor of dafachronic acid and substrate for DAF-9/CYP450 that oxidizes the 26 position carbon of lathosterone into a carboxy group to give the active dafachronic acids. Co-transfection of a daf-9 expression construct supplies enzymatic activity for the lathosterone conversion to dafachronic acid, and increasing doses of lathosterone lead to increased DAF-12 activation of the mir-241 promoter reporter (following page: Figure 42 B). I used this co-transfection assay to test if ascr#3 can interact with DAF-9/CYP450 to prevent lathosterone turn over to dafachronic acids in vivo and this way prevent developmental progression and induce dauer formation. I found that increasing doses of ascr#3 do not inhibit the DAF-9/CYP450 mediate conversion of lathosterone to dafachronic acid (following page: Figure 42 C).
6.3 \( \Delta^4 \)- and \( \Delta^7 \)-dafachronic and DAF-9/CYPp450?

It is assumed that \( \Delta^4 \)- and \( \Delta^7 \)-dafachronic acid are the natural ligands of DAF-12/NHR and the final biosynthetic products of the DAF-9/CYPp450 oxidation that show maximum DAF-12 transcriptional activation (following page: Figure 42 D). To test if this is true, or of DAF-9/CYPp450 can further oxidize dafachronic acids to higher active ligands, I measured DAF-12 and \( \Delta^4 \)-dafachronic acid dependent activation of the mir-241 promoter reporter in absence and in presence of DAF-9/CYPp450 expression vector. I found that DAF-9 co-transfection can increase \( \Delta^4 \)-dafachronic acid activation of DAF-12 (following page: Figure 42 F). This observation raises the possibility that dafachronic acids can be further modified to a yet undescribed compound that is a more potent ligand for DAF-12. However, I need to test if this result is not an artifact due to impurities of the \( \Delta^4 \)-dafachronic acid or quality variations of the DNA samples used to transfect cells.

following page: Figure 42: Ascaroside asc#3 does not interact with DAF-12 or DAF-9

A luciferase reporter containing the mir-241 promoter is strongly activated when DAF-12, DIN-1 and 400nM \( \Delta^4 \)-dafachronic acid are present, and varying concentrations of ascr#3 (panel E for molecular structure) do not change the DAF-12 and ligand dependent activation (A). Activation of mir-241 promoter luciferase reporter is strongest in presence DAF-9, DAF-12 and 10\( \mu \)M lathosterone (B). DAF-9 mediated turn over of lathosterone is not suppressed by different concentrations of ascr#3 (C). mir-241 promoter luciferase reporter activation in DAF-12 presence is <10 fold stronger with \( \Delta^7 \)-dafachronic acid then with \( \Delta^4 \)-dafachronic acid (D), and presence of DAF-9 can increase DAF-12 dependent transcriptional activation of the mir-241 promoter luciferase promoter in presence of \( \Delta^4 \)-dafachronic acid (F). Assays were repeated once with similar results.
Appendix 3: Transcriptional activation versus UTR-mediated repression

During the work with microRNA and the translational repression they cause, it became important to measure both promoter derived transcriptional activation and separately from that promoter derived transcriptional activation minus UTR-mediated translational repression. To eliminate animal-to-animal variations, the perfect system would allow measuring both values in the same animal but independently of each other. To obtain cellular resolution and measurement in live animals, fluorescent proteins regulated by the promoters and 3’UTRs of interest appeared to be the tool of choice. A previous publication showed that two GFP variants, Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) are detectable separately without significant cross-activation (Miller et al., 1999) and I decided to use these two chromophores regulated by only the promoter or the promoter together with the 3’UTR. I generated two different types of constructs: one type contains a fusion of the yfp coding region to the promoter of interest and a stable, non-repressed unc-54-UTR to generate a stable mRNA that is translated to YFP according to transcriptional activation only. The second type of construct contains a CFP instead of a YFP coding region, and a UTR that is candidate for translational repression instead of the non-repressed unc-54-UTR (Error! Reference source not found. A).

When transforming C. elegans by germline injection, the injected plasmid DNA is rearranged into long arrays and relatively stable inherited through mitosis and meiosis. To test for repeatable ratio of two plasmids injected in equimolar amounts, I used two nearly identical plasmids (A245 and A246) that only differed in a few base pairs defining the differences between CFP and YFP and measured fluorescence intensity in multiple, independently generated transgenic worm lines.

Fluorescence channel images for the CFP and for the YFP channel showed identical distribution that, when color coded and over layered gave a nearly homogenous mixed color showing equal distribution throughout all worm tissues (Error! Reference source not found. B-E). Pixel intensity measurement of the pharyngeal lobe of several animals of several independently derived transgenic worm lines also gave reproducible ration between CFP and YFP intensities (Error! Reference source not found. F). As a negative control, worm lines were generated carrying only one of the chromophore expression vectors, and animals of these strains gave strong signal in the channel corresponding to the chromophore expressed from the
injected plasmid, but no signal in the channel corresponding to the chromophore expression vector that was not injected (data not shown).

To introduce the 3’UTR-mediated repression component in this balanced system, I next injected an equimolar mix of the *yfp* plasmid with and a different *cfp* plasmid that had the un-repressed *unc-54*-UTR exchanged for a 3’UTR that is expected to mediate repression. Candidates were the *hbl-1*-UTR, the *daf-12*-UTR and the *daf-16*-UTR. I found that the newly introduced, repression-candidate UTRs led to a strong, partially tissue selective decrease of chromophore expression (Error! Reference source not found. G-J). The same decrease in chromophore expression level was observed for both chromophores when fused to the repression-candidate UTR (Error! Reference source not found. J). This repression was not alleviated by RNAi against key components of the RNAi machinery (*alg-2* and *dcr-1*, observation of YFP/CFP ratio by eye, n>10 animals per condition, RNAi positive control were severe tissue malformations on plates with RNAi against the mRNAs that were absent from plates with control vector).

I next tried to test *hbl-1*-UTR repression activity in the seam cells during L3 stage, as observed in Figure 18 of Chapter 2. Unfortunately, the *daf-12* promoter used for chromophore expression constructs activated only very weakly in this tissue, preventing a use of this system for the measurement of *hbl-1*-UTR mediated repression.

**Table 5: Double-tagging plasmid constructs**

<table>
<thead>
<tr>
<th>promoter</th>
<th>chromophore</th>
<th>UTR</th>
<th>construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 myo-3</td>
<td>cfp</td>
<td>unc-54</td>
<td>A245</td>
</tr>
<tr>
<td>2 myo-3</td>
<td>yfp</td>
<td>unc-54</td>
<td>A246</td>
</tr>
<tr>
<td>3 daf-12</td>
<td>cfp</td>
<td>unc-54</td>
<td>A433</td>
</tr>
<tr>
<td>4 daf-12</td>
<td>yfp</td>
<td>unc-54</td>
<td>A434</td>
</tr>
<tr>
<td>5 daf-12</td>
<td>cfp</td>
<td>daf-12</td>
<td>A453</td>
</tr>
<tr>
<td>6 daf-12</td>
<td>yfp</td>
<td>daf-12</td>
<td>A454</td>
</tr>
<tr>
<td>7 daf-12</td>
<td>cfp</td>
<td>hbl-1</td>
<td>A443</td>
</tr>
<tr>
<td>8 daf-12</td>
<td>yfp</td>
<td>hbl-1</td>
<td>A444</td>
</tr>
</tbody>
</table>

In this appendix, I found that extrachromosomal arrays consisting of equimolar injected identical chromophore expression plasmids yield reproducible chromophore expression ratios
that is maintained during reproduction. This observation supports the feasibility of the double-tagging approach described above. However, expression level of the repression-candidate 3’UTR plasmids did not increase when key components of the machinery were knocked out that mediates the RNAi/microRNA mediate 3’UTR-repression. From this observation I cannot conclude if the initial loss of is due to 3’UTR-mediated repression, or simply a loss of mRNA stability compared to the *unc-54* UTR or any other mechanism negatively influencing the expression in a UTR dependent way. A possible test is to cross the array containing both chromophore plasmids into *daf-12*(rh61) background and look for chromophor expression level changes, as ibserved for hbl-1-UTR constructs in Figure 18.

**following page: Figure 43: CFP/YFP tagging**

The *yfp* expression vector contains a non-repressed UTR (green) and expression intensity of the chromophore in vivo represents transcriptional activation of the promoter, while the *cfp* vector contains the repressed UTR (red) and expression intensity of the chromophore represents transcriptional activation minus UTR-mediated translational repression (A). DIC image (B) of a worm transgenic with a *yfp* and a *cfp* expression vector that carry both the *myo-3* promoter and the *unc-54*-UTR as regulatory sequences. CFP channel image (D) and YFP channel image (E) show identical chromophore fluorescence levels, and result in homogenous mixed color when colored channel images are over layered (C). The average ratio of measured pixel intensity was constant throughout three of four independently generated transgenic worm lines (CY1-2, CY5-1, CY1-3, CY5-9), transgenic with extra chromosomal arrays consisting of equimolar amounts of both *cfp* and *yfp* expression vectors containing the *daf-12* promoter and the *unc-54*-UTR (vectors A433 and A434) (F). G-I: A worm line transformed with an equimolar mix of *yfp* vector with the *daf-12* promoter and the *unc-54*-UTR, and *cfp* vector with the *daf-12* promoter and the *daf-12*-UTR (vectors A433 and A453). The YFP channel (H) shows strong expression in several tissues, while the strongly enhanced CFP channel image (I) shows loss of fluorescence in most tissues other then few cells, e.g. in the body wall (arrow). Panel G shows the corresponding DIC image. Diagram J shows the average ratio of YFP over CFP pixel intensity in a defined tissue (big pharyngeal lobe) measured in three independently generated worm lines. Worm lines C1-4 and C1-5 were transgenic with equimolar mixes of *yfp* expression vector containing the *daf-12* promoter and the *unc-54*-UTR and *cfp* expression vector containing the *daf-12* promoter and the *daf-12*-UTR. Worm line Y9-1 was transgenic with an equimolar mix of *yfp* expression vector containing the *daf-12* promoter and the *daf-12*-UTR and *cfp* expression vector containing the *daf-12* promoter and the *unc-54*-UTR, shown in G-I. B-e, G-I: n>20 animals were observed and representative images are shown, F-J: count was performed once.
Appendix 4: Conservation of cis-regulatory motifs in \textit{let-7} family microRNA promoters

To test these candidate DAF-12 dependent and independent CRMs, I analyzed the promoters by sequence comparison to related nematode species. ClustalW alignment of \textit{C. elegans}, \textit{C. briggsae} and \textit{C. brenneri} showed conservation between long regions of the \textit{myo-2} promoter that was analysed in detail (Okkema et al., 1993; Thatcher et al., 1999) (Figure 44) to test if ClustalW is functional for testing promoter conservation between these three species.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Cbrenn-myo-2 promoter & TCCCCCTTTTTTTGAGTTGAGAAAGTTGACAGACATGCATCTATGTCATCCCAACG 1435 \\
Cel-myo-2 promoter & TGGGCTCTTTTTTCTGAAATTCACTGCTATTTGCTACCAAAAAT 1446 \\
cbrigs-myo-2 promoter & ATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT 1446 \\
\hline
Cbrenn-myo-2 promoter & AGTAAAACTGACTGCTAGACACACTGCTATCTATGTCATCCCAACG 1485 \\
Cel-myo-2 promoter & AGTAAAACTGACTGCTAGACACACTGCTATCTATGTCATCCCAACG 1496 \\
cbrigs-myo-2 promoter & AGTAAAACTGACTGCTAGACACACTGCTATCTATGTCATCCCAACG 1463 \\
\hline
Cbrenn-myo-2 promoter & GAGTTAGAAAAATGGCAAGGAAGACACTTTGGAGCAACTGCTATCTATGTCATCCCAACG 1535 \\
Cel-myo-2 promoter & GAGTTAGAAAAATGGCAAGGAAGACACTTTGGAGCAACTGCTATCTATGTCATCCCAACG 1546 \\
cbrigs-myo-2 promoter & GAGTTAGAAAAATGGCAAGGAAGACACTTTGGAGCAACTGCTATCTATGTCATCCCAACG 1513 \\
\hline
Cbrenn-myo-2 promoter & TGTTATGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1583 \\
Cel-myo-2 promoter & TGTTATGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1593 \\
cbrigs-myo-2 promoter & TGTTATGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1563 \\
\hline
Cbrenn-myo-2 promoter & TTGTGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1629 \\
Cel-myo-2 promoter & TTGTGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1639 \\
cbrigs-myo-2 promoter & TTGTGAAAATGTCTGAAATTCACTGCTATCTATGTCATCCCAACG 1613 \\
\hline
\end{tabular}
\caption{Cross-species alignment of \textit{myo-2} promoter}
\end{table}

Part of the ClustalW alignment of a 2kb \textit{myo-2} promoter sequences from \textit{C. brenneri} (Cbrenn), \textit{C. briggsae} (Cbrigs) and \textit{C. elegans} (Cel). The C183 element is marked in blue, and defined transcription factor binding sites are marked in red. Nucleotide numbers on the right indicate distance to start of alignment of 2kb sequence upstream of the \textit{myo-2} start codon.

However, the same alignment strategy revealed a far lower conservation of promoters of the \textit{mir-84}, \textit{mir-241} and \textit{let-7} microRNA promoters.
Figure 45: Cross-species alignment of 84e
Part of the ClustalW alignment of mir-84 promoter sequences from C. brenneri (Cbren), C. briggsae (Cbrig) and C. elegans (Cel). Marked in blue is the sequence repeated in 84e reporter constructs. Nucleotide numbers on the right indicate distance to start of the alignment of 1.1kb sequence upstream of first nucleotide of the mir-84 seed sequence. DAF-12-like a DAF-3-like CRMs are marked in red.

Only the pharyngeal expressed 84e CRM shows high conservation surrounding the DAF-12-like CRM (Figure 32). The seam and hypodermal expressed 84d CRM is much less conserved (Figure 46).

Figure 46: Cross-species alignment of 84d
Part of the ClustalW alignment of *mir-84* promoter sequences from *C. brenneri* (Cbren), *C. briggsae* (Cbri) and *C. elegans* (Cel). Marked in blue is the sequence repeated in 84d reporter constructs. Nucleotide numbers on the right indicate distance to start of the alignment of 1.1kb sequence upstream of first nucleotide of the *mir-84* seed sequence. The DAF-12-like CRM is marked in red.

In the *mir-241* promoter, the completely daf-12 dependent 241ab CRM construct shows a much lower conservation (Figure 47) than the less daf-12 dependent 241cd CRM (Figure 48).

![Figure 47: Cross-species alignment of 241ab](image)

Part of the ClustalW alignment of *mir-241* promoter sequences from *C. brenneri* (Cbre), *C. briggsae* (Cbri) and *C. elegans* (Cel). Marked in blue is the sequence repeated in 84d reporter constructs. Nucleotide numbers on the right indicate distance to start of the alignment of 1.2kb sequence upstream of the first nucleotide of the *mir-241* seed sequence. DAF-12 and FLH-1 CRMs are marked in red.
**Figure 48: species alignment of 241cd**
Part of the ClustalW alignment of *mir-241* promoter sequences from *C. brenneri* (Cbre), *C. briggsae* (Cbri) and *C. elegans* (Cel). Marked in blue is the sequence repeated in 84d reporter constructs. Nucleotide numbers on the right indicate distance to start of the alignment of 1.2kb sequence upstream of the first nucleotide of the *mir-241* seed sequence. The DAF-12 CRMs are marked in red.

When aligning the TRE containing *let-7* promoter, I did not detect the degree of conservation reported by Johnson (Johnson et al., 2003) (Figure 49).

**Figure 49: Cross-species alignment of TRE**
Part of the ClustalW alignment of *let-7* promoter sequences from *C. brenneri* (Cbren), *C. briggsae* (Cbr) and *C. elegans* (Cel). Marked in blue is the sequence repeated in TRE reporter constructs. Nucleotide numbers on the right indicate distance to start of the alignment of 1.9kb sequence upstream of the first nucleotide of the *let-7* seed sequence. The DAF-12;like CRMs are marked in red.
I tested the repeated promoter sequences for degree of sequence conservation between closely related nematode species to find CRMs that were functionally important and therefore conserved above non-important promoter regions.

Each CRM showed a different degree of conservation between different *Caenorhabditis* species, but the degree of conservation and functionality do not appear to correlate since the non-conserved 241ab CRM drives transcription in the most DAF-12 signaling dependent fashion.

This repeats the previous observation that CEH-10 and TTX-3 (Wenick and Hobert, 2004) motifs are conserved between *C. elegans* and *C. briggsea*, but that elements that are either lost or gained between species also are functional.
Appendix 5: Dimerization might influence DAF-12 transcriptional activation

The capacity of NHRs to form homo- or hetero dimers that change their ligand dependence (Forman et al., 1995) highlight the importance of the relative orientation of nuclear receptor REs inside specific CRM-constructs. I found that point mutation of a single DAF-12 RE that is part of a repeat (IR9 = 241cd, ER23 = 84ab) increases activity in vitro in promoter full length context, while mutation of other REs, e.g. 241ab, abolish in vitro activation (data shown above) (Figure 50).

Figure 50: Luciferase assay of repeated response elements
Expression vectors for daf-12 or gfp as control were transfected into Human Embryonic Kidney cell, along with vectors containing luciferase cDNA downstream of wild type or mutated versions of the mir-84 or mir-241 promoters followed by a minimal promoter. Displayed sequences are blown up from the marked full length promoter regions, with green marking wild type sequence of DAF-12-CRMs and red marking mutated nucleotides. Point mutation of the downstream RE of a repeat in both cases led to an increase of dafachronic acid dependent fold luciferase activation, while point mutation of the separately occurring RE lead to a strong loss of activation.

NHRs commonly bind to repeats of their REs and physically interact while bound to DNA (Forman et al., 1995). By showing that transcriptional activation through DAF-12 increases after loss of one REs out of a repeat, I indicate that dimerization might play a role in modulating
DAF-12 transcriptional activation. The in vivo regulation of the IR9 containing 241cd CRM is less stringent DAF-12 and ligand dependent then regulation of the 241ab site. A possible explanation is that a non-DAF-12 NRs is involved in DAF-12 hetero dimerisation, possibly connecting other NHR cascades to the 241cd CRM, or that the CRM sequence influences DAF-12 ligand responsiveness, as observed for the glucocorticoid receptor (Meijsing et al., 2009).
### Appendix 6: List of heterochronic genes in *C. elegans*

<table>
<thead>
<tr>
<th>name</th>
<th>time point</th>
<th>tissue</th>
<th>heterochronic phenotype</th>
<th>Molecular function</th>
<th>homolog</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ain-1</strong></td>
<td>L1-Ad, L2-L3?</td>
<td>vulva, neurons</td>
<td>retarded</td>
<td>microRNA processing</td>
<td>Drosophila</td>
<td>(Ding et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GAWKY</td>
<td></td>
</tr>
<tr>
<td><strong>ain-2</strong></td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Zhang et al., 2007)</td>
</tr>
<tr>
<td><strong>alg-1/alg-2</strong></td>
<td>widely expressed from embryo to adult</td>
<td>retarded</td>
<td>RNA processing</td>
<td>Translation initiation factor 2C; Argonaute like proteins in vertebrates</td>
<td></td>
<td>(Grishok et al., 2001)</td>
</tr>
<tr>
<td><strong>cgh-1</strong></td>
<td></td>
<td>germline</td>
<td></td>
<td>germline helicase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>daf-12</strong></td>
<td>L2-L3</td>
<td>whole body, peaks at L2/L3</td>
<td>retarded</td>
<td>NHR</td>
<td>DHR96, LXR, VDR</td>
<td>(Antebi et al., 1998; Antebi et al., 2000)</td>
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<tr>
<td><strong>daf-9</strong></td>
<td>L2-L3</td>
<td>hypodermis, XXX neuron, spermatheca</td>
<td></td>
<td>CYP450</td>
<td></td>
<td>(Gerisch et al., 2001; Jia et al., 2002)</td>
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<tr>
<td><strong>dcr-1</strong></td>
<td>n.a.</td>
<td>retarded</td>
<td>RNA processing</td>
<td>DICER</td>
<td></td>
<td>(Grishok et al., 2001)</td>
</tr>
<tr>
<td>name</td>
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<td>tissue</td>
<td>heterochronic phenotype</td>
<td>Molecular function</td>
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<tr>
<td>din-1</td>
<td>L2-L3</td>
<td>whole body</td>
<td></td>
<td></td>
<td></td>
<td>SHARP (Ludewig et al., 2004)</td>
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<td>dre-1</td>
<td>L2-L3</td>
<td>whole body</td>
<td>precocious</td>
<td>ubiquitinylation</td>
<td>human</td>
<td>FBX011 (Fielenbach et al., 2007)</td>
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<tr>
<td>flh-1, -2, -3</td>
<td>embryogenesis</td>
<td>precocious</td>
<td>DNA binding</td>
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<td>FLYWCH</td>
<td>transcription factor (Ow et al., 2008)</td>
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<tr>
<td>hbl-1</td>
<td>L1, L2-L3, L4-Ad</td>
<td>hypodermal syncytium neurons later muscle</td>
<td>precocious</td>
<td>nucleic acid binding</td>
<td>hunchback</td>
<td>(Fay et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Frand et al., 2005)</td>
</tr>
<tr>
<td>Kin-20</td>
<td>L4-Ad</td>
<td>hypodemis</td>
<td>precocious</td>
<td>circadian rhythm</td>
<td>drosophila</td>
<td>timeless &amp; doubletime (Banerjee et al., 2005)</td>
</tr>
<tr>
<td>let-7</td>
<td>L4,-Ad</td>
<td>weak expression in most tissues, stronger in seam cells starting at L4</td>
<td>retarded</td>
<td>microRNA</td>
<td>let-7</td>
<td>(Pasquinelli et al., 2000; Reinhart et al., 2000)</td>
</tr>
<tr>
<td>lin-12</td>
<td>L4-Ad</td>
<td>vulva, hypodemis</td>
<td>precocious</td>
<td>notch ligand</td>
<td></td>
<td>(Solomon et al., 2008)</td>
</tr>
<tr>
<td>lin-14</td>
<td>L1-L2</td>
<td>neurons, intestine hypodemis</td>
<td>precocious</td>
<td>nuclear protein</td>
<td></td>
<td>(Ruvkun and Giusto, 1989)</td>
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<tr>
<td>name</td>
<td>time point</td>
<td>tissue</td>
<td>heterochronic phenotype</td>
<td>Molecular function</td>
<td>homolog</td>
<td>reference</td>
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<tr>
<td>lin-28</td>
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<td>precocious</td>
<td>RNA binding</td>
<td>human LIN28</td>
<td>(Arasu et al., 1991; Moss et al., 1997)</td>
</tr>
<tr>
<td>lin-29</td>
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<td>gonad at L3, hypodermis at L4, vulva &amp; goand L4</td>
<td>retarded</td>
<td>ZiFi</td>
<td>EGR2</td>
<td>(Rougvie and Ambros, 1995)</td>
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<tr>
<td>lin-4</td>
<td>L2-Ad</td>
<td>everywhere</td>
<td>retarded</td>
<td>microRNA</td>
<td></td>
<td>(Lee et al., 1993)</td>
</tr>
<tr>
<td>lin-41</td>
<td>L4-Ad</td>
<td>hypodemis pharynx pharynx, intestine at L2/L3, L4</td>
<td>precocious</td>
<td>RBBC, RING finger, B-box, coiled-coil domain, translational inhibition</td>
<td>TRIM71</td>
<td>(Slack et al., 2000)</td>
</tr>
<tr>
<td>lin-42</td>
<td>each molt</td>
<td>seam cells, hyp. syncitium,</td>
<td>precocious</td>
<td>transcriptional regulator</td>
<td>period</td>
<td>(Jeon et al., 1999)</td>
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<td>retarded</td>
<td>Scaffolding protein</td>
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<tr>
<td>lin-58</td>
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<td>see mir-48</td>
<td>precocious</td>
<td>mir-48 gf</td>
<td>let-7</td>
<td>(Abrahante et al., 1998; Li et al., 2005)</td>
</tr>
<tr>
<td>name</td>
<td>time point</td>
<td>tissue</td>
<td>heterochronic phenotype</td>
<td>Molecular function</td>
<td>homolog</td>
<td>reference</td>
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</tr>
<tr>
<td>Lin-66</td>
<td>L2/L3</td>
<td>body vulva muscle, intestine, neurons, hypodermis</td>
<td>retarded</td>
<td>novel</td>
<td>nematode specific</td>
<td>(Morita and Han, 2006)</td>
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<td>L2-L3</td>
<td>widely expressed after L2</td>
<td>retarded</td>
<td>microRNA</td>
<td>let-7</td>
<td>(Abbott et al., 2005)</td>
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<td>(Abbott et al., 2005)</td>
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<td>mir-84</td>
<td>L2-L3, L4-Ad</td>
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<td>retarded</td>
<td>microRNA</td>
<td>let-7</td>
<td>(Abbott et al., 2005)</td>
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<td>nhl-2</td>
<td></td>
<td>neurons, body muscle, pharynx</td>
<td></td>
<td>microRNA processing</td>
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<td>(Hammell et al., 2009)</td>
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<td>nhr-23</td>
<td>L4-Ad</td>
<td>germline during embryogenesis, intestine and pharynx of all stages, peaks during intermolts</td>
<td>retarded</td>
<td>NHR, molting</td>
<td>Drosophila DHR3, ecdysone responsive</td>
<td>(Frand et al., 2005; Gissendanner et al., 2004)</td>
</tr>
<tr>
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<td>Tissue</td>
<td>Heterochronic Phenotype</td>
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<tr>
<td><em>nhr-25</em></td>
<td>L4-Ad</td>
<td>widely expressed during all stages, peaks during molts, succeeding <em>nhr-23</em> peaks</td>
<td>retarded</td>
<td>NHR, molting</td>
<td><em>Drosophila</em> Ftz-F1, human LRH-1</td>
<td>(Frand et al., 2005; Gissendanner et al., 2004)</td>
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<td><em>sop-2</em></td>
<td>L4-Ad</td>
<td>whole body</td>
<td>?</td>
<td>DNA binding HOX gene expression</td>
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<td>(Zhang et al., 2004)</td>
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<td>L4-Ad</td>
<td>hypodermis</td>
<td>precocious</td>
<td>circadian rhythm</td>
<td>mammalian timeless / <em>Drosophila</em> doubletime</td>
<td>(Banerjee et al., 2005)</td>
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Nuclear Hormone Receptor Regulation of MicroRNAs Controls Developmental Progression

Axel Bethke,1,2 Nicole Fielenbach,1 Zhu Wang,1 David J. Mangeldorf,3 Adam Antebi1,4

In response to small-molecule signals such as retinoids or steroids, nuclear receptors activate gene expression to regulate development in different tissues. MicroRNAs turn off target gene expression within cells by binding complementary regions in messenger RNA transcripts, and they have been broadly implicated in development and disease. Here we show that the Caenorhabditis elegans nuclear receptor DAF-12 and its steroid ligand directly activate promoters of let-7 microRNA family members to down-regulate the microRNA target tba-1, which drives progression of epidermal stem cells from second to third larval stage patterns of cell division. Conversely, the receptor without the ligand represses microRNA expression during developmental arrest. These findings identify microRNAs as components of a hormone-coupled molecular switch that shut off earlier developmental programs to allow for later ones.

In unfavorable environments, endocrine systems control is exerted by microRNAs. MicroRNAs are ~20- to 22-nucleotide-long RNA molecules that bind to the 3'UTRs of target messenger RNAs (mRNAs) and decrease their expression (6–8). Null mutants for several microRNA genes show tissue-selective failure of progression from one stage-specific program to the next, generally described as heterochronic phenotypes. These phenotypes are most visible in the hypodermis, where hypodermal seam cells undergo invariant asymmetric stem cell division patterns, in which one daughter cell fuses to the hypodermal syncytiurn, whereas the other retains stem cell character and its capacity to divide (9). Only during L2 do seam cells undergo one proliferative division before stem cell division, and repetition or loss of this program leads to changes in overall seam cell number in later stages. Finally, seam cell division ceases altogether by adulthood. Seam cells in animals with mutation of the microRNA lin-4 repeat L1 programs of asymmetric cell division during L2 stage; a triple deletion of the let-7 microRNA homologs mir-48, -84, -241 (referred to as -let-7) repeat L2 programs of cell proliferation during L3 stage; and let-7 null mutants repeat larval stage divisions and molting behavior in adults (10–12).

Lipophilic hormones coordinate organism-wide developmental progression in metazoans by binding to nuclear hormone receptors (NHRs), converting the presence or absence of ligand into changes in gene expression patterns (1). This regulation is conserved in the nematode Caenorhabditis elegans, where the nuclear hormone receptor DAF-12, a homolog of vertebrate liver X and vitamin D receptors, regulates developmental progression or arrest in response to the environment (2,3). In favorable environments, activation of transforming growth factor-β (TGF-β) and insulin/insulin-like growth factor (IGF) signaling cascades results in production of the DAF-12 steroid ligands, the dafachronic acids (e.g., 4,5-DA), which promote rapid progression through four larval stages (L1 to L4) to reproductive adults (4). In unfavorable environments, endocrine systems are suppressed, and DAF-12 without the ligand causes arrest at a stress-resistant, long-lived alternative third larval stage, called the dauer diapause (L3d) (5).

A more cell-intrinsic level of developmental control is exerted by microRNAs. MicroRNAs are ~20- to 22-nucleotide-long RNA molecules that bind to the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs) and decrease their expression (6–8). Null mutants for several microRNA genes show tissue-selective failure of progression from one stage-specific program to the next, generally described as heterochronic phenotypes. These phenotypes are most visible in the hypodermis, where hypodermal seam cells undergo invariant asymmetric stem cell division patterns, in which one daughter cell fuses to the hypodermal syncytiurn, whereas the other retains stem cell character and its capacity to divide (9). Only during L2 do seam cells undergo one proliferative division before stem cell division, and repetition or loss of this program leads to changes in overall seam cell number in later stages. Finally, seam cell division ceases altogether by adulthood. Seam cells in animals with mutation of the microRNA lin-4 repeat L1 programs of asymmetric cell division during L2 stage; a triple deletion of the let-7 microRNA homologs mir-48, -84, -241 (referred to as -let-7) repeat L2 programs of cell proliferation during L3 stage; and let-7 null mutants repeat larval stage divisions and molting behavior in adults (10–12).

1Huffington Center on Aging, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. University of Osnabrück, Fachbereich Biologie/Chemie, Barbarastrasse 11, 49069 Osnabrück, Germany. Department of Pharmacology and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, 6001 Forest Park Road, Dallas, TX 75390, USA. Max-Planck-Institute for Biology of Ageing, Gerakentstrasse 50, D-50931 Cologne, Germany.

*To whom correspondence should be addressed. E-mail: aantebi@bcm.edu

Fig. 1. DAF-12 and dafachronic acid (4,5-DA) activate microRNA promoters in vitro. (A) Activation of microRNA promoters in HEK293T cells. Promoters of let-7 homologs, mir-84, and mir-241, were strongly activated in the presence of DAF-12 and 400 nM 4-DA, whereas other microRNAs were relatively unaffected. Luciferase assays were measured in triplicate and are shown with SD. E1OH, ethanol vehicle control; ptk, empty luciferase vector. (B) Mutation analysis of mir-237p and mir-48p reveals DAF-12−ΔDA−activating elements. Deletion analysis of the mir-241p showed that the highest relative induction occurs with fragment 4, which contains four DAF-12 REs, 241a, b, c, and d. Deletion or point mutation of 241ab elements (in red) abolished activation (blue bars). Similarly, point mutation of DAF-12 REs in mir-84p, 84a and b, reduced expression (red bars). (C) Gel mobility shift assay of DAF-12 and mir-241p. 32P-radiolabeled oligomers containing the WT 241b element were shifted (●) by nuclear extracts expressing DAF-12:FLAG and supershifted (○) in the presence of FLAG-specific antibody. Unlabeled WT 241b-oligonmer prevented the shift, but addition of an oligomer with a point mutation did not.

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daf-12(hb61r412) null mutants exhibit a heterochronic phenotype similar to triple deletion of let-7 family members mir-48, mir-241(nDf51); mir-84p(n46937), resulting in extra seam cells at the L3 stage (table S1) (2, 11). This observation suggested that daf-12 might directly activate the let-7s microRNAs. To test this hypothesis, we fused the microRNA promoters mir-241p and mir-84p to the luciferase gene and co-transfected the reporters with DAF-12 into human cells (13).

DAF-12 and Δ4-DA strongly activated mir-241p and mir-84p, whereas other promoters gave little or no signal (Fig. 1A). Deletion analysis of the mir-241p revealed that, whereas several deletion mutations retained transcriptional activity in the presence of DAF-12 and Δ4-DA, fragment 4 produced the highest fold induction, and its removal substantially reduced activity (Fig. 1B). This fragment contained two pairs of DAF-12 response elements (REs), as described by Shostak (14). In the full-length promoter context, mutation of one RE pair in mir-241p and two REs in mir-84p led to a decrease in activation of about seven- and threefold, respectively. Gel mobility shift assays confirmed in vitro binding of DAF-12 to these REs, whereas mutated versions abolished the interaction (Fig. 1C).

To examine microRNA expression in vivo, we generated transgenic worms containing the microRNA promoters fused to green fluorescent protein (GFP). Wild-type (WT) worms containing mir-241p::GFP gave a broad expression pattern as described (15). However, daf-12 nulls showed decreased expression, most noticeably in the excretory cells (exc), as well as in muscles, pharynx, and intestine, although neuronal expression seemed less affected, which reveals the tissue selectivity of daf-12 regulation (Fig. 2, A to D). Null mutants of...

**Fig. 2.** DAF-12 and Δ4-DA regulate microRNA promoters in vivo. (A to K) mir-241p::GFP. Images show representative L3 animals, with indicated cell types (white arrowheads and exc, excretory cell; outlined arrowheads, neu, nervous; exc, muscle; int, intestinal; ph, pharynx). Bar graphs alongside the images quantify the percentage of worms with excretory cell GFP expression as either strong (green), weak (yellow), or off (red) (two independent experiments, left and right, n = 10 animals each). For mir-241p::GFP expression level in worms grown without ligand (EIOH) or with ligand (Δ4-DA). (A and B) WT (N2-type). (C) Expression was decreased in daf-2(e1368);N2 wild-type (N2) animals, (D) strongly repressed in daf-9(cy42);CYP450 null, (E) not activated in daf-2(e1368);CYP450–SHARP double null, but (F and H) was rescued nearly to WT level by growth on 250 nM Δ4-DA. In WT (B) or daf-12 null (D) animals, Δ4-DA had no effect. (I to K) Point mutation of all four DAF-12 REs abolished differences of tested genetic backgrounds or ligand [see also (fig. S1)]. (L and O) mir-84p::GFP. Epidermal seam cells (arrowheads) expressed mir-84p::GFP in (J) WT N2, but (M) not in daf-12 nulls. (N) Seam cell expression was absent in hormone-deficient daf-9;cin-1 animals, but (O) restored to nearly WT levels by Δ4-DA supplementation. (Left) Percentage of L3 animals showing weak or no seam cell expression (two independent experiments, n = 20 animals each). (P) Relative quantification of microRNAs by QPCR. MicroRNA expression was decreased in daf-12, daf-9;cin-1 and repressed in daf-9 mutants. In daf-9 genotypes, expression was Δ4-DA–dependent. QPCR was carried out using the TaqMan system (see (13) for data analysis).

**Fig. 3.** MicroRNA regulation by dauer signaling pathways. mir-241p::GFP showed high expression in continuously growing WT (A), but low expression in daf-2(e1372);N2 larvae (B), mir-48p::GFP showed high expression in the pharynx of continuously growing WT (C) but low expression in daf-2(e1368);N2 dauer larvae (D). mir-48p::GFP seam cell expression (E) was elevated in daf-2 and daf-9 mutants during dauer stage (F and H) and was even higher in daf-7 mutants during reproductive growth at 20°C (G). Penetrant seam expression was reversed by 500 nM Δ4-DA in daf-7, but not daf-2, during reproductive growth (B to M). Animals were assayed during L3 and/or L4 stages, n > 20. Red bars, ethanol vehicle; green bars, Δ4-DA (SEM).
transcriptional regulation of the let-7s is also reflected in the abundance of total mature microRNAs as measured by TaqMan fluorescence-based quantitative real-time polymerase chain reaction (qPCR). daf-12 mutants and daf-9;lin-1 animals showed decreased levels compared with WT, whereas daf-9 nulls showed tight repression of let-7 family members of microRNAs (Fig. 2P and figs. S3 to S5). As expected, expression in daf-4, daf-9;lin-1, but not daf-12, mutants was rescued by daf-9;lin-1.

The dauer signaling pathways work upstream of daf-9 to govern organismal developmental progression and 3-DA production. We therefore wanted to see if let-7s expression was affected by dauer constitutive mutant backgrounds of daf-7—TGF-β, daf-2 insulin/IGF-I receptor, and daf-9; CYP450. mir-241p::GFP expression was nearly completely repressed in these dauer mutants, whereas mir-84p::GFP was down-regulated in some tissues, such as the pharynx (Fig. 3, A to D), but consistently up-regulated and more penetrant in others such as the seam (Fig. 3, E to H). Even in reproducibly growing L3 larvae, seam cell expression was more penetrant in WT, daf-9 null, and daf-9; lin-1 doubles with or without ligand (Fig. 2, I to K, and fig. S1), which revealed that these REs mediate both activation and repression. Thus, DAF-12 with the ligand works through its REs to mildly activate mir-241p::GFP expression back to WT levels (Fig. 2F). Tight repression was also relieved in daf-9; dh149; din-1(SB1149) double-null mutants lacking the co-repressor, with mir-241p::GFP expression levels similar to daf-12 nulls (Fig. 2G and H). Unlike daf-12 nulls, however, 3-DA supplementation of daf-9;lin-1 worms restored mir-241p::GFP expression back to WT levels. Point mutation of all four daf-12 REs in mir-241p resulted in the same weak expression level in WT, daf-12 null with ligand, as well as daf-9 null, and daf-9;lin-1 doubles with or without ligand (Fig. 2, I to K, and fig. S1), which revealed that these REs mediate both activation and repression. Thus, DAF-12 with the ligand works through its REs to mildly activate mir-241p::GFP in some tissues, whereas DAF-12 without the ligand, together with DIN-1, tightly represses expression in nearly all tissues.

As reported, mir-84p::GFP was expressed in pharynx, somatic gonad, seam cells, vulva cells, and, occasionally, in the intestine (15, 19). Seam cell expression was absent in daf-12 null and daf-9;lin-1 null worms, and expression was increased almost to WT levels in daf-9; lin-1 animals by addition of 3-DA (Fig. 2, L to O), which explains daf-12 heterochronic phenotypes as a failure to activate the microRNAs in temporally patterned tissues. By contrast, expression in other tissues, such as the pharynx, was less affected, which shows again tissue-specific daf-12 regulation (Fig. S2).

The zinc finger protein hbl-1 (hunchback) is responsible for L2 proliferative programs of seam cells (11). hbl-1 loss leads to a loss of proliferative programs and, hence, a decreased seam cell number (table S1). let-7s are proposed to inhibit hbl-1, through microRNA-mediated repression of its 3’UTR, because let-7 mutants have both increased seam number and hbl-1; GFP expression at L3 (11, 20). Given that DAF-12 activates let-7s, we examined interactions with hbl-1. Consistent with the notion that hbl-1 inhibition is promoted by NHR signaling, daf-12 mutants and daf-9;lin-1 double-mutants have extra seam cells, the latter reversed by 3-DA (table S1). Moreover, the penetrant extra seam phenotype of the daf-12(her1) ligand-binding domain mutant was dependent on functional hbl-1(+)::GFP, placing daf-12 upstream of hbl-1 by genetic epistasis. Accordingly, we observed consistent up-regulated hypodermal expression of hbl-1::GFP::hbl-1::3-UTR during L3 in daf-12(her1), but not in WT (Fig. 4, A and B). This up-regulation was likely due to loss of UTR-mediated repression, as exchange of the hbl-1::3-UTR for a nonexpressed unc-54::3-UTR led to equal hypodermal expression levels in both WT and the daf-12(her1) background (Fig. 4, C and D).

In this work, we show that the NHR DAF-12 directly regulates the let-7 relatives, mir-84 and mir-241, and connects organism-wide commitment to cell intrinsic programs (Fig. 4E). These studies suggest a model whereby, in unfavorable environments, down-regulated insulin/IGF-I and TGF-β pathways suppress dafachronic acid production and DAF-12 without the ligand together with co-repressor DIN-1 repress microRNA expression in most tissues and specify developmental arrest. Conversely, in favorable environments, stimulation of insulin/IGF-I and TGF-β growth signaling pathways results in dafachronic acid production. DAF-12 with the ligand activates let-7 homologs, which, in turn, down-regulate their target, hbl-1, and allows L2 to L3 transitions in the hypodermis. The use of this NHR-microRNA-coupled molecular switch to turn off earlier programs to allow for later ones is a function likely to be conserved and may be a paradigm for understanding hormone-dependent developmental progression, stem cell differentiation, maturation, or tumor formation in metazoans. In particular, activation of new programs and inhibition of earlier ones are critical for the fidelity of distinct developmental states, which may be less apparent in more complex animals whose cell lineages are unknown. In fact, DAF-12 itself is down-regulated by let-7 at later stages, which suggests that both feedforward and feedback loops drive transitions (21). In Drosophila melanogaster, the steroid hormone ecdysone and its cognate receptor regulate developmental progression in part via mir-14, but it is not known whether regulation is direct or indirect (22). Our studies also reveal the intricacy of NHR signaling in an organismal context. They give visible evidence that receptors with the ligand can activate their targets, whereas receptors without the ligand can repress them, with vastly different outcomes for progression or arrest. This hormone-dependent modulation of target gene expression around basal transcription mirrors that seen with the vertebrate homolog LXRx (23). Finally, DAF-12-NHR regulation of the microRNAs is highly tissue- and stage-specific, implicating other transcription factors, coregulators, and chromatin factors in the control of microRNA expression.
Evidence for Cardiomyocyte Renewal in Humans

Olaf Bergmann,† Ratan D. Bhadrwal,‡ Samuel Bernard,§ Sofia Zdunek,‖ Fanie Barnabé-Heider,† Stuart Walsh,‡ Joel Zupich,§ Kanar Aliaks,§ Bruce A. Buchholz,§ Henrik Drud,† Stefan Jövinge,*,‡ Jonas Frisen‡

It has been difficult to establish whether we are limited to the heart muscle cells we are born with or if cardiomyocytes are generated also later in life. We have taken advantage of the integration of carbon-14, generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans. We report that cardiomyocytes renew, with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75. Fewer than 50% of cardiomyocytes are exchanged during a normal life span. The capacity to generate cardiomyocytes in the adult human heart suggests that it may be rational to work toward the development of therapeutic strategies aimed at stimulating this process in cardiac pathologies.

Myocardial damage often results in chronic heart failure due to loss and insufficient regeneration of cardiomyocytes. This has prompted efforts to devise cardiomyocyte replacement therapies by cell transplantation or by the promotion of endogenous regenerative processes. The development of cell transplantation strategies is advancing rapidly, and some are currently being evaluated in clinical trials (1, 2). Stimulating endogenous regenerative processes is attractive as it potentially could provide a non-invasive therapy and circumvent the immunosuppression required for allografts. However, it is unclear whether such regenerative strategies are realistic because it has been difficult to establish whether cardiomyocytes can be generated after the perinatal period in humans.

Stem/progenitor cells with the potential to generate cardiomyocytes in vitro remain in the adult rodent and human myocardium (3, 4). Moreover, mature cardiomyocytes have been suggested to be able to reenter the cell cycle and duplicate (5). However, studies over several decades in rodents with labeled nucleotide analogs have led to conflicting results, ranging from no to substantial generation of cardiomyocytes postnatally (6). A recent genetic labeling study, which enabled detection of cardiomyocyte generation by stem/progenitor cells (but not by cardiomyocyte duplication), demonstrated cardiomyocyte renewal after myocardial injury, but not during 1 year in the healthy mouse (7). It is possible that humans, who live much longer than rodents, may have a different requirement for cardiomyocyte replacement. Cell turnover has been difficult to study in humans because the use of labeled nucleotide analogs and other strategies commonly used in experimental animals cannot readily be adapted for studies in humans owing to safety concerns. The limited functional recovery after loss of myocardium and the fact that primary cardiac tumors are very rare indicate that postnatal DNA synthesis in the human heart (8). Several studies have described the presence of molecular markers associated with mitosis in the human myocardium (9). This includes limited information because it is difficult to determine the future fate of a potentially dividing cell in terms of differentiation and long-term survival.

We have measured carbon-14 (14C) from nuclear bomb tests in genomic DNA of human myocardial cells, which allows retrospective birth dating (9–11). 14C concentrations in the atmosphere remained relatively stable until the Cold War, when aboveground nuclear bomb tests caused a sharp increase (12, 13). Even though the detonations were conducted at a limited number of locations, the elevated amounts of 14C in the atmosphere rapidly equalized around the world as 14CO2. After the Limited Nuclear Test Ban Treaty in 1963, the 14C concentrations dropped exponentially, not primarily because of radioactive decay (half-life of 5730 years), but by diffusion from the atmosphere (14). Newly created atmospheric 14C reacts with oxygen to form 14CO2, which is incorporated by plants through photosynthesis. Humans eat plants, and animals that live off plants, so the 14C concentration in the human body mirrors that in the atmosphere at any given time (15–18). Because DNA is stable after a cell has gone through its last cell division, the concentration of 14C in DNA serves as a date mark for when a cell was born and can be used to retrospectively birth date cells in humans (9–11).

We first carbon-dated left ventricle myocardial cells, including cardiomyocytes and other cell types, to determine the extent of postnatal DNA synthesis in the human heart. DNA was extracted, and 14C concentrations were measured by accelerator mass spectrometry (see tables S1 and S2 for 14C values and associated data). The cellular birth dates can be inferred by determining the time at which the sample’s 14C concentration corresponded to the atmospheric concentration (Fig. 1A). 14C concentrations from all individuals born around or after the nuclear bomb tests corresponded to atmospheric concentrations several years after the subjects’ birth (Fig. 1B), indicating substantial postnatal DNA synthesis. Analysis of individuals born before the period of nuclear bomb tests allows for sensitive detection of any turnover after 1955, due to the marked increase in 14C concentrations. By analyzing individuals born at different times before 1955 it is possible to establish the age up to which DNA synthesis occurs, or whether it continues beyond that age.
Syntheses and Biological Evaluation of B-Ring-Modified Analogues of Dafachronic Acid A

Simon Giroux,† Axel Bethke,‡ Nicole Fielenbach,‡ Adam Antebi,‡ and E. J. Corey*,†

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, and Huffington Center on Aging, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

corey@chemistry.harvard.edu

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ABSTRACT

Synthesis and testing of dafachronic acid A (1) and its derivatives 2 and 3 have revealed that 1, and not a further oxidation product, is the natural ligand for the DAF-12 receptor of Caenorhabditis elegans.

Remarkably, the life span of the nematode Caenorhabditis elegans can be increased significantly by loss of function of a handful of genes that affect endocrine function. Among them, the daf-9 gene encodes a cytochrome P450 enzyme which is responsible for the biosynthesis of the bile acid-like steroid, dafachronic acid A (1). Based on various analytical techniques, it has been recently proposed by Mangelsdorf and Antebi that 1 is the major ligand for the nuclear receptor DAF-12, which in its ligand bound form regulates genes that prevent entry into the dauer stage, a long-lived quiescent mode. However, synthesis of the proposed ligand remained elusive until a later work, in which the 25-(S) structure of 1 and its 25-(R)-diastereomer were made.2,3 In this research, we address the question of whether

dafachronic acid A is the true ligand for the nuclear hormone receptor DAF-12 or just a precursor of a further biooxidation product which is the actual ligand. We were intrigued by the fact that dafachronic acid A, with its Δ7-olefinic linkage, might be further oxidized biologically to a seco acid structure resembling that of glycinoeclepin A, a potent hatching factor for the eggs of the nematode Heterodera glycines.
Figure 1). Consequently, we became interested in exploring the biological activity of the \(\beta\)-seco dafachronic acid A derivative 2, as an analogue of glycinoeclepin A, which might even be a more active metabolite of 1. In this paper, we describe the synthesis and biological evaluation of 2. For comparison, we have also synthesized the 7,8-dihydro derivative of dafachronic acid A, 3, which would be expected to be devoid of activity if the seco acid 2 were the real ligand for DAF-12, rather than dafachronic acid A (1).

The synthesis of the diketo diacid 2 started with the previously reported 6-keto steroid 4. Baeyer–Villiger oxidation of 4 with trifluoroacetic acid ((CF\(_3\))\(_2\)CO:H\(_2\)O, 0 °C, CHCl\(_3\)) afforded the desired 7-membered lactone 5 in 94% yield and as a sole regioisomer. Lactone 5 was cleaved to a ketoacid intermediate by treatment with Jones' reagent (2 equiv, 23 °C, acetone) which was esterified by diazomethane (CH\(_2\)N\(_2\), Et\(_2\)O) to give ketoester 6 in essentially quantitative yield over two steps. Saponification of the \(\beta\)-acetate, oxidation of the resulting alcohol to the ketone, and hydrolysis gave the diketo diacid 2 in 52% overall yield (three steps, Scheme 1). Our initial strategy for the synthesis of 2 involved the oxidation of the \(\Delta^7\)-olefinic linkage in 1 by various methods. Surprisingly, all attempts to directly oxidize the \(\Delta^7\) bond to the diketo diacid 2 using \(\text{O}_3\) then \(\text{H}_2\text{O}_2\), \(\text{KMnO}_4\), \(\text{NBu}_4\text{MnO}_4\), and \(\text{RuCl}_3\)–\(\text{NaIO}_4\) were unsuccessful.

To synthesize the 7,8-dihydro analogue 3, we have also used an intermediate from our synthesis of 1. Thus, the \(\Delta^5\)-double bond in 7 was reduced (H\(_2\), 1 atm, Pd–C, EtOAc) to give the fully saturated steroid, and the same three steps as above were performed to give analogue 3 in 33% overall yield for the four steps. It should also be mentioned that the hydrogenation of 1 to 3 failed under several conditions. Next, samples of the synthetic dafachronic acid A 1, the seco-diacid 2, and 7,8-dihydrafachronic acid A 3 were evaluated for their bioactivity. First, the ability of synthetic ligands to rescue daf-9 hormone biosynthetic mutants from the dauer state was measured. Consistent with 1 being a natural ligand for DAF-12, dafachronic acid A rescued dauer formation in the nanomolar range, with half-maximal activity of 18.5 nM (Figure 2). Similarly, the 7,8-dihydro analogue 3 also gave substantial rescue with half-maximal rescue at 292 nM. By contrast, the seco-diacid 2 was found not to rescue C. elegans from the dauer state, indicating that it is not a ligand. Second, the ability of synthetic ligands to activate DAF-12 in transcriptional assays on a target gene,
lit-1, was measured. To do this, plasmid constructs containing the daf-12 gene and the lit-1 gene fused to a luciferase reporter were cotransfected into human embryonic kidney cells (HEK293T) and treated with various doses of the compounds and luciferase induction measured by light emission.\(^1\) In accord with the dauer rescue results, \(^2\) showed no activity even at 100 \(\mu\)M concentration (Figure 3), whereas 7,8-dihydrodafachronic acid A (\(^3\)) showed activity similar to that of dafachronic acid A (\(^1\)). Specifically, measurement of the dose–response revealed EC\(_{50}\) values for daf-12 activation to be 114 nM for 7,8-dihydrodafachronic acid A and 26 nM for dafachronic acid A.

These results taken together allow the following conclusions: (1) dafachronic acid A is a natural ligand for DAF-12 nuclear receptor, (2) in contrast to the soybean nematode case, ring B oxidative cleavage products are not the active agents for gene activation of \(C.\) elegans DAF-12, and (3) the \(\Delta^7,8\) double bond is not essential for dafachronic acid activity on \(C.\) elegans.

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**Supporting Information Available:** Experimental protocols, characterization for all new compounds, and methods for performing the dauer assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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7. Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich / unentgeltlich geholfen.

1. Nicole Fielenbach, a postdoctoral fellow of the Antebi Lab, contributed to genetics to generate worm strains and to analyzing GFP intensities.

2. Zhu Wang is a graduate student in David Mangelsdorf's lab and assisted in performing initial luciferase assays.

3. David Mangelsdorf provided the Δ4-dafachronic acid.

4. Adam Antebi supervised the study and supervised the text writing for the publication, but also contributed directly by generating worm strains and analysis of GFP intensities.

5. For Chapter 2, Mary Wiese, a graduate student of the Antebi lab and the Zheng lab at Baylor College of Medicine contributed by germline injection of several constructs.

6. For Chapter 4, Linyan Meng, a rotation student in the Antebi Lab, contributed to luciferase assays and GFP intensity analysis.

7. For Appendix 2, the Corey lab at Harvard University provided Δ7-dafachronic acid and all tested derivatives. The Schroeder lab at Cornell University provided ascaroside ascr#3.

8. For Appendix 3, Dongling Li, research assistant of the Antebi lab, contributed by germline injection of some of the constructs.

9. Christopher Henseley, an undergrad student of the Department of Biology of the Rice University in Houston, Tx, contributed to the molecular biology required to generate various
reporter construct plasmids used for Appendix 3 and to germline injection to transform the generated constructs into C. elegans.


Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ahnlicher Form einer anderen Praeferungsbuerde vorgelegt.

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(Ort. Datum)                                     (Unterschrift)

7.1 Publication of Data presented in Chapter 2

Axel Bethke, Nicole Fielenbach, Zhu Wang, David J. Mangelsdorf, Adam Antebi:
"Direct nuclear hormone receptor regulation of microRNAs controls developmental progression"

7.2 Publication of Data presented in Appendix 2

Simon Giroux, Axel Bethke, Nicole Fielenbach, Adam Antebi, and E. J. Corey:
“Syntheses and Biological Evaluation of B-Ring-Modified Analogues of Dafachronic Acid A”


8. References


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