2 INTRODUCTION

2.1 Model Systems

2.1.1 Overview

Basic biomedical research in the past 30 years has concentrated on a small number of model systems like *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevi, Mus musculus*, mammalian cells in culture, and primates. In spite of obvious differences between the systems, a remarkably high degree of congruence on the molecular level has been revealed. For example, data base alignments predict that 40% of the ca.19000 *C. elegans* genes have homologs in other organisms (The *C.elegans* sequencing consortium, 1998).

2.1.2 *C. elegans* is a powerful model for genetic analyses of development and diseases

The soil saprozoic nematode *C. elegans* is one of the best-studied organisms in the world. The adult hermaphrodite consists of only 959 cells that are constant in number and position in each individual (eutely). The origin and fate of each single cell during development has been traced in large part by John Sulston and coworkers, giving rise to a defined cell lineage and fate map (Sulston and Horwitz, 1977). *C. elegans* offers a great potential for genetic analyses because of its rapid life cycle (3 days), small size (1, 5 mm-long adult), transparency of the body, eutely, large broods (300-350 per animal) and the ease of laboratory cultivation on agar plates seeded with an *E. coli* lawn. Moreover, the natural way of *C. elegans* breeding is by self-fertilizing hermaphrodites, as well as crossing with rarely occurring males (1 male/500 animals) (Figure 1A, B). Selfing and crossing at will facilitates genetic analyses, and the isolation and characterization of mutants (*C. elegans* II, Riddle, 1997).

2.2 Genome

C. elegans is the first metazoan organism whose complete genome has been sequenced (*C. elegans* Sequencing Consortium, 1998). The knowledge of the *C. elegans* genomic sequence, together with genetic, developmental and anatomic data provides a powerful resource applicable also for research in other systems. Its genome is relatively small (100

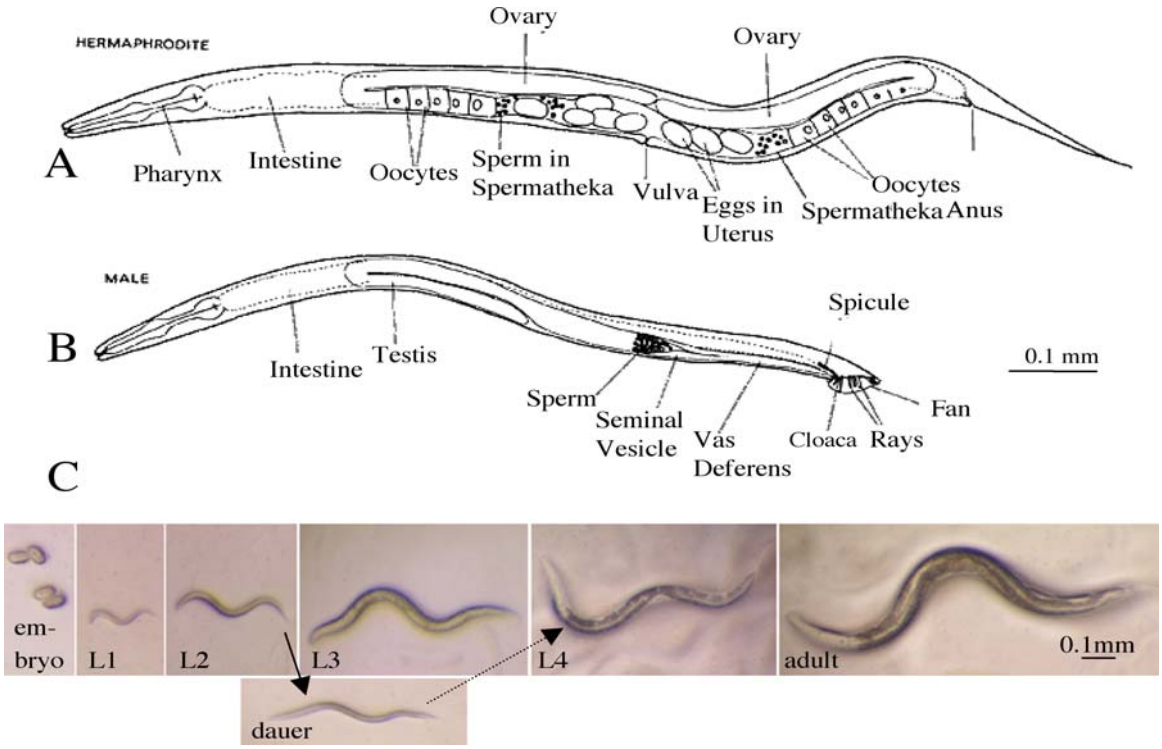

Figure 1) The nematode *Caenorhabditis elegans.* A) Adult hermaphrodite; **B**) adult male (adapted from Riddle, *C. elegans* II, 1997); C) *C. elegans* larval stages from embryo to adult including optional L3 dauer larvae.

million base pairs), only 20 times that of *E. coli*, and approximately half of *Drosophila melanogaster* and *Homo sapiens* (International Human Genomem Sequencing Consortium, 2001). It is organized in 5 autosomes and one X-chromosome. Sex determination is achieved by the copy number of the X-chromosome; hermaphrodites have two copies (XX), males only one (X0) (*C. elegans* II, Riddle, 1997).

2.3 Life history

The life history of *C. elegans* is well described, and is influenced by environmental inputs. *C. elegans* has six recognized developmental stages: Embryo, first (L1) through fourth (L4) stage larvae, and adult, with options at L1 and L3 for a reversible arrest (Figure 1C). Larval life stages are marked by molting of the cuticle and also by specific patterns of cell division, leading to the formation of stage-specific cell types (Sulston and Horvitz, 1977). In abundant food, *C. elegans* develops to reproductive adult in 3 days and then lives for another 2-3 weeks (Albert and Riddle, 1997). However, when worms sense imminent starvation, they arrest development, either at L1 or at L3 diapause. In particular, the L3 dauer diapause (Golden and Riddle, 1984) is a well-studied example of a regulated, modified life program dependent on environmental inputs.

2.4 Dauer Diapause

The dauer diapause is caused by several unfavorable environmental conditions including dwindling food, high temperature and high concentrations of pheromone signaling caused by high population density. Notably, dauer larvae are sexually immature, non-feeding and long-lived; they can survive four to eight times longer than the normal two week life span of reproductive animals (Klass and Hirsh, 1976). When environmental conditions improve, dauer larvae recover to normal reproductive adults with then normal life spans (Golden and Riddle, 1984). Dauer larvae have been shown to be stress resistant and tolerant against oxidative damage (Honda and Honda, 1999; Vanfleteren, 1993). They are morphologically, metabolically, and behaviorally specialized for dispersal and long term survival (Cassada and Russel, 1975). Dauer larvae derive energy from stored fat accumulated in intestinal and hypodermal cells before entry into dauer (Popham and Webster, 1979; Albert and Riddle, 1988). They are thin and dense due to shrinkage of the hypodermis at the dauer-specific molt (Cassada and Russel, 1975; Albert and Riddle, 1988). The dauer cuticle is thicker and has lateral ridges (alae) that are not present on L2, L3 and L4 cuticles. Pharyngeal pumping is suppressed (Cassada and Russel, 1975), and several sensory neurons exhibit altered position or dendrite orientation (Albert and Riddle, 1983). Taking all this into account the question arises how environmental signals like food availability can cause these dramatic changes in the *C. elegans* anatomy and behavior.

2.5 The dauer pathways converge on the nuclear hormone receptor DAF- 12

2.5.1 Molecular overview

Products of the dauer formation genes (Daf-genes) evaluate sensory information and subsequently select programs of diapause or reproductive growth. Molecular, genetic, and cellular analyses of the Daf loci reveal a network of neuroendocrine pathways, including insulin/insulin growth factor I (IGF-I) (Morris, 1996; Kimura, 1997; Ogg, 1998; Ogg, 1997; Paradis, 1999; Pierce, 2001) transformation growth factor-ß (TGF-β) (Estevez and Riddle, 1993; Georgi, 1990; Inoue, 2000; Ren, 1996; Schackwitz, 1996) cyclic guanosine monophosphate (cGMP) (Birnby, 2000; Coburn, 1998) and serotonergic (Sze and Ruvkun, 2000) signaling. Mutants in the Daf-genes fall into two general classes: Daf-c mutants always form dauers, whereas Daf-d mutants never form dauers independent of environmental inputs (Riddle et al., 1981). Genetic epistasis experiments suggest that the

dauer pathways converge on the cytochrome P450 DAF-9 (Gerisch et al., 2001; Figure 2). DAF-9 is likely involved in the production of a steroid-like hormone that regulates the activity of the nuclear hormone receptor DAF-12. Nuclear receptors such as DAF-12 are non-membrane bound transcription factors responding to lipophilic ligands like steroid hormones and regulate gene expression. It is thought that DAF-12 promotes reproductive development in the presence of the postulated hormone, whereas in the absence of hormone, reproductive development is repressed and dauer formation occurs. How *daf-12* actually works to activate or repress gene expression is yet unknown and is a key question of this study.

2.5.2 Heterochronic pathways

In addition to its function in the dauer signaling, *daf-12* also acts in the heterochronic pathway (Figure 2). The heterochronic genes specify developmental age and select stage-appropriate developmental programs (Ambros, 1997). Mutations in these genes can advance or delay the expression of stage specific developmental programs. Those heterochronic genes that have been molecularly characterized encode transcriptional (e.g. *lin-29*, Bettinger et al., 1996) or translational regulators (e.g. *let-7, lin-4*; Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Jeon et al., 1999). *daf-12* mutants with heterochronic phenotypes inappropriately repeat L2 programs in gonadal and extragonadal tissues during L3. By interference, *daf-12(+)* is required to promote L3 and later fates (Antebi et al., 1998).

Thus, DAF-12 acts at the intersection of dauer and heterochronic pathways, coupling environmental conditions to the proper succession of life stages (Figure 2). Two of the heterochronic genes, *lin-4* and *let-7* encode 22 nt RNAs that regulate early and late larval transitions, respectively (Figure 2; Feinbaum and Ambros, 1999). They belong to the small temporal RNA (stRNAs) class of regulators. stRNAs act as modulators of target mRNA translation and stability by binding to complementary sites in the 3`UTR. *lin-4* and *let-7* are processed from 70 nt precursor DNA stem loop structures to 22 nt stRNAs. Interestingly, the stRNA cleavage and processing is performed by *dcr-1, alg-1* and *alg-2* (Grishok et al., 2001), homologs of the nuclease complex components that mediate RNA interference (RNAi) processes in *C. elegans* (Fire, 1998), *Drosophila* and probably in all metazoans (Tuschel et al., 2001). In the heterochronic pathways they are thought to mediate translational control through *lin-4* and *let-7.* Consistent with this, *alg-1, alg-2* and *dcr-1* phenotypes resemble those of *lin-4* and *let-7 (*Grishok and Mello, 2001). This

indicates that the RNAi machinery and the regulation of developmental timing in the heterochronic pathway are closely coupled. RNAi mediates the post transcriptional gene silencing (PTGS) and is thought to be a defense mechanism against alien double stranded RNA. *daf-12* acts at a central position within the heterochronic pathway and possesses potential *lin-4* and *let-7* binding sites in the 3`UTR (Antebi, personal communication). Thus, it would be interesting to know if *daf-12* is regulated by stRNAs or if it is itself involved in the regulation of these factors.

2.5.3 Aging

It has been shown that several genes controlling dauer formation also regulate the rate of aging in *C. elegans* (Kenyon, 2001). In particular, mutations in components of the insulin signaling pathway, including the insulin/IGF like receptor *daf-2* or the kinases *age-1* and *pdk-1* (see below) live 2 to 3 times longer than wild type (Age-phenotype; Kenyon, 1993; Kenyon, 1995; Larsen and Riddle, 1995). *daf-12,* which acts downstream or parallel to insulin signaling, can modify the longevity phenotypes of *daf*-2. *daf-12* weakly suppresses some long-lived mutants (*daf-2*, class 1, see below), but enhances others (*daf-2,* class 2). *daf-12* on its own weakly shortens life span (Gems, 1998; Antebi et al., 2000). This indicates an influence of *daf-12* in the regulation of life span.

Interestingly, insulin signaling is closely coupled to other processes that are thought to influence life span also in mammals, such as metabolism, caloric restriction or free radical protection (Finch and Ruvkun, 2001). The free radical theory of aging predicts that free radicals cause severe damage in the cell, including the genome. It is thought that an increasing number of mutations or macromolecular lesions would arise, subsequently causing dysfunction and cell death. Consistent with this, mutations in a cytosolic catalase result in a short life (Taub et al., 1999). Dauer larvae have elevated levels of catalase (*ctl-1*) and SOD (Larsen, 1993). Moreover, *daf-2* mutants are resistant to oxidative stress and have elevated levels of the manganese superoxide dismutase (*sod-3*) (Honda, 1999). Conceivably, an upregulation of insulin signaling leads to decreased expression of *ctl-1* and *sod-3.* As a consequence, the number of free radicals in the organism would increase, causing damage, mutations, and early cell death in animals undergoing reproductive growth.

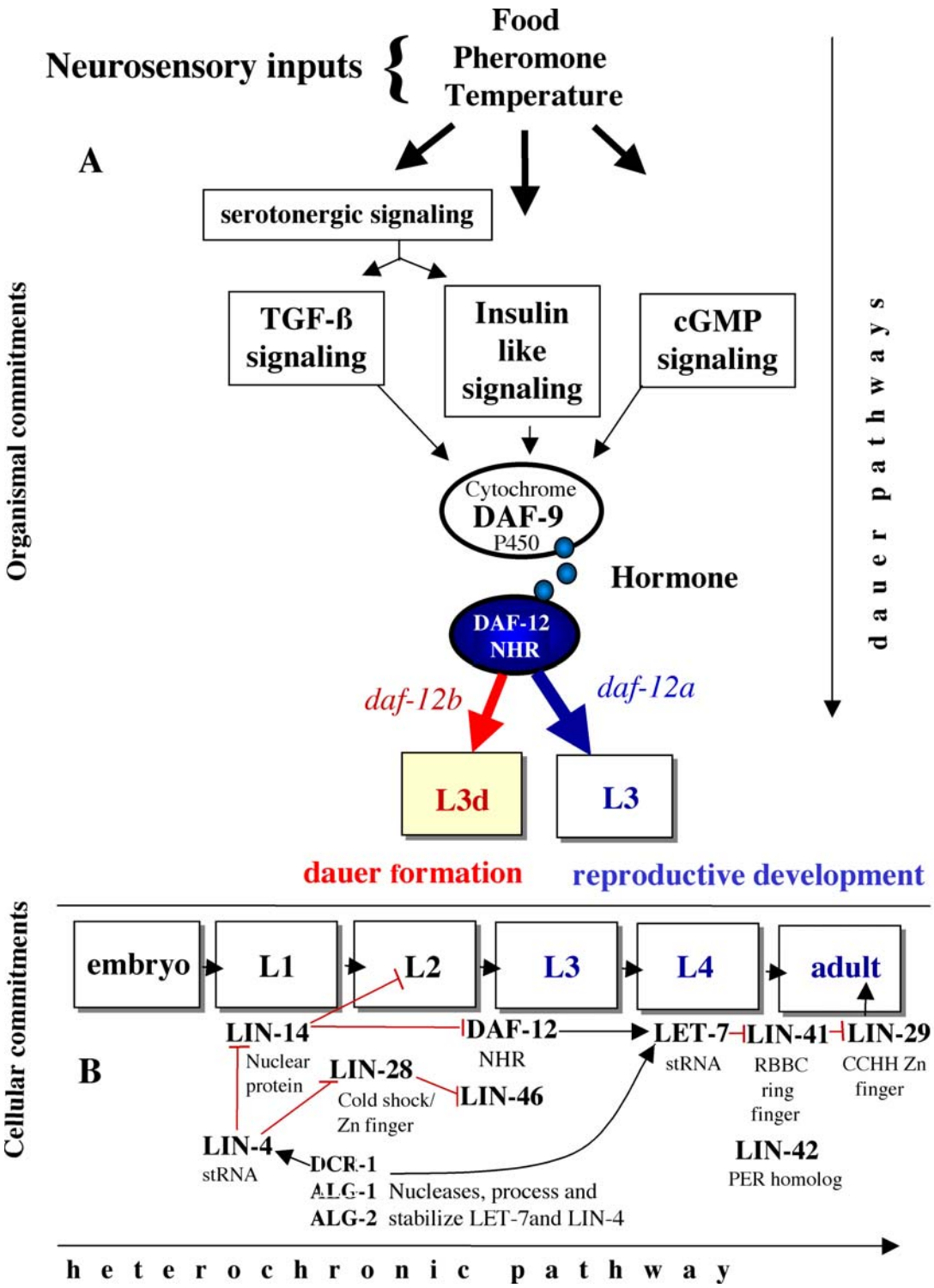

Figure 2) ***daf-12*** **acts at the intersection of heterochronic and dauer pathways**. **A)** In the dauer pathways, genetic epistasis experiments place *daf-12* downstream of serotonergic-, insulin/IGF-, TGF-ß-, cGMP- and P450 DAF-9 signaling. Together, these pathways select between *daf-12 a* and *daf-12 b* activity at the L2/L3 transition. A simple model suggests that inputs from the dauer pathways control synthesis or availability of a retinoid or steroid hormone which then acts through *daf-12* and perhaps other receptors to select *daf-12a* or *daf-12b* activity. **B)** In the heterochronic pathways, *daf-12* is placed downstream of *lin-14* and in aproximity to *lin-28*. Heterochronic genes act in the target tissues to select stage- appropriate programs.

2.6 *daf-12* features

2.6.1 Molecular features

daf-12 is located on the X-chromosome. It contains domains typical of nuclear receptors, including a DNA binding domain (DBD) a hinge region and a ligand binding domain (LBD) (Antebi, 2000). A ligand dependent activation function (AF-2) is localized in the very C-terminal region of *daf-12,* which is thought to trigger the transcription of target genes (Laudet and Gronemeyer, 2002). Its closest relatives include vertebrate vitamin D and pregnane X-receptors (Baker et al., 1988; Bertilsson et al., 1998; Kliewer et al., 1998) and the *Drosophila melanogaster* DHR96 (Fisk and Thummel, 1995). *C. elegans* paralogs include *nhr-8* and *nhr-48. daf-12, nhr-8* and *nhr-48* and DHR96 form a subgroup of nuclear hormone receptors, called the ESCKA family, defined by a 13 aa identical stretch within the DNA recognition helix (Antebi et al., 2000).

2.6.2 Phenotypic complexity

daf-12 mutants show great phenotypic complexity. Null alleles or DBD mutants are dauer defective (Daf-d). LBD mutants are dauer constitutive (Daf-c) or Daf-d and in addition, some alleles exhibit delayed heterochronic phenotypes, arising from developmental arrest (Antebi et al., 1998). The phenotypic complexity of *daf-12* mutants represents at least two *daf-12* activities, *daf-12a* and *daf-12b* (Figure 2). *daf-12a* promotes third stage reproductive development and short life span under reproductive growth conditions, and inhibits dauer formation. In the contrary, *daf-12b* promotes third stage dauer diapause and long life under dauer inducing conditions (Antebi et al., 1998, Antebi et al., 2000). A single hormone that activates *daf-12* target gene expression could instruct *daf-12a* activity. In the absence of hormone, *daf-12b* activity promotes the dauer diapause, perhaps by a repressor function (Figure 2). In this view, the *daf-12* Daf-c phenotypes result from loss of hormone binding, leading to a constitutive repression of the genes for reproductive growth. In addition, *daf-12b* could actively promote dauer genes. It is also possible that two distinct hormones could specify a and b activity.

2.6.3 Functional redundancy

Null mutants of *daf-12* are fully dauer defective but have impenetrantly delayed heterochronic phenotypes. In contrast, *daf-12* LBD mutants often display penetrant heterochronic phenotypes. Presumably, *daf-12* LBD mutants can still bind to the promotor

regions of *daf-12* target genes, but subsequent gene expression is inhibited (Antebi et al., 1998). Therefore, *daf-12* is necessary for dauer formation (*daf-12b*), but only partly required for the promotion of reproductive growth (*daf-12a*) and appropriate developmental timing. These findings strongly suggest that overlapping functions must work together with *daf-12* to specify reproductive growth. In genetic screens for mutants that enhance the heterochronic phenotype of *daf-12* null alleles, the *daf-12* redundant factor 1 *dre-1* could be identified, displaying a strong heterochronic phenotype in a *daf-12* background (Fielenbach, personal communication).

2.7 The Daf- genes and homologs regulate fundamental processes during the development

2.7.1 Dauer signaling

The dauer formation genes and their homologs in other species have important functions in the regulation of basic cellular and developmental processes. Here, we give a more detailed account of these pathways in *C. elegans* and other species.

2.7.2 Insulin/IGF Pathway

Insulin signaling genes are conserved throughout metazoan taxa. In mammals, insulin signaling regulates glucose and amino acid uptake, increases synthesis of glycogen and fat, and inhibits glucose synthesis, lipolysis, and glycogen breakdown (White and Kahn, 1994). The involvement of insulin-like hormones in the regulation of *C. elegans* life span suggests that hormones controlling vitality and mortality may regulate the plasticity in life spans in general (Finch and Ruvkun, 2001). The key tissue where insulin-like signaling and free radical protective pathways regulate life span in *C. elegans* is the nervous system. Life span determining genes could act in neuroendocrine cells in diverse animals.

An insulin-like/IGF signaling pathway with many signal transduction components homologous to proteins in higher organisms regulates metabolism, development, and life span in *C. elegans* (Morris et al., 1996; Kimura et al., 1997; Paradis and Ruvkun, 1998). Molecular and genetic studies of epistasis as well as synergy suggest a model for insulin signal transduction that is summarized in Figure 3.

In brief, in abundant food, neurosensory cells produce insulin-like molecules. In cellular targets, insulin binds to the DAF-2 insulin-like receptor (Kimura et al., 1997) recruiting PI 3-kinase AGE-1 (Morris, 1996; Gottlieb and Ruvkun, 1994). AGE-1 generates PIP3 that binds to the kinases AKT-1 and AKT-2 (Paradis and Ruvkun, 1998), subsequently

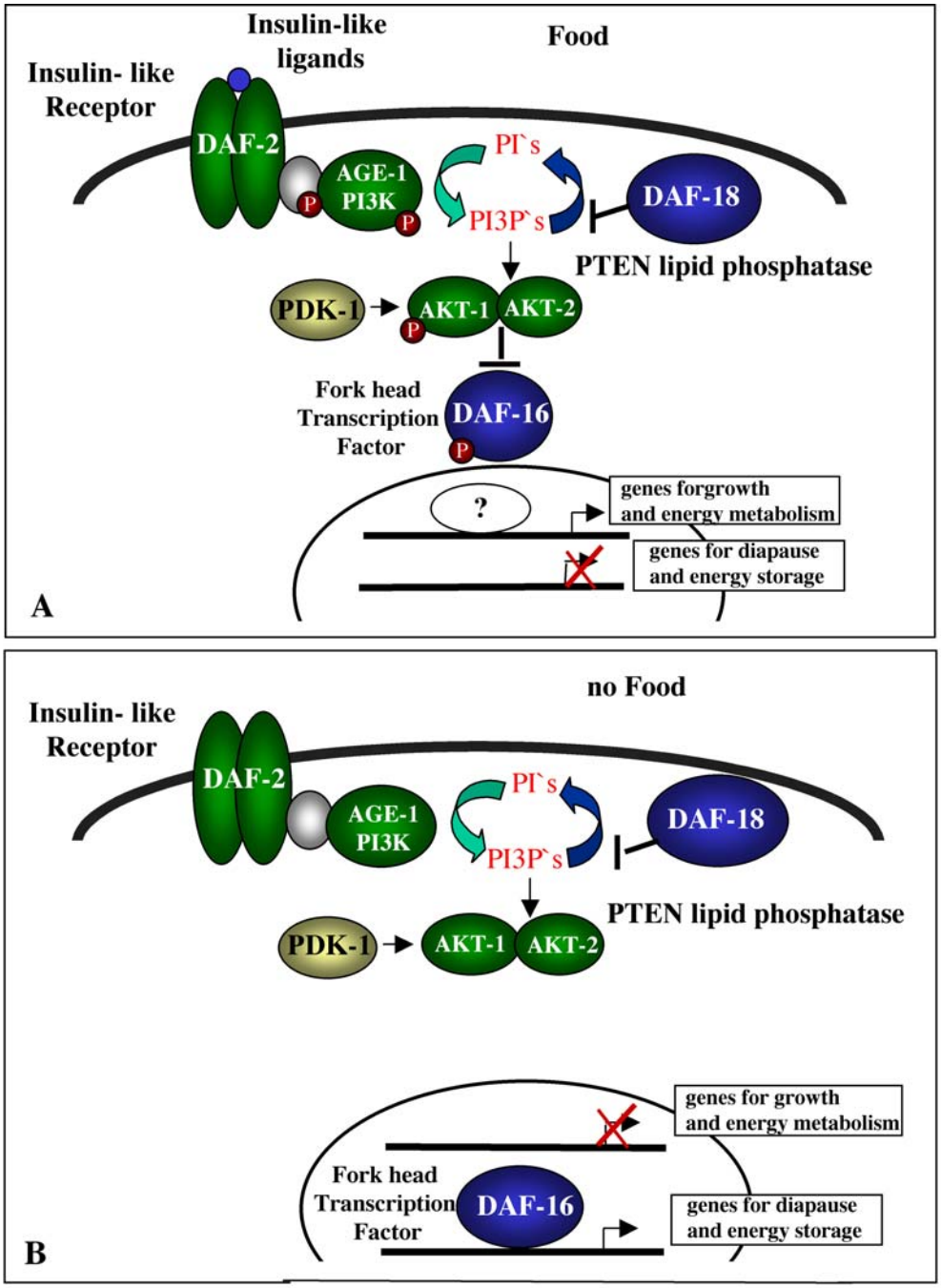

Figure 3) (Finch and Ruvkun 2001, modified) **A molecular model for insulin like regulation of gene expression in *C. elegans.* A)** High insulin signaling activates the Insulin/IGF like DAF-2 receptor recruiting the phosphatidylinositol 3-kinase (PI-3K) p110 catalytic subunit AGE-1 upon activation. AGE-1 generates phosphatidyl inositol phosphate P3 (PIP3), a membrane bounded signaling lipid. PIP3 binds to the pleckstrin homology domains in the serine/threonine kinases AKT-1 and AKT-2, homologs of mammalian Akt/PKB, subsequently activating their kinase activity. The PTEN lipid phosphatase DAF-18 downregulates PIP3 signals via phosphatase activity. For activation, AKT-1 and AKT-2 need to be additionally phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1). AKT-1 and AKT-2 negatively regulate the DAF-16 transcription factor by phophorylation, causing cytoplasmatic localization of DAF-16 with the consequence that genes necessary for dauer diapause stress resistance and long life span stay turned off. DAF-16 is closely related to human FKHRL1, FKHR, and AFX. **B)** In the absence of insulin like ligand these kinase cascades are inactive and DAF-16 can actively enter the nucleus where it represses genes required for reproductive growth and short life span and/or it activates genes required for dauer arrest and long life span.

activating their kinase activity. AKT-1 and AKT-2 need to be phosphorylated by PDK1 (Alessi et al., 1997). Activated AKT-1 and phosphatase DAF-18 (Ogg and Ruvkun, 1998) downregulates PIP3 signals. AKT-1 AKT-2 then negatively regulate the fork head transcription factor DAF-16 by phosphorylation (Lee and Ruvkun, 2001). Phosphorylated DAF-16 remains in the cytosol and target genes stay turned off (Figure 3A). However, in the absence of an insulin signal, the kinase cascade is inactive, DAF-16 is unphosphorylated and enters the nucleus, where it activates its target genes, (Figure 3B; Paradis and Ruvkun, 1998; Lin and Kenyon, 1997; Ogg et al., 1997; Murakami and Johnson, 1996).

The documented insulin pathway mutants that affect life span and dauer formation - *daf-2, age-1* and *pdk-1*- are Daf- c and long lived (Kenyon, 1993). *akt-1* and *akt-2* are Daf-c, but their Age phenotype is undetermined (Paradis and Ruvkun, 1998). In contrast, *daf-18* and *daf-16* are dauer defective and short lived. Beside the Daf-c and longevity phenotypes, *daf-2* mutants exhibit decreased metabolic rates and energy metabolism is shifted dramatically toward energy storage (Vowels and Thomas 1992). Two classes of daf-2 mutants are distinguished (Gems et al., 1998). Class I alleles affect dauer formation and life span. In addition to these phenotypes, class II alleles cause embryonic and L1 arrest, abnormal gonads, narrower adult body, reduced adult brood size, late progeny and premature death.

2.7.3 TGF-ß pathway

TGF-ß signaling plays a role in the control of differentiation, proliferation, apoptosis, and state of activation of many different cell types (Kingsley, 1994; Hogan, 1996). Moreover, TGF-ß family members have potent immuno suppressor activities. Mutations in TGF-ß family ligands cause several human diseases (Massagué, 2000) and several components of the TGF-ß signaling pathways are mutated in cancer (Massagué, 2000). Transformation growth factor ß (TGF-ß, in *C. elegans* encoded by *daf-7*) is the prototypic member of a large family of structurally related pleiotropic-secreted cytokines (Kingsley, 1994; Hogan, 1996). SMAD (for *small* and *mad*) proteins are also conserved in metazoans. Three classes of SMAD proteins are distinguished. First, the R-SMADs (e.g. DAF-8 and DAF-14, Figure 4), that are phosphorylated by type I receptors, which stimulates them to accumulate in the nucleus. In the nucleus, R-SMADs tend to heteromerize with the second class of SMADs, the Co-SMADs, and with other cofactors and transcription factors. A third class of SMADs negatively regulates gene expression (e.g. DAF-3, Figure 3). The complexity and

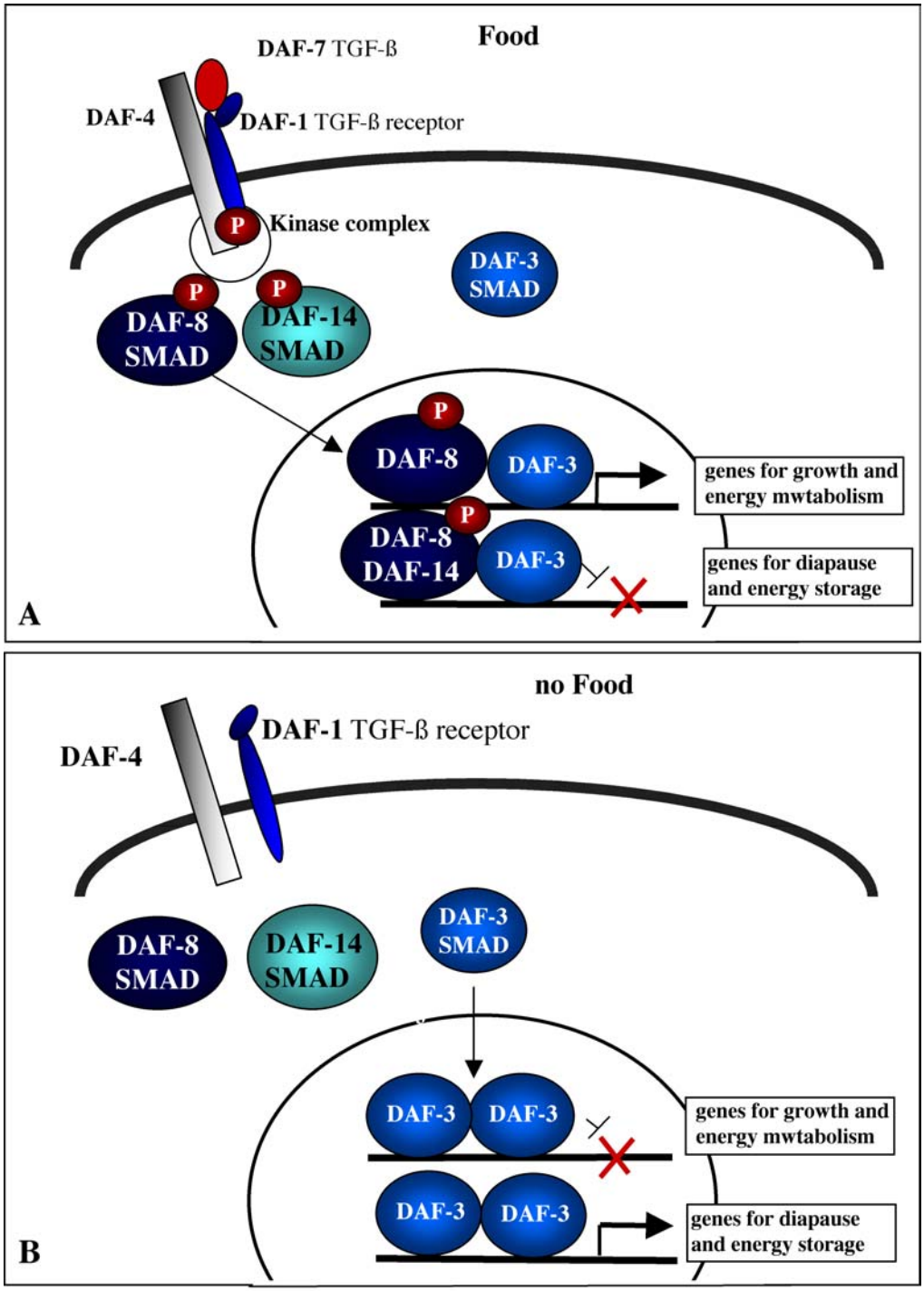

Figure 4) A molecular model for TGF-ß dependent regulation of gene expression in *C. elegans.* (Patterson et al.,1997, modified).**A)** Under reproductive conditions, TGF-ß binding to the receptors DAF-1 and DAF-4 initiates the formation of a transmembrane kinase receptor complex that phosphorylates DAF-8 and DAF-14 that enter the nucleus, where they act as transcription factors. **B)** In absence of a TGF-ß ligand, DAF-3 enters the nucleus and forms homodimers, that promote the dauer formation and to repress genes for reproductive growth.

variability of SMAD responses is determined first, by dimerization and second, by the cross talk to other signaling pathways like MAPK pathway or insulin signaling. (Murakami

et al., 2001; Moustakas et al., 2001). In *C. elegans*, the components of the TGF-ß signaling pathway form a neurosensory signal transduction pathway that is summarized in Figure 4.

Under reproductive conditions and in response to an environmental signal, TGF-ß is produced in the chemosensory ASI neuron (Ren et al., 1996). TGF- ß binds to the receptors DAF-1 (Georgi et al., 1990), and DAF-4, (Estevez et al., 1993) and induces their dimerization. This leads to a phosphorylation of DAF-1 by DAF-4 and to the subsequent formation of a heteromeric transmembrane serine/threonine kinase receptor complex. (Attisano et al., 1992). This complex phosphorylates cytoplasmatic SMADSs DAF-8 and DAF-14 (Estevez et al.,1993), that enter the nucleus where they act as transcription factors to activate genes promoting reproductive growth (Chen et al., 1996; Lagna et al., 1996; Mascias-Silva et al., 1996; Zhang et al., 1996, 1997, Figure 4A).

However, in the absence of a TGF-ß, DAF-3 enters the nucleus, where it forms a homo- or multimer that induces dauer formation (Figure 4B). As *daf-3* and *daf-4* are expressed in the tissues that are remodeled during dauer formation, these genes probably control morphogenic and metabolic changes for dauer formation. Whereas DAF-3 homodimerization is critical for maintenance of dauer formation, heterodimerization with DAF-14 and DAF-8 inhibits DAF-3 and disrupts dauer formation (Patterson et al., 1997).

Mutants in *daf-1, 4, 7, 8, 14* are Daf-c whereas *daf-3* is Daf-d (Patterson, 1997). *daf-3* appears to act at the end of the TGF- ß pathway suppressing the Daf-c phenotypes of upstream components. *daf-5* (not shown) is also Daf-d and encodes a homolog of the ski/sno co-repressor (Riddle, personal communication).

2.7.4 cGMP and serotonergic signaling pathways

In mammals, guanyl cyclases and cGMP-mediated signaling cascades play a central role in the regulation of neuronal functions like long-term potentiation, long term depression, and in vascular smooth muscle motility, intestinal fluid and electrolyte homeostasis, and retinal phototransduction (Lucas et al., 2000).

Stimulation of guanylyl cyclases regulates signaling cascades through downstream effectors, including cGMP-dependent protein kinases (Lohmann et al., 1984, Uhler, 1993), cGMP-regulated phosphodiesterases (Francis et al., 1980), and cyclic nucleotide-gated ion channels (Guy et al., 1991). G-protein linked receptors depend on complex cascades providing numerous opportunities for amplifying the responses to extracellular signals (Alberts, 1995). Guanyl cyclases comprise membrane-bound and soluble isoforms that are expressed in nearly all cell types. They are regulated by diverse extracellular agonists that

include peptide hormones, bacterial toxins, and free radicals, as well as intracellular molecules, such as calcium and adenine nucleotides.

C. elegans cGMP signaling pathways regulate chemosensory transduction in several types of sensory neurons. Ligand binding to a membrane bound G-protein coupled receptor is proposed to activate the *C. elegans* Gα- proteins *gpa-2* and *gpa-3* (Zwaal et al., 1997), each of which can activate a guanyl cyclase, catalyzing the conversion of GTP to the second messenger cGMP. A well characterized *C. elegans* guanyl cyclase is encoded by *daf-11* that acts in neuroendocrine signaling (Birnby et al., 2000). Also *daf-21*, encoding the heat-shock protein 90 (Hsp90), acts at the same step of the pathway as *daf-11,* indicating that they have closely related functions (Riddle and Albert, 1997). *C. elegans tax-2* and *tax-4* act further downstream in cGMP signaling. They encode cyclic nucleotide gated channels with function in dauer formation and sensory transduction (Hedgecock and Russel, 1975).

Regulation of dauer formation is also mediated by amphid sensory neurons. ADF and ASI neurons, which mediate the TGF-ß signaling, repress dauer formation in favorable conditions (Bargmann and Horvitz, 1990; Schackwitz et al., 1996; Thomas et al., 1993). In contrast, the ASJ neurons promote dauer formation (Schackwitz et al., 1996). In *daf-11* and *daf-21* mutants, the Daf-c phenotype probably results from activation of ASJ (Schackwitz et al., 1996), indicating that DAF-11 and DAF-21 could function in the sensory endings to mediate an early step in chemosensory signal transduction (Vowells and Thomas, 1992).

daf-11 and *daf-21* mutants are Daf-c, and they have defects in specific chemosensory responses mediated by several classes of sensory neurons. *tax-2* and *tax-4* are also Daf-c, *gpa-1, 2* have reduced sensitivity to dauer pheromone.

Another pathway acting upstream of the three described dauer pathways is the serotonergic signaling. Serotonin regulates a large variety of neural processes in vertebrates including feeding behavior. The key enzyme for serotonin biosynthesis is the tryptophan hydroxylase (*tph-1*). The *C. elegans tph-1* mutant shows abnormalities in behavior and metabolism that are normally coupled with the sensation and ingestion of food. Rates of feeding and egg laying are decreased; large amounts of fat are stored, reproductive life span is increased, and some animals arrest at the dauer stage. This metabolic dysregulation is in part due to down regulation of transforming growth factor- and insulin-like neuroendocrine signals. The action of the *C. elegans* serotonergic system in metabolic control is similar to mammalian serotonergic input to metabolism and obesity (Sze et al., 2000).

2.8 Nuclear hormone receptor complexes

2.8.1 Nuclear hormone receptors

DAF-12 belongs to the family of nuclear hormone receptors (NHR), defined as transcription factors that respond to lipophilic ligands like steroid hormones, or certain vitamins and subsequently regulate gene expression. Their major role is the integration of upstream acting signaling pathways and the regulation of downstream signaling cascades. NHRs can either repress or activate the transcription of subsequent target genes, depending on the molecular context. NHRs are involved in many important biological processes, including embryonic development, homeostasis, fertility, memory and cognition (Tetel, 2000) and the regulation of cholesterol metabolism. They can be divided into several subfamilies. Type I receptor comprise the classic steroid receptors for estrogens (ER), progestins (PR), androgens (AR), glucocorticoids (GR) and mineralocorticoids (MR). They bind to DNA in the presence of ligand. Type II comprises receptors for thyroid hormone (TR), vitamin D3 (VDR), all-*trans* retinoic acid (RAR) and 9-*cis* retinoic acid (RXR), which bind to DNA in the absence of ligand. A third type, the orphan nuclear receptors have no known ligands (Mangelsdorf et al., 1995). NHRs contain conserved domains, including LBD and DBD, which are linked by a less conserved hinge region. Typically, an N-terminal autonomous transcriptional activation region (AF-1) and a ligand-dependent C-terminal activation function (AF-2) can trigger the transcription of target genes (summarized by Laudet and Gronemeyer, 2002).

The overall nuclear receptor LBD contains 12 helices (H1 to H12). The binding surface for the co-activator peptide is formed by H3, H4, part of H5, and H12. The position of H12 is regulated by a ligand. In the liganded receptor, H12 folds back to form part of the co-activator binding surface. By contrast, H12 inhibits corepressor binding to RXR and other NHRs (Schulman, 1996; Zhang, 1999). The corepressor interaction surface also requires H3, H4, and H5, thereby overlapping the coactivator interaction surface (Hu, 1999; Nagy, 1999; Perissi, 1999).

In mammals, 48 members of this protein family are known. However, *C. elegans* contains 270 nuclear receptors, more than four times the number identified in other organism (Sluder, 2001; Enmark and Gustaffson, 2000). The majority are expressed at least at the RNA level (Sluder, 1999), but many of these receptors lack known homologs in other species. Nuclear hormone receptors recruit large regulatory complexes to target genes (McKenna, 1999; Glass and Rosenfeld, 2000).

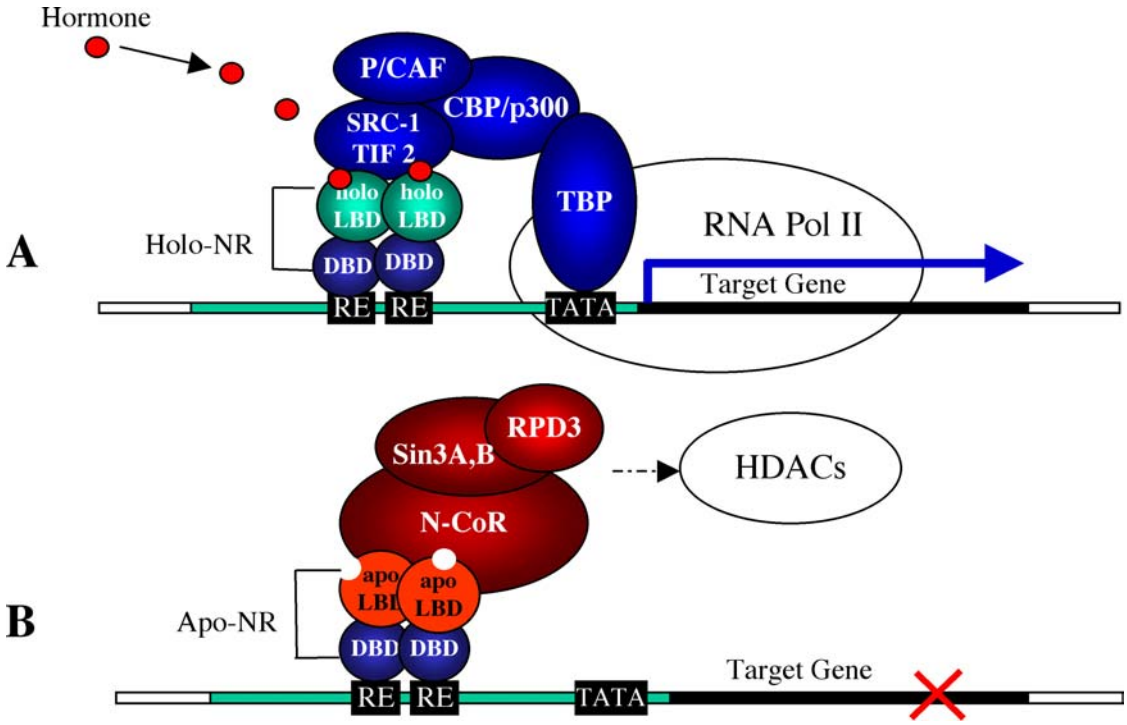

Figure 5) Gene expression is regulated by recruitment of Co- repressor and Co- activator complexes to DNA bound nuclear hormon receptors. A) Holo nuclear receptors recruit coactivator complexes consisting of i.e. Transcription Initiation Facor 2 (TIF-2), Steroid Receptor Cofactor 1 (SRC-1), p300/CREB binding protein-associated factor (P/CAF), CREB binding protein (CBP/p300), or TATA Binding Protein (TBP). RNA Pol II= RNA Polymerase II; RE= Responsive Element; TATA= TATA box; Holo- NR= Holo Nuclear Receptor, holo LBD= ligated nuclear hormone receptor ligand binding domain; DBD= DNA Binding Domain. **B**) Apo nuclear receptors recruit corepressor complexes consisting of i.e. Nuclear Corepressor N-CoR or Sin3A, or the transcriptional regulator Rpd3. HDACs= Histone Deacytelases; Apo- NR= Apo Nuclear Receptor; apo LBD= unligated nuclear hormone receptor ligand binding domain, white circles indicate the unliganded LBD.of the nuclear hormone receptor.

Liganded nuclear hormone receptors (holoreceptor, Figure 5A) assemble multisubunit coactivator complexes that trigger transcription. Unliganded receptors (aporeceptors, Figure 5B) recruit a corepressor complex, thereby shutting off target gene transcription. In many cases, nuclear hormone receptors form homodimers (ER, GR, MR, AR) or dimerize with other nuclear hormone receptors. These dimers are able to recognize and bind to distinct DNA sequences, the hormone response elements (HRE, Becker et al., 1986), located within the promotor region upstream of the target gene (Laudet and Gronemeyer, 2002). Various interactions of nuclear receptors with other nuclear receptors (RXR-RAR: Leid et al., 1992; RXR-PPAR: Bardot et al., 1993; RXR-VitD3: Kliewer et al., 1993), coactivators (SRC-1: Yao, 1996; TIF-2: Voegel, 1996; CBP-P300: Arany, 1994; pCAF: Blanco, 1998), corepressors (N-CoR: Kurokawa, 1995; SMRT: Chen and Evans, 1995; SMRTER: Tsai, 1999; ALIEN: Goubeaud, 1996) have been described previously.

2.8.2 Nuclear receptor coactivators

Nuclear receptor coactivators are proteins that interact with nuclear receptors and enhance their transcriptional activity (McKenna et al., 1999; Glass and Rosenfeld, 2000; Figure 5A). They act as bridging proteins between the receptor and the basal transcriptional machinery. Coactivators contain typical LxxLL motifs (Heery, 1997) in their receptor interaction domains that are recognized by the receptor. The association of the co-activator complex triggers association with histone acetyltransferases like pCAF (HAT complex, Spencer et al., 1997) and subsequent decondensation of the chromatin via acetylation of the histones. Some coactivators like CBP/p300 possess intrinsic histone acetyltransferase activity (Ogryzko, 1996).

Acetylation of the coactivator complex leads to dissociation of the HAT complex and promotes the establishment of the transcriptional initiation complex at the target gene, comprised of basal factors, TATA box binding protein and RNA polymerase II, resulting in transcription of the target gene.

2.8.3 Nuclear receptor corepressors

Corepressors are nuclear receptor-interacting proteins that actively decrease the transcriptional activity of the receptor. During transcriptional repression, nuclear hormone receptors actively recruit histone deacytelases (HDACs) like Rpd-3 to the corepressor complex. The HDACs are thought to maintain the condensed state of chromatin structure by deacetylation of the histones H2 and H4 thus hindering the establishment of the transcriptional machinery at the target genes. Nuclear receptor corepressors such as N-CoR (Kurokawa, 1995), or SMRT (Chen and Evans, 1995) are large modular proteins that contain separable N- terminal repression domains and C-terminal interaction domains. The N-terminal repression domains interact with histone deacetylase complexes (HDAC) that interact with histones (Guenther, 2000; Heinzel, 1997; Huang, 2000; Kao, 2000; Li, 2000; Nagy, 1997). The C-terminal domain contains two interaction domains, ID1 and ID2 (Cohen, 1998; Seol, 1996; Zamir, 1996). Motifs called CoRNR boxes (corepressor-nuclear receptor) within ID1 and ID2 are responsible for the interaction with NHRs (Hu, 1999; Nagy, 1999; Perissi, 1999). Each CoRNR box contains a motif of the form L/IxxL/IL/I or I/LxxIVI (Hu et al., 2001), or LxxI/HIxxxI/l (Rosenfeld, 2001), which are very similar to the LXXLL motifs (NR box) found in coactivator proteins (Heery, 1997). CoRNR1 and CoRNR2 interactions bind in different positions to the coregulator-binding surface,

mediated by distinct regions of different NHRs. In the case of CoRNR1, particular residues are required to interact with specific NHRs. This suggests remarkable specificity for functional NHR-CoRNR-corepressor combinations (Hu et al., 2001).

In *C. elegans*, only few nuclear receptor repressors have been identified so far, for example *unc-37* encodes a groucho corepressor (Miller et al., 1993). *spr-1* (suppressor of presenilin) encodes a conserved transcriptional repressor that plays a role in LIN-12/Notch signaling (Jariault and Greenwald, 2002).

2.9 Techniques

2.9.1 Yeast-two hybrid System

The yeast-two hybrid system is an in vivo system to assay protein-protein interactions (Fields and Song, 1989). It is based on the reconstitution of the DBD and the activation domain (AD) of a transcription factor to a functional protein. In our system we used the lex-A transcription factor. The activation of a reporter gene in yeast is still possible if both domains are expressed in different hybrid fusion proteins. One fusion protein consists of the DBD and a bait protein X. The other fusion protein contains the AD and prey protein Y. If the fusion proteins are interacting, a functional transcription factor is reconstituted. The DBD binds to a LexA operator sequence that is fused to reporter genes (HIS-3, URA-3, and lacZ). The AD activates RNA polymerase II complex resulting in transcription of the reporter genes. Typically, the AD is fused to random clones derived from a cDNA library of the organism, tissue and developmental stage sought-after that is screened against one single bait protein. In this study, DAF-12 bait constructs are screened against *C. elegans* mixed stage cDNA libraries.

2.9.2 RNA interference (RNAi)

With the RNAi technique (Fire, 1998), gene expression can be knocked down gene-specifically by a post-transcriptional gene silencing mechanism. It is thought that RNAi is initiated by cleavage of long dsRNA into 21-25 nucleotide (nt) double-stranded fragments, termed small interfering RNAs (siRNAs) (Elbashir, 2001; Hamilton and Baulcombe, 1999; Hammond, 2000; Zamore, 2000; Bernstein, 2001) by the enzyme Dicer, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein, 2001). These siRNA duplexes are then incorporated into the RNA interference silencing complex (RISC) (Hammond, 2000; Bernstein, 2001). The RISC can recognize and cleave a target RNA complementary to the guide strand of the siRNA (Bernstein, 2001; Hammond, 2001). The cleaved RNA fragments can target other complementary RNAs, resulting in an efficient

and specific knockdown of the target gene. In our studies, we applied RNAi by feeding *C. elegans* with *E. coli* strains expressing siRNAs directed against the target genes identified in the Two-Hybrid Screens under the control of a T7 promotor.

2.10 Thesis question

daf-12 acts at the nexus of pathways regulating diapause, developmental timing and life span. Many of the molecular components of these pathways and their physiological functions are evolutionarily conserved. Thus the identification of conserved connecting components between the pathways might improve the knowledge of how specifically *daf-12* works to promote these distinct functions and of how nuclear receptors are interfering with cellular and developmental processes in general. In particular, molecular genetic dissection of nuclear hormone signaling pathways enables the physiological function to be revealed in the context of the entire organism.

According to its mammalian homologs it is likely that DAF-12 recruits coactivator and corepressor complexes, to either promote gene expression of target genes or to block it. However, no direct interactors of DAF-12 have been detected so far. In order to identify postulated DAF-12 regulator complexes, we applied the yeast-two hybrid method. In addition to transcriptional cofactors, we intended to identify components involved *in daf-12`*s regulation, modification and transport.

The resulting yeast- two hybrid clones were evaluated by data base analyses for a function in the *daf-12* context. The most interesting candidates encoded putative posttranstricptional regulators, transcriptional regulators, transcriptional repressors or proteins possibly involved in a ligand transport pathway. We selected seven of them for further studies. To connect the candidates to DAF-12 on a functional level, we knocked down their activity in RNAi feeding assays. The F1 generation was scored for all kinds of phenotypes and in particular for enhancement or suppression of *daf-12* specific phenotypes like appropriate developmental timing and dauer formation. We also tested different dauer pathway mutants for suppression or enhancement of previously described phenotypes and additional effects caused by RNAi.

In these studies, one of our candidates, *din-1,* turned out to be a potent suppressor of Daf-c and heterochronic phenotypes of *daf-12* mutants and of mutants in all known dauer pathways, including insulin- and TGF-ß signaling. Therefore, we focused our studies on the examination of this so far uncharacterized protein. BLAST searches revealed that the closest homologs of DIN-1 are large nuclear proteins that act as transcriptional regulators.

To characterize *din-1* features in more detail, we clarified its genomic structure by analyzing cDNA clones. It turned out, that *din-1* has at least four different splice variants and originates from two transcriptional units.

To gain stable *din-1* alleles, we performed EMS mutagenesis screens. Resulting mutants were mapped, crossed with *daf-12*- and dauer pathway mutants and phenotypes were scored and compared to those observed before with *din-1* RNAi.

Since DAF-12 was shown to increase and diminish Age phenotype in *daf-2* mutant background, we subsequently tested if *din-1* alleles would influence life span as well. To examine the expression pattern of *din-1* in *C. elegans*, we microinjected GFP tagged *din-1* constructs and scored the expression in different stages and tissues.