5 DISCUSSION

5.1 DAF-12 interactors

Nuclear hormone receptors regulate development, metabolism, and homeostasis in metazoans, often coordinating programs throughout the body via lipophilic hormones. As large transciptional machines, they turn on gene expression by assembling coactivator complexes, and turn off gene expression by assembling corepressor complexes. Moreover, their activities are modulated by inputs from kinase cascades as well as by colocalization with other transcription factors at the site of promoters or enhancers (Laudet and Gronemeyer, 2002).

A genetic analysis of nuclear receptor function in *C. elegans* has revealed that the DAF-12 nuclear receptor has multiple activities and physiological functions. In response to environmental conditions, DAF-12 mediates the choice between dauer diapause and reproductive growth within larval stage L3 (Antebi et al., 1998; Antebi et al., 2000; Gerisch et al., 2001). In addition, DAF-12 regulates expression of L3 and later stage programs within the heterochronic circuit, a regulatory pathway that controls developmental timing (Antebi et al., 1998; Antebi et al., 2000). Moreover, it influences lipid metabolism, fertility, and adult life span (Antebi et al., 2000; Gems et al., 1998; Larsen et al, 1995).

To better understand the biochemical activities and physiological functions of DAF-12 we investigated DAF-12 interacting proteins by the yeast-two hybrid method. We have identified 22 putative DAF-12 interacting proteins. At least seven of these are possibly involved in DAF-12 transcriptional complexes (Tables 5-8, Figure 9). One candidate, DIN-1 (F07A11.6) is the major focus of this study. It is homologous to a conserved family of coregulators for nuclear receptors and other transcription factors. *din-1* also genetically interacts with *daf-12* and other pathway components for regulation of diapause, life stages and life span. The other six, which we did not analyze in detail, are discussed in Part 14 of this chapter.

5.2 DIN-1 and homologs

The DAF-12 interacting protein number 1, DIN-1 (F07A11.6) shows a similar architecture and structural homology to several large nuclear proteins from other species, namely the

human SHARP (Shi et al., 2001), the mouse homolog MINT (MSX-2 interacting nuclear target protein (Newberry et al., 1999) and *Drosophila's* SPEN (split ends) (Rebay et al., 1999). Notably, these proteins have three to four RNA recognition motifs at their N-terminus, a long hinge region, and a conserved C-terminal domain. DIN-1 shares these structural features (Figure 14). In addition, they all contain predicted nuclear localization sites (Figure 14), as well as several consensus phosphorylation sites for AKT kinase. We call this family of proteins the SPEN family for the founding member.

Another related class of proteins, dubbed the mini-SPENs, contain three N-terminal RRM domains and the C-terminal homology region, but lacks the long hinge region. One such *C. elegans* gene, F29C4.7, contains RRM motifs and a C-terminal domain that are similarly related to SPEN and SHARP as DIN-1. However, we consider the size and architecture of DIN-1 to be more similar overall. One of human mini-SPEN genes, RBM15, is implicated in acute megakaryoblastic leukemia, and results from a chromosomal rearrangement bringing the RBM15 and the SAP DNA binding domain together (Ma et al., 2001).

BLAST alignments revealed some additional conserved features within DIN-1 (Figure 15). We identified an N-terminal string of amino acids with homology to various heat shock 90 proteins, including that encoded by *daf-21* of the cGMP pathway (Thomas et al., 1993). Interestingly, Hsp 90 binds to apo steroid receptors. Ligand binding to the receptor causes a conformational change and dissociation of Hsp 90 from the receptor (Tetel, 2000). Hsp genes also respond to oxidative stress (Fukuda et al., 1996). Conceivably, this motif may help stabilize associated proteins within the nuclear scaffold. In exon 13 there is also 47% homology with a mammalian voltage gated Na^+ channel protein. The functional significance of these homologies is not yet known.

5.3 *din-1* physiological function

To understand the physiological function of *din-1*, we reduced gene activity both by using RNAi and by generating conventional Mendelian alleles. *din-1* reduction-of-function on its own had no obvious effect on larval growth and development. However, it potently suppressed the Daf-c phenotypes of reduced insulin/IGF (*daf-2*), TGF-ß (*daf-7*) and cGMP(*daf-11*) signaling, as well as the Daf-c and heterochronic phenotypes of various *daf-9* CYP450 and *daf-12* nhr mutants (Figure 17, 18, Table 13, 14). Suppression of *daf-9* and *daf-*

12 phenotypes was particularly efficient. For example, *daf-9* null mutants, which alone form 100% Daf-c dauer larvae, were completely suppressed by *din-1*, suggesting that *din-1* acts proximal to *daf-9* in the dauer pathways (*daf-9(dh6)* in Table 14). By contrast, suppression of *daf-2* phenotypes was incomplete. We found that *din-1RNAi* as well as *din-1* alleles suppressed weak *daf-2* (class 1, narrow spectrum), but not strong *daf-2* (class 2, wide spectrum) alleles (Gems et al., 1998) for their Daf-c phenotypes (Figure 17, Table 14). These data support the hypothesis that *daf-2* signals through two different pathways, one *din-1* dependent, the other *din-1* independent. Similarly, *din-1* failed to completely suppress *daf-7*. Altogether, our findings suggest that *din-1* acts downstream or parallel to insulin/IGF, TGF-ß and cGMP signaling pathways, as well downstream or at the same point as nuclear hormone receptor signal transduction. This genetic position is consistent with the detected DAF-12-DIN-1 two-hybrid interaction.

5.4 Biochemical and physiological functions of DIN-1 homologs

Important clues to DIN-1's potential biochemical activity come from studies on the closest *Homo sapiens* homolog, SHARP, a steroid inducible cofactor that integrates nuclear receptor repression and activation (Shi et al., 2001). SHARP is a transcriptional corepressor that interacts directly via its C-terminal domain with SMRT corepressor and five members of the NuRD complex, HDAC1, HDAC2, MTA2, MBD3 and RbAp48. These factors form a complex that is proposed to cause a local change in the chromatin structure, resulting in transcriptional repression of target genes (Heinzel, 1997). SHARP was also shown to bind to two nuclear receptors, namely unliganded RAR (Shi et al., 2001) and PPAR delta (Shi et al., 2002). By inference, DIN-1 may act in a similar manner.

Several other observations suggest that this class of molecules work more generally as corepressors. Recently, SHARP has been shown to form a corepressor complex with CBF-1, the human homolog of *Drosophila* Su(H), by recruiting histone deacetylase complexes in the absence of intracellular Notch-IC (Oswald, 2002). In addition, mouse MINT was originally isolated as an interactor of the Msx-2 homeobox repressor (Newberry et al., 1999). Presumably, repression depends on interaction with MINT.

5.5 Is DIN-1 is a corepressor or coactivator?

In the absence of a well-characterized *daf-12* target gene, it is not possible to *a priori* determine whether DIN-1 functions as a corepressor, coactivator or both. However, several pieces of evidence are consistent with the idea that DIN-1 could act as a transcriptional corepressor.

First*, daf-12* mutants that truncate the receptor within the LBD or disrupt ligand binding are predicted to have repressor activity based on studies in vertebrates (Yoh et al., 1997). DIN-1 physically interacts with such mutant proteins as shown in the two-hybrid assay (Figure 16). Moreover, loss of *din-1* activity suppresses most of the phenotypic effects of these LBD mutants (Figure 18A, C, Table 13). Second, the minimal interaction domain in DAF-12 corresponds to the DAF-12 LBD helices H1-H6. This region has been demonstrated to be specifically critical for the interaction of nuclear hormone receptors with corepressors (Hu, 1999; Perissi, 1999). Third, DIN-1 contains L/IXXL/IL/I and I/LXXI/VI motifs, typical of corepressors (Figure 13, 14; Hu et al., 2001, Rosenfeld et al., 2001). These motifs have been shown to be critical for the interaction with nuclear receptors of well-characterized nuclear receptor corepressors, SMRT and N-CoR. It remains to be determined whether the L/IXXL/IL/I like motifs perform a similar function in DIN-1.

The human DIN-1 homolog SHARP was shown to interact with nuclear corepressors NCoR and SMRT (Shi et al., 2001). No clear *C. elegans* homologs of these proteins are found in the genome. If *C. elegans* functional analogs exist, they may have evolved so rapidly as to be unrecognized by typical BLAST searches. However, in genetic screens for suppressors of the MIG phenotype in *daf-12* LBD mutants, we identified two extragenic suppressors, wich were not linked to *dpy-10* (Figure 20). This indicates that they are *din-1* and *daf-12* independent suppressors of the *daf-12* Mig phenotype, which might act together with DIN-1. It will be interesting to identify their localization and molecular features. In addition to binding the corepressors NcoR and SMRT, SHARP also interacts with the NurD complex and HDACs. In this study, we were unable to observe obvious dauer phenotypes with *C. elegans* HDAC and Nurd complex homologs in RNAi feeding assays (Table 11).
This could be because some of the components have early embryonic phenotypes that obscure the effect on dauer formation (e.g. K07A1.12= *lin-53;* Ahringer et al., 2000). In other

cases, RNAi did not efficiently produce phenotypes (e.g.F02E9.4= *pqn-28;* Ahringer et al., 2000). Ultimately, genetic tests using Mendelian alleles of these genes, or biochemical tests, measuring association with DIN-1 may give more definitive answers.

A handful of other *C. elegans* corepressors have been identified. They include the *unc-37* groucho homolog, which interacts with the *unc-4* homeodomain protein to regulate synaptogenesis (Miller et al., 1993), and *spr-1* and *spr-5* suppressors of presenilin, which encode components of the CoRest corepressor complex that antagonizes Notch signaling (Jarriault and Greenwald, 2002). Interestingly, SHARP also associates in a corepressor complex with the human homolog of Su(H),CBF-1, implicating SHARP in the human Notch signaling pathway (0swald et al., 2002). Furthermore, *Drosophila* SPEN also influences Notch signaling by reducing levels of Su(H), which may underlie some of the neuronal defects found in these mutants (Chen and Rebay, 2000; Kuang et al., 2000). In this work, we did not detect phenotypic abnormalities typical of altered Notch signaling among existent alleles. Moreover, in screens for suppressors of the *C. elegans* presenilin, none of the identified loci mapped near *din-1* (Wen et al., 2002). Conceivably, this function has been lost or is masked by functional redundancy in *C. elegans*. Understanding both the distinct and shared aspects of corepressor function will be important to clarify.

The N-terminal RRMs of SHARP have been shown to modulate nuclear receptor activity in another way. They sequester an identified coactivator RNA, the Steroid RNA Activators (SRA) (Figure13, 14; Shi et al., 2001), which works together with various steroid receptors to promote ligand independent activation. It is thought that when RRM binds SRA it squelches activation, resulting in an indirect suppression of steroid receptor activity. In *C. elegans* DIN-1, RRMs could also attenuate transcriptional activation by binding similar RNAs. Since nuclear receptor transcription also influences downstream splicing events, it is also possible that the RRMs function in this regard (Auboeuf et al., 2002; Honig et al., 2002).

Besides its function as a corepressor, DIN-1 could work as a transcriptional activator, positively regulating the expression of dauer formation genes. Newberry and coworkers reported that the RRM region of MINT protein positively regulates the HSV promoter, although it is unknown if this is direct. Beside the three L/IXXL/IL/I like motifs, that are characteristic for repressor receptor interactions, we identified three LXXLL motifs in DIN-1, sites typical for receptor coactivator interaction (Heery, 1997). In DIN-1 isoform D, we

found two of such motifs and another eight motifs of the form LxxL that cluster in exon 18, which might indicate an activator function. Whether DIN-1 acts as a corepressor or coactivator will ultimately be resolved by biochemical analysis of transcription at identified target genes.

5.6 *din-1* isoforms

The *din-1* locus has a complex genomic structure consisting of 22 exons, arranged in at least two transcriptional units. In total, we identified 4 different *din-1* isoforms: Isoform A lacks exon18, isoform B lacks exons 13 and 18, isoform C lacks exons 1, 2, 3 and 18 and the isoform D lacks exons 1-17 (Figure 18). Several lines of evidence suggest that the short isoform D, which is the only isoform containing exon 18, might arise from alternate splicing and / or from an alternate promoter. First, we failed to amplify exon 18 using primers from flanking exons (Figure 11, lane 4), whereas PCR with exon 18 specific primers down to the *din-1* 3'-end gave a product of the predicted size (Figure 11, lane 3), suggesting alternate splicing. Moreover, isoform D is spliced to the SL1 splice leader to yield two products, one of which is joined to the exon 18 splice junction and another that is joined to the upstream intron (Figure 11, lane 1, 2). Notably, the upstream intron does not contain an open reading frame, suggesting that it corresponds to a 5'UTR (Figure 10, Table 10). By contrast, the first methionine of the D isoform is preceded by a run of four adenines, the hallmark of a good initiator methionine (Kozak, 1995). Finally, at the exon 18 junction, we detected a non-canonical splice acceptor.

In addition to these structural features, two pieces of functional evidence support the notion of a second transcriptional unit. Microinjections with a plasmid encoding the predicted *din-1* isoform D and 3.4kb of upstream sequence gave a partial rescue (i.e. anti-suppression) of the Daf-c phenotype in *din-1;daf-2* double mutants. This indicates that DIN-1D alone is at least partly sufficient to promote dauer formation. Moreover, allele *din-1(sa1262)* maps within the predicted isoform D promoter/UTR region, not in a coding region. *din-1(sa1262)* suppresses the Daf-c phenotypes of *npc-1; npc-2* double mutants, much like our mutants localized in exon 18. These data altogether demonstrate the in vivo relevance of the D isoform.

Isoform D contains the DAF-12 interacting and the C-terminal conserved regions, but lacks the RRM and long hinge regions. Although DIN-1 isoform D contains no explicit NLS, Psort predicts 60% probability for nuclear expression. Protein homology BLAST searches with exon 18 revealed no homologies. However, one of three L/IXXL/IL/I motifs in DIN-1D is found in exon 18, consistent with its role in binding to nuclear receptors. Moreover, exon 18 also contains 2 LxxLL motifs, characteristic for receptor coactivators interactions (Figure 12, 14). In addition, 3 out of 4 DAF-12 interacting *din-1* clones picked up in the yeast two-hybrid system map in exon 18 (Table 5).

DIN-1 isoforms A, B and C may also be able to bind to nuclear receptors, because they contain two L/IXXL/IL/I motifs and one of the yeast two-hybrid clones maps in the very C-terminal region of DIN-1, that is present in all isoforms (Table 5). All of the identified nuclear localization sites are found in these long isoforms (Figure 12, 14), strongly indicating a function in the nucleus. Consistent with this prediction, DIN-1A is nuclearly localized (see below). Also the RRMs are found in DIN-1 A, B and C that could contribute to DNA binding and / or to binding of different subtypes of nucleic acids.

DIN-1 isoforms A and C contain three AKT/PKB phosphorylation sites. AKT is a key regulator of insulin/IGF signal transduction. Speculatively, DIN-1 activity could be modulated by kinases like AKT-1, consistent with its placement downstream of insulin/IGF signal transduction by genetic epistasis experiments. The DIN-1 homologs SHARP, SPEN and MINT contain four to six AKT phosphorylation sites. It will be interesting to see if DIN-1 homologs in other species have any role in insulin/IGF signal transduction. DIN-1 homologs also exist as various isoforms (i. e. in MINT, Newberry et al., 1999), although none of the reported isoforms correspond to the short DIN-1 isoform D. Clearly, it will be important to dissect the specific functions, expression patterns and regulation of these isoforms.

5.7 *din-1* mutants

All the *din-1* alleles that have been isolated in this work strikingly cluster within exon 18 of isoform D, which encodes the major DAF-12 interaction surface. Thus, it is likely that they severely affect isoform D, and largely disrupt association with DAF-12. This also indicates,

that none of the *din-1* mutants is a null, because isoforms A, B and C are not affected. Moreover, in our RNAi experiments, only *din-1* isoform D was knocked down, because the original fragment used for the RNAi construct corresponded to exon 18. Conceivably, the complete *din-1* knock out has more severe phenotypes. For example, stronger suppression of *daf-7* and *daf-2* mutants may become visible with a null allele. In addition, new roles in *C. elegans* development might become apparent. It will be useful to knock out the other isoforms, using RNAi directed to specific as well as common regions, or generate deletion alleles by published methods (Epstein and Shakes, 1995).

Although *din-1* RNAi and mutant alleles exhibited similar phenotypes, there were some notable differences. For example, *din-1* RNAi more efficiently suppressed *daf-7* Daf-c phenotypes and *daf-12* L3 seam phenotypes. It is possible that existent alleles are not null for isoform D, or that the *din-1* RNAi somehow affects other isoforms.

In the mutagenesis screens for *din-1* alleles, we selected for revertants of the *daf-12* Mig phenotype only. Our findings indicate, however, that *din-1* is probably not acting solely in the gonad, since *din-1* can suppress heterochronic phenotypes in the epidermal seam cells, as well as Daf-c phenotypes through most of the body. Surprisingly then, we found that injection of the *din-1* isoform D in *daf-2; din-1* double mutants partially restores the Daf-c phenotype seen in *daf-2* mutants in nearly all tissues except the gonad. This may indicate that while *din-1D* is important for the promotion of dauer formation, it may require the activity of other loci in a tissue-specific manner. Alternatively, isoform D constructs might not contain all the promoter elements for complete expression in the gonad.

5.*8 din-1* expression pattern

The *din-1* expression pattern reveals that it is widely expressed, localized in the nucleus, and broadly overlaps that of *daf-12::gfp*, consistent with a role as a DAF-12 interactor (Figure 26, 27). In particular, constructs containing the *din-1A* isoform are expressed in most tissues including muscle, intestine, nervous system, epidermis, somatic gonad and pharynx, which are largely remodeled during dauer formation. Moreover, it is expresssed in tissues, such as the gonadal distal tip cell and the epidermal seam cells (Figure 26C, D), which are phenotypically affected in *daf-12* LBD mutants with heterochronic defects. With expression from late embryo to adult, as well as in dauer larvae, *din-1* might act cell autonomously, and

have broad roles throughout the life stages, consistent with influences on life span (see below). DIN-1 homologs in other species are also nuclear localized and widely expressed (Shi et al., 2001; Newberry et al., 1999). Many of the observed *din-1* phenotypes are mediated by the *din-1D* isoform. Experiments are in progress to GFP tag this isoform as well. Based on the phenotypic spectrum, it is predicted to be broadly expressed and nuclear, too.

5.9 Heterochronic phenotypes

daf-12 null mutants, affected in their DBD, have impenetrant delayed heterochronic phenotypes. In contrast, *daf-12* LBD mutants display penetrant heterochronic phenotypes. With DBD intact, *daf-12* LBD mutants can presumably still bind to the promoter regions regulating *daf-12* target gene expression, but subsequent gene expression is inhibited (Antebi et al, 1998; Antebi et al., 2000). A simple model is that overlapping functions must work in parallel with *daf-12* to specify third larval stage reproductive programs. DIN-1 together with non-liganded DAF-12 probably represses reproductive development, since *din-1* suppresses the *daf-12* heterochronic phenotypes in gonad and epidermal seam cells. Similarly, the *daf-9* gonadal Mig phenotype is suppressed in *daf-9; din-1* double mutants (Figure 18, Table 12, 13), indicating, that the DAF-12 activating hormone is critical for appropriate gonadal development.

The *daf-12* redundant function *dre-1* was identified in genetic screens for mutants that enhance the gonadal heterochronic phenotypes of *daf-12* null alleles. (Fielenbach, personal communication). On its own, *dre-1* is only weak Mig. However, *daf-12; dre-1* showed an enhancement of the Mig phenotype, indicating that the *dre-1(+)* is required in parallel with *daf-12(+)* to promote normal gonadogenesis. Similarly, *dre-1* grown on *din-1RNAi* showed an enhanced Mig phenotype. One explanation could be that *din-1* positively regulates *daf-12* expression. Then in *din-1;dre-1* mutants, *daf-12* activity is reduced resulting in the Mig phenotype. However, in vivo experiments with gfp tagged DAF-12 showed that DAF-12 levels appear stable in the *din-1* mutant background. Another possibility could be that *din-1* itself acts as a transcription factor or as a transcriptional cofactor acting at the same point or downstream of *daf-12* in the heterochronic pathway to positively regulate reproductive programs. We also noted that *din-1(dh127); dre-1* double mutants did not show an enhanced

Mig phenotype, indicating that *din-1RNAi* again exhibits more severe phenotypes than existent *din-1* alleles.

5.10 *daf-12* phenotypic complexity

daf-12 mutants display remarkable phenotypic complexity, falling into 6 different classes. Mutants can display Daf-d, Daf-c, as well as weak or strong heterochronic phenotypes depending on allele). Moreover, tissue-specific heterochronic phenotypes are seen in gonad and extragonadal tissues. In addition, *daf-12* shows complex Age phenotypes depending on genetic background (Antebi et al, 1998, Antebi et al., 2000). Evidently, some of this complexity can be explained by postulating that *daf-12* has two activities, *daf-12a* which promotes L3 and later reproductive programs, and *daf-12b,* which promotes the dauer diapause. Whereas *daf-12b* is absolutely required for diapause, *daf-12a* is only partly responsible for reproductive growth, a function assumed in conduction with functionally redundant loci. The molecular identities of nuclear hormone signaling components provide a plausible framework to interpret this complexity. A simple model is that *daf-12*a and *daf-12b* activities reflect activation and repression, respectively. In this study we have shown in particular that *din-1* can modulate primarily *daf-12b* activity. *daf-12* DNA binding domain (DBD) as well as null mutants are Daf-d and have weak heterochronic phenotypes. Presumably unable to bind to target genes, neither repression nor activation is expected. However, because *daf-12a* is not essential, reproductive programs must be turned on by other transcription factors, i.e. *daf-12* redundant functions (*dre*). Because *daf-12b* is essential for dauer formation, the ability to repress reproductive growth and promote diapause is entirely lost (Figure 28A).

daf-12 ligand binding domain mutants have the opposite phenotype. They tend to be Daf-c, have strong heterochronic and developmental arrest phenotypes. Non-liganded DAF-12 binds the DNA, probably maintaining the interaction with a corepressor. As a consequence, the genes for reproductive growth are constitutively blocked (Figure 28B).

In our studies we have shown that *daf-12LBD; din-1* double mutants no longer display Daf-c and strong heterochronic phenotypes, suggesting that DIN-1 corresponds to a DAF-12 corepressor function (Figure 28B). Similarly, the Daf-c phenotypes of *daf-9, daf-2, daf-11* and *daf-7* mutants are suppressed in double mutants with *din-1*. We propose that all these

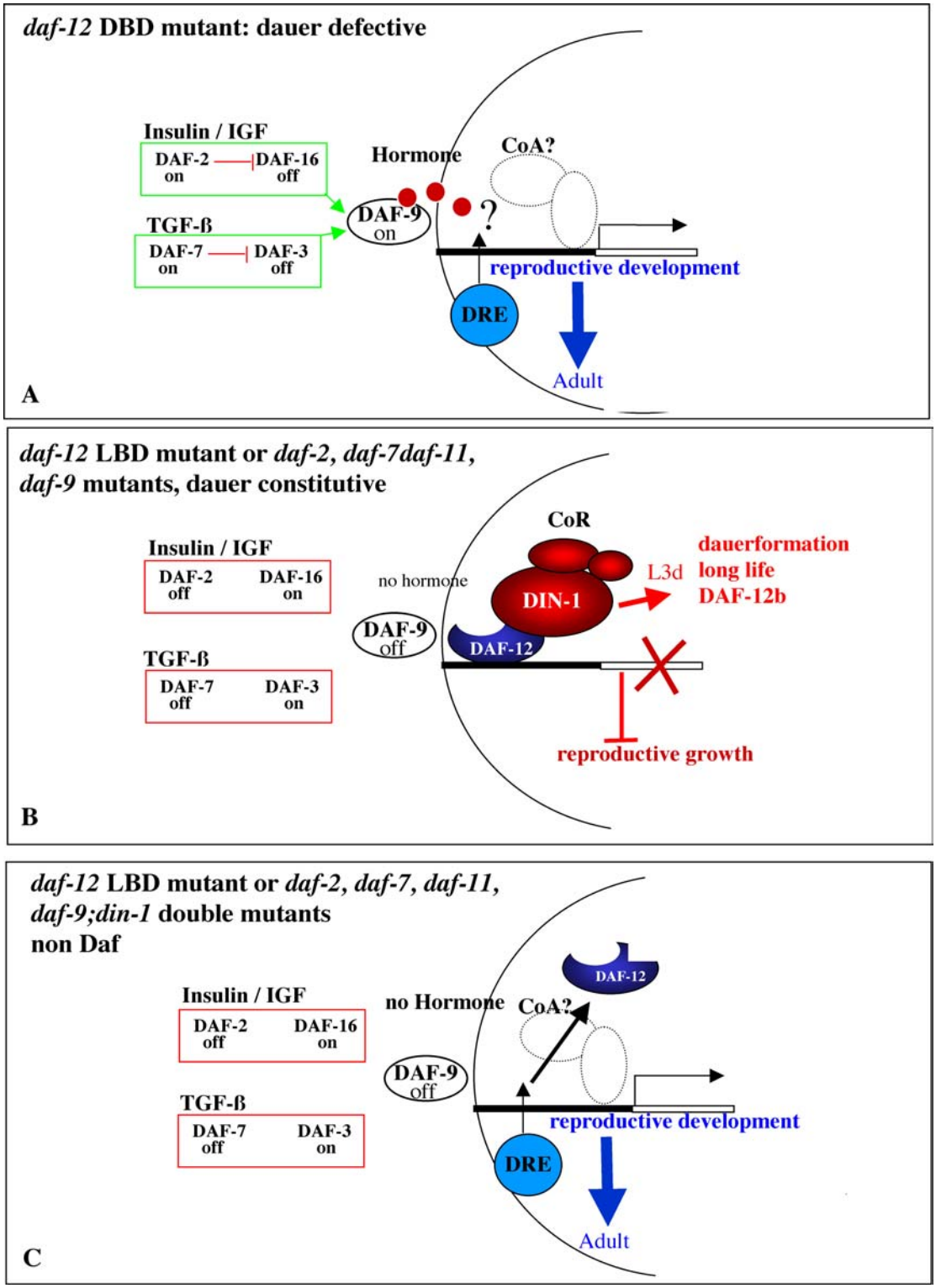

Figure 28) Models for the regulation of the dauer diapause in *daf-12*DBD mutant (**A**), in daf-12LBD mutants (**B**) and in dauerpathway;din-1 double mutants (**C**). The black half cycle indicates the membrane of the nucleus. Details are explained in the text.

Daf-c mutants ultimately reduce production of the DAF-12 regulating hormone (Figure 28 C), or reduce the affinity of the DAF-12 LBD for hormone. In the absence of hormone, a corepressor complex forms, inhibiting genes of reproductive growth. Because DIN-1 is not absolutely required for diapause, presumably other coregulators, e.g. DAF-16, DAF-3 also assemble with DAF-12 to arrest reproductive growth and promote dauer formation (Figure 29B).

In summary, hormone is critical for DAF-12´s ability to regulate the choice between dauer formation and reproductive growth, between activation and repression. DIN-1 is one component of several corepressor complexes that, together with non-liganded DAF-12 (*daf-12b*), block genes for reproductive growth and promotes diapause.

5.11 Dauer Model

We suggest the following model for regulation of diapause. In abundant food, insulin/IGF, TGF-beta and cGMP signal transduction pathways are active. In particular, sensory cues stimulate production and release of insulin/IGF and TGF-beta growth factors from sensory neurons and other endocrine tissues. They act through their respective signal transduction pathways to turn on DAF-9, which produces a hormone for DAF-12. In the presence of hormone, DAF-12 assembles postulated coactivator complexes to promote reproductive growth (DAF-12a, Figure 29A) When worms sense imminent starvation, insulin/IGF, TGF-ß and cGMP signal pathways are shut down, resulting in the repression of DAF-9 and its produced hormone. In the absence of hormone (or in the presence of an alternate hormone), DAF-12b-DIN-1 coregulator complexes inhibit programs of reproductive development and promote diapause. Presumably additional coregulator complexes also modulate DAF-12 activity (Figure 29B). As a consequence of improved environmental conditions, an increase of the DAF-12 ligand finally could allow coactivators to promote exit from diapause and reproductive growth again. Although we have stressed the function as corepressor, it may be that DIN-1 is more of coregulator, since it could also coactivate DAF-12 in some contexts.

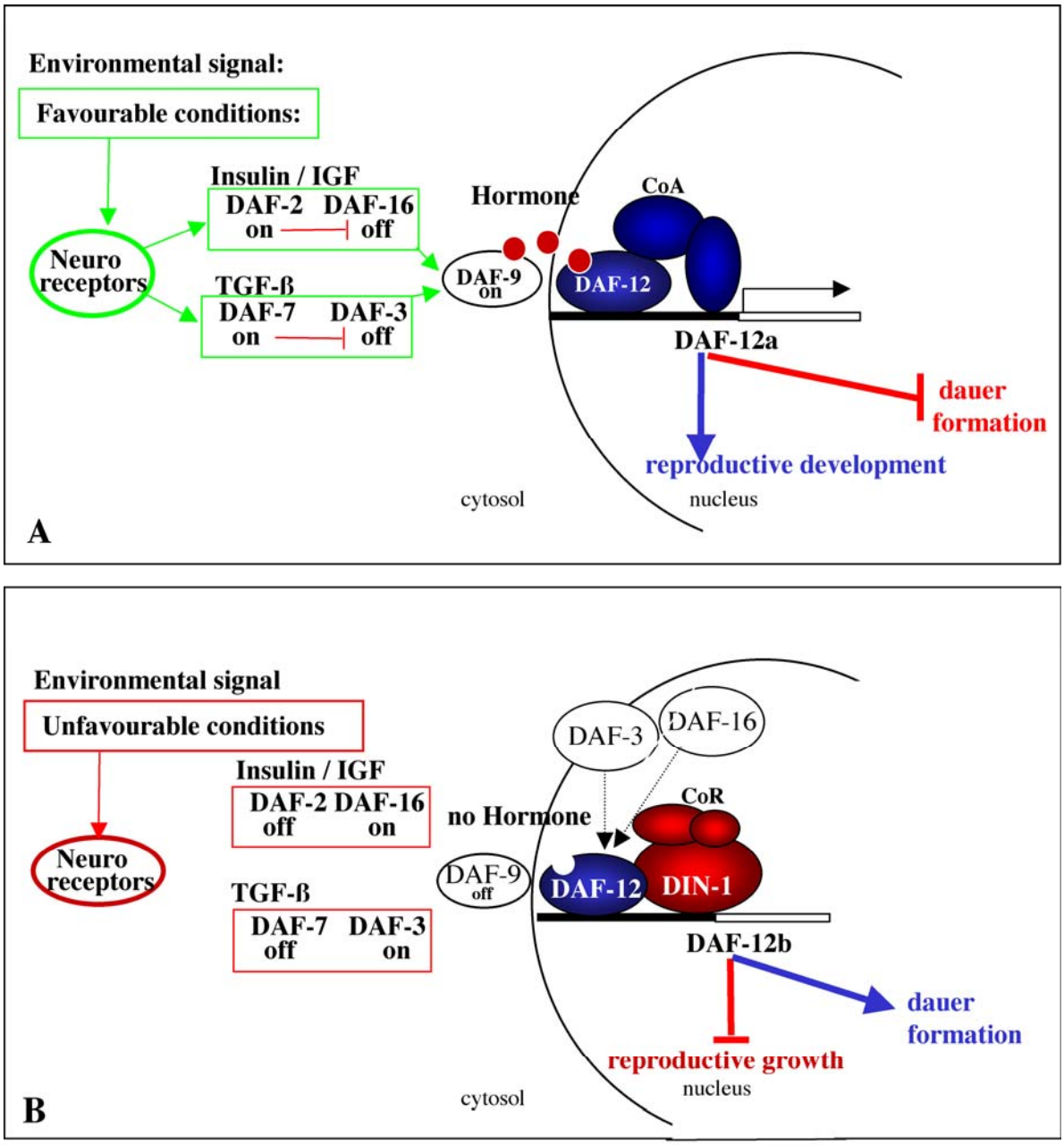

Figure 29) Model for the regulation of the dauer diapause. **A)** favorable conditions, **B)** unfavorable conditions. Details are explained in the text.

5.12 Life span

Mutations in *daf-2 daf-16* (insulin like pathway), *daf-9* and *daf-12* (steroid hormone pathway) influence *C. elegans* life span. The Age phenotype of *daf-2* class 1 and 2 alleles is cleanly suppressed by *daf*-16 (Lin et al., 2001). *daf-1*2 null and *daf-9* hypomorphic alleles weakly suppress *daf-2* class 1 Age phenotype and enhance that of *daf-2* class 2 two fold (Gerisch et al., 2001; Antebi et al., 2000; Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995). This suggests that insulin-like and lipophilic hormonal signals may regulate life span somewhat independently, although interactions between the pathways are likely.

In this study, we have shown that *din-1* on its own has no influence on *C. elegans* life span. However, in *daf-2* (class-2);*din-1(dh127)* double mutants, mean life span was increased by 35% compared with *daf-2* (class-2) mutants alone. Similarly, *daf-12* null mutants enhance the longevity of *daf-2* class 2 alleles, suggesting that the DAF-12;DIN-1 corepressor complex somehow antagonises the longevity of *daf-2* mutants. When either is lost, a further increase in life span is observed. For these life span studies only 10% of *daf-2(e1370);din-1 (dh127)* mutants survived past the reproductive stage, because of internal hatching. These were excluded from the analysis, as is done throughout the literature (Sulston and Horvitz, 1988). Internal hatching or matricide is an adaptation that enables larvae to feed off the maternal carcass in times of food deprivation. The cause of matricide in this genotype is unknown, but may actually obscure the full potential of life span extension.

5.13 SPEN proteins

Collectively DIN-1 and homologs MINT and SHARP in mammals, and SPEN in *Drosophila* can be considered as a novel class of transcriptional regulators, the SPEN proteins. All are large nuclear proteins, and broadly expressed. Evidently, they act as corepressors in most contexts, but may also work as coactivators or even transcription factors in others. Some of them have been shown to associate with the nuclear matrix (Newberry et al. 1999), suggesting a function as nuclear scaffold proteins organizing transcriptional complexes. Notably, all these proteins have been studied from different aspects, and have been connected with various pathways and physiology.

In particular, SHARP functions as a corepressor with unliganded nuclear receptors, RAR and pPAR delta (Shi et al., 2001, Shi et al., 2002), as well as with CBF-1 in the notch

signalling pathway (Oswald et al., 2002). Its role as corepressor depends on the ability to recruit of HDAC and Nurd components. Mouse Mint likely acts as a corepressor with Msx2 in cra-facial development, but may also promote transcription directly from the HSV gene (Newberry et al., 1999). *Drosophila* SPEN has been shown to act in Ras/Raf, Hox and Notch signal transduction, influencing head and body plan development, eye morphology, neurogenesis and axon outgrowth (Rebay et al., 1999; Chen et al., 2000; Kuang et al., 2000). *C. elegans* DIN-1 acts principallly within an identified nuclear hormone signaling pathway, downstream of insulin/IGF signal transduction in dauer formation. Whether SPEN family members function within all these pathways in a given species needs to be more fully explored. Based on the *C. elegans* physiology, we are particularly intrigued at the possibility that human SHARP may act within or downstream of insulin/IGF signal transduction.

We speculate that these large nuclear proteins integrate several signaling pathways by binding a variety of transcription factors in their scaffold domain. Generally, SHARP could centrally coordinate corepression, much in the same way that P300 centrally coordinates coactivation. Once tethered, transcription factors could coassemble with HDAC, Nurd and other components that mediate transcriptional repression. Perhaps the large difference in size of these proteins found between species reflects rapid evolution as they create or removemodular transcription factor binding sites. Conceivably, the alternative splicing of an interaction interface with the nuclear hormone receptor DAF-12 may exemplify a more general mechanism to create or destroy interactions with such a central scaffolding coregulator of transcription.

5.14 Other putative DAF-12 cofactors

Beside *din-1,* we identified six other candidate DAF-12 interacting proteins in the yeast-two hybrid screens for DAF-12 interacting factors.

In particular, we found F4611.2 (Table 6, Figure 9A), a cold shock protein that contains DNA binding regions (Doninger et al, 1992) and according to other proteins of this class, it could be regarded as transcriptional regulator. Cold shock proteins are thought to help the cell to survive in temperatures lower than optimum growth temperature. F4611.2 has reasonable ($7e^{-8}$) homology with *lin-28* (Ambros, 1984), another cold shock domain protein, that is thought to act in approximately to DAF-12 in the heterochronic pathway. The DAF-

12- F4611.2 interaction domain is spanning big parts of the predicted cold shock domain within the detected *C. elegans* protein (Figure 9A), indicating a function as transcriptional regulator in a DAF-12 cofactor complex.

M03F4.7 is a calumenin like calcium binding protein that shares homologie with a mammalian vitamin D receptor associated factor (Table 6, Figure 9B). The Vitamin D receptor is a close *daf-12* homolog in humans (52% identity within the DBD; Baker et al., 1988); it promotes calcium and phosphate absorption (Haussler et al., 1998). M03F4.7 includes several EF hand motifs that are characteristic for signaling proteins or buffering/transport proteins. It is possible that M03F4.7 acts as a carrier protein that is involved in calcium transport. This could be a hint that *daf-12* as its mammalian homologs Vitamin D receptor and PXR has also a role in calcium metabolism.

T05C1.6 shows homology with transcription activators from *Arabidopsis* and *Brassica napus* (Table 7, Figure 9C). T05C1.6 contains a conserved IPT/TIG domain (PF01833), found in intracellular transcription factors, and is involved in DNA binding (Collesi et al., 1996). Another predicted PFAM domain in T05C1.6 is the ankyrin repeat (PF00023), which is present in a large number of functionally diverse eukaryotic proteins like p53 binding protein 53BP2 or NF–kappaBinhibitory protein IkB alpha (Gorina, 1996). These data suggest a role for T05C1.6 in transcriptional regulation. Previous RNAi studies with T05C1.6 revealed an embryonic lethality of 11% (Maeda et al 2001).

M04B2.1 is homologue to human C2H2 type zinc finger protein (Table 7, Figure 9D). C2H2 Zinc fingers can bind to RNA and DNA (Evans and Hollenberg, 1988). The region of interaction between DAF-12 and M04B2.1. comprises the three N-terminal C2H2 zinc finger motifs out of six within the predicted protein. Another motif is an ATP-dependent DNA ligase domain. M04B2.1 corresponds to an identified mutant locus *mep-1* (MOG interacting and Ectopic P-granules, Belfiore et al, 2002). MEP-1 is an RNA binding zinc finger protein that binds MOG (for masculinization of the germline, Graham and Kimble, 1993). *mep-1* mutants arrest early in larval development; heterozygotes reach adulthood and are sterile in that they are defective in oogenesis. *mep-1* mutants produces less germ cells than wild type animals and thy are defective in somatic gonadal arm elongation. The latter phenotype could be interpreted as a heterochronic delay, which is also seen in some *daf-12* mutants. MEP-1 might correspond to a new factor acting together with DAF-12 in a nuclear complex.

Together with MOG proteins, it thas been shown to control the sperm/oocyte switch by repressing the 3' mRNA of *fem-3*.

ZK270.1 = *ptr-23* has similarity to the human Niemann-Pick C disease protein (NPC, a fatal childhood onset neurodegenarative disorder) and to numerous patched-related proteins from different species (Table 8, Figure 9E). Patched is a receptor for the morphogene Sonic Hedgehog. The mouse homolog may play a role in epidermal development. PTR-23 is a transmembrane protein, the region of interaction with DAF-12 spans the transmembrane domains within PTR-23, that are supposed to have key roles in different aspects of cholesterol homeostasis or cholesterol-linked signaling such as sterol-regulated movement or the trafficking of specific cargoes (Loftus 1997). Thus, ZK270.1 could have a bridging function between DAF-9 hormone synthesis and DAF-12 in hormone transport, acting in the membrane of the ER/nuclear membrane. Consistent with that, Psort predicts a 74% probability for membrane, 22% for endoplasmatic reticular and 4,3% nuclear localisation.

Several lines of evidence indicate that DAF-12 is a target of a *C. elegans* npc-like pathway: First, for *npc-1, npc-2* double mutants it was shown in genetic epistasis experiments that their Daf-c phenotype depends on DAF-12 but not on the dauer pathways. Second, a screen for repressors of the *npc-1; npc-2* Daf-c phenotype revealed DIN-1, a DAF-12 interacting repressor and main target of this study (Li and Thomas, 2000 (abstract) andpersonal communication) and third, in this work, we identified *ptr-23* as a *daf-12* interacting protein, that is also involved in the npc- pathway.

R144.7 is homologous to a La related protein from *Drosophila.* The La protein associated with the 3' termini of many newly synthesized small RNAs. Binding by the La protein protects the 3' ends of these RNAs from exonucleases. This La-mediated stabilization is required for the normal pathway of pre-tRNA maturation, facilitates assembly of small RNAs into functional RNA-protein complexes, and contributes to nuclear retention of certain small RNAs. (Wolin and Cedervall, 2002). Human LA protein was shown to be involved in posttranscriptional down regulation of Hepatitis B virus RNA (Horke, 2002). Speculatively, R144.7 could be involved in the stabilization of DAF-12 regulating RNAs and subsequently connect them with their target.

In conclusion, the seven candidate DAF-12 interacting proteins could refer to different DAF-12 functions, F4611.2 and T05C1.6 could be involved in transcriptional regulation,

M04B2.1 and R144.7 might be post transcriptional regulators, M03F4 and ZK270.1 could be part of the ligand transport pathways and F07A11.6 probably is a transcriptional repressor.

Remarkably, we did not observe any overlapping clones within the four yeast- two hybrid screens. An explanation for this could be that we used three different baits that were screened against two different cDNA libraries. It is possible that the tested DAF-12 constructs might recruit alternative sets interacting factors. Moreover, the used cDNA libraries might contain different clones due to differences in their production and amplification. However, when we tested the libraries by PCR amplifying genes with few or only one copy in the *C. elegans* genome (i.e. *nhr-48*), we obtained the expected PCR fragments. We also did restriction digestions with randomly picked clones, and obtained inserts of different sizes. These data indicate that the applied cDNA libraries probably represented a big part of the *C. elegans* genome.

With the yeast- two hybrid method it is generally difficult to reach saturation in screens for protein- protein interactions in *C. elegans*, since this method does not reflect the *C. elegans in vivo* situation. For example, stRNAs remain undiscovered, because *S. cerevisiae* lacks the components to process them efficiently. We would have expected to detect certain proteins as DAF-12 interacting factors, like *daf-16* or *daf-3*, which have been placed in close aproximity to DAF-12 by previous genetic and epistatic analyses. However, the explanation that they did not occur in the two- hybrid- screen could be that either the genetic interaction did notcorrespond with a direct molecular interactions or that an interaction was to weak under the applied conditions and thus remained undetected. Furthermore, not all the positive clones havebeen analyzed. For all that reasons, we presume that we only identified a part of *daf-12* interacting proteins.

Nonetheless, we have reasonable evidence that the genes picked up in the yeast- two hybrid screens are real DAF-12 interacting proteins: Most candidates have been picked up more than once, most candidates are predicted to be nuclear localized and all of them fit in the context of DAF-12 function. However, all the interactions with DAF-12 remain to be confirmed in an in vivo assay like immunoprecipitation or GST pulldown assay.

5.15 Future Prospects

To confirm the DIN-1 DAF-12 interaction, we will perform GST pulldown assays and Co-immunoprecipitations. To do that, we raised epitopic antibodies against the DIN-1 C-terminus and the DIN-1 N-terminal region (Figure 13). Moreover, we are going to HA-tag the C-terminus of genomic *din-1*.

To further ilucidate *din-1* functions, it will be interesting to know if DIN-1 directly controlls the activity of DAF-12 target genes or target genes of other nuclear hormone receptors. To test this hypothesis, we will measure target gene expression in an appropriate inducible system.

In addition to our mutants affecting DIN-1 isoform D only, we want to create mutants affecting more N-terminal DIN-1 regions that would knock out the isoforms A, B and C by creating *din-1* deletion allelels. Double mutants with *din-1D* alleles then should reveal the knock out phenotype, which might be more severe than a *din-1D* knock out alone. We als want to direct *din-1RNAi* against other parts of the gene.

We will further look at expression patterns of DIN-1 isoforms A, B, C and D at different stages and to compare them with those presented in his work and those from a genomic GFP::*din-1* construct in wild typ and in dauer- and aging pathway mutant background.

To underline the hypothesis that SPEN proteins share some of their characteristic features, we want to investigate if DIN-1 can take over SHARP functions in mammalian cell culture systems or vice versa, if SHARP can also suppress TGF-ß and insulin like signals in *C. elegans*. Moreover, yeast- two hybrid screens with different DIN-1 domains should reveal components of pathways that have been shown to interact with large nuclear scaffold proteins like Raf, Hox and Notch or homologs of components of the NurD complex.