5. Discussion

Part I - *dre-1*

5.1. *dre-1* is a novel player in *C. elegans* developmental timing

dre-1 encodes an evolutionary conserved F-box protein that affects postembryonic developmental age of *C. elegans*. DRE-1 wild type activity is required for the promotion of late larval fates in extragonadal and gonadal tissues. Moreover, DRE-1 is involved in specification of chronological age since it affects molting and hatching. Hence, DRE-1 is required at multiple times during *C. elegans* development and acts within separate timing pathways. The finding that *dre-1* encodes a F-box protein that may function in the ubiquitin pathway is consistent with a possible regulatory role in development. The next section will give an overview of how F-box proteins work and highlight some processes they regulate.

5.2. F-box proteins function in the ubiquitin system

The identification of *dre-1* as an F-box protein suggests that it is likely to play a role in the ubiquitin pathway, and thus in protein degradation. F-box proteins are characterized components of SCF (Skp1, Cullin and F-box) complexes that act as E3 ubiquitin ligases (Bai et al., 1996; Pickart, 2001; Skowyra et al., 1997; Tyers and Jorgensen, 2000; Zheng et al., 2002). In a SCF complex the F-box component is required for substrate recognition. The next section illuminates the ubiquitin system with focus on the E3 ligase SCF complex and the role of F-box proteins.

A critical step in ubiquitin dependent protein degradation is the recognition and subsequent ubiquitination of target proteins. This step is facilitated by the E3 ubiquitin-ligases. Ubiquitin, a small (76 aa), highly conserved peptide, is transferred to an E3 ligase bound substrate via two enzymes. The first step covalently links ubiquitin to the E1 ubiquitin-activating enzyme (Haas et al., 1982). In the second step, ubiquitin is transferred to the E2 ubiquitin conjugating enzyme. In the last step, ubiquitination of target proteins is achieved through binding of the E2 enzyme to the E3 ubiquitin ligase whereby ubiquitin is transferred to lysine side chains in the target protein by an isopetide linkage (Passmore and Barford, 2004; Pickart, 2001). Repetition of this “cycle” results in polyubiquitination of substrates that are then degraded by the 26S proteasome (Hershko and Ciechanover, 1998). Delivery of the ubiquitinated substrate to the 26S proteasome is achieved through direct interaction of either E3 ligases (Xie and Varshavsky, 1999) among them SCF subunits (Davy et al., 2001; Verma et al., 2000; Xie and Varshavsky, 2000) or E2 enzymes (Tongaonkar et al., 2000) with components of the 26S proteasome.

The 26S proteasome consists of the proteolytically active 20S core and 19S particles consisting of a “base” with ATPase activity, responsible for unfolding the substrate, and the “lid”, responsible for substrate recognition (Doherty et al., 2002; von Arnim, 2001).

Other components of the ubiquitin pathway are ubiquitin-like modifier proteins. The two best studied examples are SUMO and NEDD8. However, in contrast to ubiquitination, sumoylation of proteins (covalent attachment of SUMO) does not result in degradation of targets, but rather mediates nuclear import or protein-protein interactions. NEDD8 exclusively binds to cullins and may play a regulatory role of E3 ligase activity (Hochstrasser, 2000; Jentsch and Pyrowolakis, 2000). Conjugation of ubiquitin-like proteins to their substrates is mediated by the E1, E2 and E3 protein cascade (Hochstrasser, 2000).

In contrast to E1 and E2 enzymes that contain recognizable protein motifs of high conservation, E3 proteins are structurally diverse (Tyers and Jorgensen, 2000). To date two types of E3 ligases have been defined (Pickart, 2001): The HECT (Homology to the papillomavirus E6-associated protein Carboxyl Terminus) domain ligases that e.g. function in ubiquitination of p53 (Huibregtse et al., 1995; Scheffner et al., 1995) and the Ring finger domain containing ligases (Joazeiro and Weissman, 2000).

The largest group within the Ring finger ligase family is represented by the SCF complexes (Figure 5.1.) (Craig and Tyers, 1999; Deshaies, 1999). This multiprotein complex consists of a core component and a interchangeable substrate recognition component. The core component includes a Skp1-like protein, a cullin and a RING finger (e.g. Rbx1 (Kamura et al., 1999)) (del Pozo and Estelle, 2000; Jackson and Eldridge, 2002). The substrate recognition component is defined by an F-box protein. In yeast, for example, three characterized SCF complexes that share same core elements, but contain different F-box proteins (Cdc4, Met30, Grr1), are involved in distinct biological processes (Goh and Surana, 1999; Kaiser et al., 2000; Li and Johnston, 1997; Patton et al., 2000). Hence, F-box proteins must provide substrate specificity of SCF complexes and are required for the protein turnover of a wide variety of functionally and structurally diverse proteins.

Consistent with this observation is that copy numbers of F-box proteins are high throughout phyla whereas numbers for E1 and E2 enzymes are comparably low. *C. elegans* encodes for as many as 386 F-box proteins, but only 23 Ubiquitin-conjugating (E2) and only one ubiquitin activating enzyme (E1) (WormBase Release WS120). Moreover, E3 ligase components such as Skp1 like proteins and Cullins are represented in low copy numbers as well. The *C. elegans* genome encodes for 21 (WormBase Release WS120) SKP-1 like proteins and 6 different Cullins (WormBase Release WS120).

Target recognition by F-box proteins is mediated through second protein-protein interaction domains generally N-terminally located. Based on the second protein interaction motif, F-box proteins have been categorized into three major classes FBL, FBW and FBX. FBL proteins

contain Leucine-Rich Repeats (LRR), FBWs contain WD repeats and FBXs contain other protein-protein interaction motifs or no motif (Cenciarelli et al., 1999).

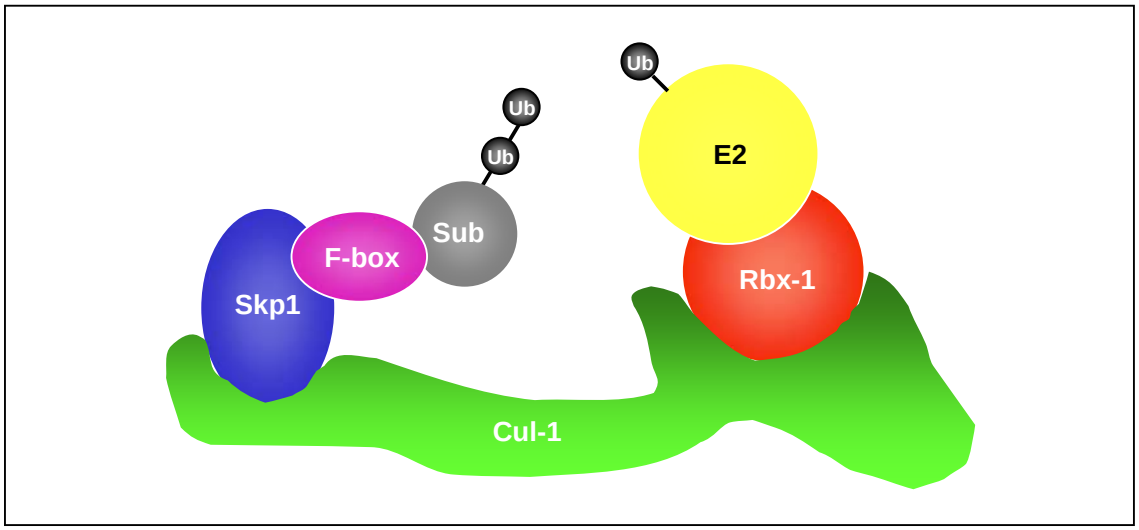

Figure 5.1. SCF E3 ubiquitin ligase complex
A cartoon of an example of a SCF complex illustrating interactions of SCF components (Zheng et al., 2002). The cullin Cul1 serves as a scaffold for the SCF core complex. With its C-terminus it interacts with a Ring finger containing protein (e.g. Rbx1) and with its N-terminus with Skp1 (through a BTB domain fold). Skp1 binds to the N-terminal F-box motif of an F-box protein. The F-box protein contains an additional C-terminally located protein-protein interaction motif through which it recognizes and binds the substrate (Sub). Linkage of E2 conjugating enzyme to the SCF complex through the Ring finger protein results in covalent ubiquitin transfer onto the substrate. Ubiquitinated targets are prone for degradation.

Other motifs include zinc fingers, cyclin domains, leucine zippers, ring fingers, tetratricopeptide (TPR) repeats, and proline rich regions. Due to the great variety of second protein interaction motifs, it is believed that F-box motifs have been somehow inserted into existing proteins in evolution (Kipreos and Pagano, 2000). FBL, FBW and some FBX proteins show conservation from yeast to human (Kipreos and Pagano, 2000). Moreover, various members of all F-box classes FBL, FBW and FBX have been found to interact with Skp1 *in vitro* and *in vivo* (Cenciarelli et al., 1999).

In *C. elegans* the high copy number of F-box proteins makes the F-box the fourth most prevalent protein domain encoded by the genome. However, more than half of the predicted F-box proteins contain a nematode-specific DUF38 (Domain of Unknown Function 38) or FTH (FOG-2 homology domain) (Clifford et al., 2000; Kipreos and Pagano, 2000).

5.2.1. F-box proteins are involved in diverse biological processes

F-box proteins are the interchangeable substrate recognition components of E3 ubiquitin ligases and their great abundance facilitates ubiquitination and subsequent degradation of a great variety of substrates.

However, substrate recognition by F-box proteins must be highly specific and involves sequence and structural features of the substrate (Laney and Hochstrasser, 1999). Oftentimes F-box proteins recruit phosphorylated substrates (Orlicky et al., 2003; Skowyra et al., 1997). Phosphorylation is ideal for fast intracellular signaling. This corresponds to the nature of some processes for which SCF complexes are required, like major cell cycle transitions. Degradation of key regulatory proteins such as cyclin-dependent kinase inhibitors, mitotic cyclins and anaphase inhibitors must occur fast and irreversibly. However, F-box proteins also function in diverse biological processes like control of development, signaling pathways, plant pathogen interaction and amino acid biosynthesis by e.g. mediating the proteolysis of transcriptional regulators.

To illustrate this diversity we highlight here a few examples of the use of F-box proteins within various species. In yeast, 11 F-box proteins have been identified. The F-box protein Cdc4 is involved cell cycle transitions (Blondel et al., 2000; Feldman et al., 1997; Henchoz et al., 1997; Mathias et al., 1996; Patton et al., 1998; Verma et al., 1997). Additionally, it targets replication factors (Drury et al., 1997), kinetochore components (Kaplan et al., 1997) and transcription factors (Meimoun et al., 2000). Grr1 is involved in glucose signaling (targeting a transcriptional repressor) and cell cycle regulation (targeting the cyclins Cln1 and Cln2 for destruction) (Barral et al., 1995; Flick et al., 2003; Li and Johnston, 1997; Skowyra et al., 1999), Met30 is involved in methionine biosynthesis and cell cycle progression (Kaiser et al., 1998; Patton et al., 2000; Thomas et al., 1995).

In fungi *N. crassa* and *A. nidulans* the Scon proteins are involved in the Sulfur regulatory network (Kumar and Paietta, 1995; Kumar and Paietta, 1998; Natorff et al., 1998; Sizemore and Paietta, 2002). In plants, F-box proteins are required for floral organ development photomorphogenesis, auxin response, and defence mechanisms (del Pozo and Estelle, 2000; Durfee et al., 2003; Feng et al., 2003; Ruegger et al., 1998; Wang et al., 2003; Xie et al., 1998). Interestingly, in *A. thaliana* the F-box protein ZTL plays a role in the photocontrol of circadian period (Somers et al., 2000).

In *Drosophlia* 33 F-box proteins have been identified. Slimb is involved in the Hedgehog and Wingless and the dorsal/NFκB signaling pathway (Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Spencer et al., 1999; Theodosiou et al., 1998). It is also implicated in the Hedgehog pathway controlling *Drosophila* eye development (Ou et al., 2003; Wang et al., 1999). Furthermore it is involved in progression through mitosis (Margottin-Goguet et al., 2003) and the initiation of DNA replication (Heriche et al., 2003). Finally, Slimb is implicated in regulation of circadian rhythm (Grima et al., 2002; Ko et al., 2002).

In human, a total of 36 F-box proteins have been identified, but only a few have been assigned a function (Cenciarelli et al., 1999; Winston et al., 1999a). Skp2 is involved in the

control of cell cycle entry (p27 degradation), NFB42 is highly enriched in neurons and induces growth arrest (Carrano et al., 1999; Erhardt et al., 1998; Kamura et al., 2003; Sutterluty et al., 1999; Tsvetkov et al., 1999). β-TrCP is implicated in the IKappa and β-catenin pathway, in Wnt/Wingless and Hedgehog pathways and for CD4 degradation in HIV infected cells (Maniatis, 1999; Margottin et al., 1998; Winston et al., 1999b).

C. elegans contains 386 F-box proteins. However, only a few members have been shown to be involved in biological processes. SEL-10 influences Notch signaling, triggering degradation of the nuclear form of the intracellular Notch1 transcription factor. The Notch pathway specifies many cell fate decisions and perturbation can lead to cancer and impaired development (Gupta-Rossi et al., 2001; Wu et al., 2001). FOG-2 is involved in spermatogenesis in the hermaphrodite germline (Clifford et al., 2000) and LIN-23 regulates cell cycle exit (Kipreos et al., 2000).

5.2.2. Regulation of F-box protein and DRE-1

F-box proteins are regulated by transcriptional and post-translational mechanisms. For example, yeast Grr1, is unstable and post-translational modification by ubiquitin and subsequent degradation is mediated by an autocatalytic mechanism through the SCF complex (Galan and Peter, 1999; Wirbelauer et al., 2000; Zhou and Howley, 1998).

Additionally, Grr1 activity is controlled by nutritional inputs. High glucose levels enhance the Grr1-Skp1 interaction to negatively regulate a repressor of glucose induced gene expression. Moreover, Grr1 regulates the proteolysis of cell cycle components. Hence, it couples nutrient availability to transcriptional regulation and cell cycle control (Li and Johnston, 1997). Speculatively, DRE-1 may be regulated by sugar availability, since it contains CASH and PbH1 domains that are employed in carbohydrate binding and sugar hydrolysis (Ciccarelli et al., 2002; Jenkins et al., 1998).

Additionally, DRE-1 contains a NosD domain. In bacteria nitrous oxide (N_2O) respiration characterizes dentrification. In this process the NosD protein is required for copper insertion into the N_2O reductase, termed NosZ (Holloway et al., 1996). Interestingly, the NosD protein of *Pseudomonas stutzeri* also contains several CASH domains (Ciccarelli et al., 2002).

5.2.3. The role of F-box protein in other biochemical contexts

In some contexts, F-box proteins have been shown to have functions different from SCF dependent proteolysis. For example, the *C. elegans* F-box protein FOG-2 is involved translational repression of *tra-2,* a process important for male sex determination in the hermaphrodite germline. FOG-2 binds a RNA binding protein that is associated with the *tra-2* mRNA through its FTH domain. However, the function of the F-box remains to be

determined (Clifford et al., 2000). Another example is Elongin A, which controls transcriptional elongation activity of the RNA polymerase II in a complex with Elongin C, a protein homologous to Skp1 (Kamura et al., 1998). It is not clear, however, whether these processes are not somehow connected to protein degradation.

5.2.4. DRE-1 may be a component of a SCF complex

DRE-1 is a F-box protein containing a Zinc finger as a potential second protein-protein interaction motif, and by definition is a member of the FBX class of F-box proteins. Notably, the DRE-1 Zinc finger belongs to the N-recognin class of Zinc fingers of the UBR1 protein family. UBR1 proteins are E3 ligases (so called N-recognin) of the N-end rule pathway in which the N-terminal residue of a substrate-protein determines its *in vivo* half-life. In particular, the Zinc finger of yeast Ubr1 is essential for binding basic N-terminal residues Arg, Lys and His in substrates (Kwon et al., 1998). These findings suggest that DRE-1 may be a component of an SCF E3 ligase complex that functions in protein degradation.

To test this hypothesis we performed an RNAi screen with putative SCF complex components. In particular we examined the effect of RNAi knock down of *C. elegans* Skp1 and Cullin homologs in *dre-1* and *daf-12* mutant backgrounds. We hypothesized that such components alone would have *dre-1* like phenotypes, and in *daf-12* background would result in SynMig phenotypes. Notably, *skr-1* (Skp1 homolog) and *cul-1* (cullin homolog) gave rise to a SynMig phenotype in *daf-12*, thus specifically resembling *dre-1*. These genetic data suggest that DRE-1 functions in an SCF E3-ligase complex containing CUL-1 and SKR-1. To confirm these data, we are currently testing for interaction of candidates by the yeast two hybrid method. Furthermore, we are analyzing additional putative components of the complex by RNAi.

Although, VIT-1, the human ortholog of DRE-1, has not been found to interact with human Skp1 *in vitro* (Cenciarelli et al., 1999; Winston et al., 1999b) other human F-box proteins, containing a Zinc finger have been shown to interact with Skp1 *in vitro* and *in vivo.*

5.2.5. DRE-1 may interact with a BTB domain containing protein

Recently, a novel E3 ligase complex has been identified containing an BTB protein that unifies features of the Skp1 and F-box components. The BTB protein binds a cullin through its BTB domain and recognizes the substrate through another protein-protein interaction domain (e.g. the MATH domain) (Pintard et al., 2003; Xu et al., 2003a). Vidal and co-workers (Li et al., 2004) found DRE-1 to interact with a predicted BTB protein (C05C6.8) in protein interaction studies of *C. elegans*. However, preliminary genetic data suggest that in gonad development DRE-1 may not function together with this BTB protein *in vivo*. Specifically, reduction of function assays of C05C6.8 in different mutant backgrounds failed to show *dre-1*

like gonadal phenotypes. It will be very interesting to analyze whether DRE-1 functions with the BTB protein C05C6.8 in other developmental processes.

5.2.6. Functional analysis of the *C. elegans* ubiquitin system

The *C. elegans* genome encodes 23 E2 ubiquitin conjugating enzymes, three of which (LET-70, UBC-9 and UBC-12) are required for embryogensis. In yeast, Ubc9p, an ortholog of *C. elegans* UBC-9, is involved in the transfer of SUMO; and Ubc-12p, an ortholog of *C. elegans* UBC-12, is involved in the transfer of Rub1/Nedd8. In *C. elegans,* E1 ubiquitin activating enzymes and the ubiquitin-like proteins NED-8 and SUMO are also needed for embryogenesis (Jones et al., 2002). Reduction-of-function of *ubc-9*, *ubc-12* and *nedd-8* leads to diverged or branched adult alae (Jones and Candido, 2000; Jones et al., 2002) and body morphology defects (*ubc-9* and *ubc-12*) (Jones and Candido, 2000), phenotypes found in *dre-1* reduction-of-function as well. Since conjugation of ubiquitin-like proteins to their substrates is mediated by the E1, E2 and E3 protein cascade (Hochstrasser, 2000), DRE-1 could also play a role in modification of substrates by ubiquitin-like proteins.

5.3. DRE-1 orthologs

DRE-1 is highly conserved from nematode to human. Orthologs are found in *C. briggsae*, *D. melanogaster*, *R. norvegicus* and humans. Remarkably, DRE-1 and orthologs share an almost identical protein architecture. However, DRE-1 orthologs are uncharacterized proteins with unknown biological functions. Hence, the finding that DRE-1 is involved in the regulation of developmental timing suggests that DRE-1 orthologs may function in temporal control of development as well.

The human ortholog of DRE-1, VIT-1 (Vitiligo associated protein 1) plays a role in vitiligo, which is under control of multiple genes (Le Poole et al., 2001). Vitiligo is a skin disease that affects 0.5% - 1% of the world population and is characterized by absence of the pigment-producing melanocytes from the epidermis, leading to depigmentation of the skin. Although different theories have been expounded, the molecular mechanism underlying vitiligo remains unknown. The *vit-1* gene is weakly expressed in various skin cell types such as melanocytes, keratinocytes and fibroblasts (Le Poole et al., 2001). *vit-1* message is downregulated in vitiligo lesions. Interestingly, the *vit-1* transcript may form RNA-RNA hybrids with the mismatch repair gene hMSH6 and might mediate posttranscriptional repression (Le Poole et al., 2001).

Given the high degree of homology of the human ortholog, we asked whether the human VIT-1 could substitute for DRE-1 function in the control of developmental timing in *C. elegans.* Preliminary results indicate that human VIT-1 cannot rescue the *dre-1* phenotypes (data not shown). However, given the fact that we experienced difficulties getting

the worm gene to rescue mutant phenotypes, it may be even harder to make the human gene work.

The closest *C. elegans* homolog of DRE-1, BE0003N10.3 may not be required in *C. elegans* developmental timing. Although both proteins share a similar protein architecture, BE0003N10.3 lacks the functionally important F-box domain. In addition, reduction-of-function assays of BE0003N10.3 failed to show *dre-1* like phenotypes singly and in the *daf-12* mutant background. Together these data suggest that BE0003N10.3 may function in other processes.

5.4. *dre-1* functions in developmental timing in *C. elegans*

dre-1 is involved in the control of postembryonic temporal development in *C. elegans,* where it is essential for the promotion of late larval programs in extragonadal and gonadal tissues. It was found to interact genetically with other heterochronic regulators in epidermal seam cells and the gonad. Its proposed function in protein degradation suggests that *dre-1* might play a negative regulatory role.

5.4.1. *dre-1* promotes S4 programs in the epidermis

In the epidermis DRE-1 wild type activity is required for the promotion of S4 and/or the inhibition of adult (SA) programs in the hypodermal seam cells at the L3/L4 stage. Mutations in *dre-1* or depletion of *dre-1* by RNAi lead to the expression of adult fates in the juvenile body. Epidermal adult fates such as seam cell fusion and adult alae formation occur precociously at the L3 molt, one stage earlier than wild type. Additionally, *dre-1(lf)* adult animals exhibit gaps in the adult alae. Conceivably, adult alae are resynthesized at the L4 to adult molt and some seam cells may fail to execute this process, leading to visible gaps within this structure. *dre-1::gfp* is predominantly expressed in the nucleus and to a weaker extent in the cytoplasm of seam cells, and therefore *dre-1* is likely to act cell autonomously within this tissue. Epistasis and synergy experiments positioned *dre-1* within the heterochronic seam cell pathway downstream of microRNA *let-7* and upstream of the transcription factor *lin-29,* at the same step as *lin-41, lin-42* and *hbl-1.*

5.4.2. *dre-1* acts upstream of *lin-29*

The transcription factor *lin-29* is the latest acting gene within the epidermal circuit of the heterochronic gene cascade. LIN-29 represses the expression of larval and promotes that of adult programs on the transcriptional level (Liu et al., 1995; Rougvie and Ambros, 1995). Hence, *lin-29* is epistatic to all heterochronic genes and all genes must act through *lin-29* to temporally control seam cell development (Abrahante et al., 2003; Ambros, 1989; Jeon et al., 1999; Lin et al., 2003; Slack et al., 2000).

In genetic epistasis experiments *lin-29* was found to act downstream of *dre-1.* Double mutants of *dre-1* and *lin-29* resembled the *lin-29* delayed phenotype and failed to show the *dre-1* specific precocious phenotype. These data indicate that *dre-1* may negatively regulate *lin-29* to inhibit precocious activation of adult programs at the L4 stage. Hence, LIN-29 protein accumulation in the *dre-1* mutant background should prove DRE-1 this point. Inhibition of LIN-29 may be direct or indirect. For direct interactions of *dre-1* and *lin-29*, there are two possibilities. First, LIN-29 protein may be a substrate of DRE-1, and thus triggered for degradation. Transcription factors are common substrates of SCF E3 ligase complexes. For example, the yeast F-box protein Cdc4 targets the transcription factor Gcn4p for degradation (Kornitzer et al., 1994). Since LIN-29 protein levels accumulate during the L4 stage (Bettinger et al., 1996), DRE-1 may be required for its degradation until the L4 stage. However, preliminary two-hybrid data suggest that DRE-1 and LIN-29 may not interact directly (data not shown).

Yet, the second possibility of direct interaction involves translational repression of the *lin-29* message. *lin-29* regulation is proposed to occur on the translational level since LIN-29 expression is restricted to the L4 stage, but *lin-29* mRNA accumulation initiates by the L2 stage (Bettinger et al., 1996; Rougvie and Ambros, 1995). Hence, the DRE-1 protein may together with other proteins repress *lin-29* translationally. Notably, the F-box protein FOG-2 represses translation of a target through association with a RNA binding protein (Clifford et al., 2000). Translational repression of *lin-29* involves another heterochronic regulator, the RNA binding protein LIN-41 (Slack et al., 2000). Despite acting at the same stage, DRE-1 and LIN-41 function in parallel as indicated by synergy experiments (see below). Therefore, inhibition of *lin-29* until the L4 stage may be redundantly controlled by distinct post-transcriptional repression systems involving LIN-41 and possibly DRE-1.

Finally, DRE-1 could negatively regulate *lin-29* indirectly through other heterochronic regulators such as *C. elegans hunchback* homolog *hbl-1,* since HBL-1 is suggested to inhibit LIN-29 (Lin et al., 2003). Consistent with both nuclear and extranuclear mechanisms, DRE-1 is detected in cytoplasm and nucleus, with a higher concentration in the nucleus.

5.4.3. *dre-1* acts downstream of *let-7*

In epistasis experiments *dre-1* was found to act partially downstream of *let-7.* First, *dre-1* partially suppresses *let-7* lethality. Second, *dre-1* partially suppressed the *let-7* delayed seam cell phenotype. Double mutants of *dre-1* and *let-7* exhibited the *dre-1* specific precocious seam cell fusion at the L3 molt. However, double mutants formed adult alae only weakly compared to *dre-1* single mutants at the L4 molt. Therefore, suppression of *let-7* by *dre-1* may be only partial. In addition, *let-7;dre-1* double mutants on occasion failed to undergo complete seam cell fusion at the L4 molt, again suggesting that *let-7* suppression is

incomplete. Together these results indicate that other heterochronic regulators must act in parallel to *dre-1*.

Accordingly, other heterochronic genes that act downstream of *let-7* such as *lin-41, lin-42 and hbl-1* also suppress *let-7* only partially (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000). These heterochronic loci contain *let-7* binding sites in their 3'UTR and some of them have been shown to be translationally repressed by *let-7* (Reinhart et al., 2000). For example, *lin-41* is negatively regulated by *let-7* in the seams (Slack et al., 2000; Vella et al., 2004) and *hbl-1* in the nervous system (Abrahante et al., 2003; Lin et al., 2003). However, *dre-1* is unlikely to be a direct target of *let-7* since its 3'UTR lacks *let-7* binding sites.

5.4.4. *dre-1* acts in parallel to *lin-41* and *lin-42*

In developmental timing, the last step, the larval to adult switch is controlled by at least four distinct heterochronic loci *lin-41, lin-42, hbl-1* and *dre-1.* They prevent precocious adult program expression at late larval stages through negative regulation of LIN-29 (Abrahante et al., 2003; Jeon et al., 1999; Lin et al., 2003; Slack et al., 2000). Although the relationship between these regulators is still unclear, several lines of evidence suggest that these factors function in parallel. First, none of them exhibit fully penetrant precocious phenotypes. Second, they likely have different biochemical functions. For example, *hbl-1* is a transcription factor, whereas *lin-41* is thought to be involved in translational control (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). Below, an overview of reported synergistic interactions is given and synergy experiments with *dre-1* are described.

dre-1(lf) mutant animals exhibit precocious alae that are weak and indistinct compared to wild type alae. However, a large fraction of mutant animals show the precocious seam cell fusion, but only half of the seam cells display the phenotype. Moreover, only a small fraction of mutants exhibit precocious alae. In average only a third of the seam cells synthesize precocious alae in an affected animal. In contrast all *hbl-1(RNAi)* animals exhibit and a large fraction of seam cells express wild type like precocious alae (Abrahante et al., 2003; Lin et al., 2003). *lin-41* mutant animals exhibit a wild type like precocious alae. However, only half of *lin-41(lf)* animals exhibit the phenotype, and moreover only one third of the seam cells express precocious adult alae (Slack et al., 2000). *lin-42* mutant animals exhibit a wild type like precocious alae. Nearly all *lin-42(lf)* animals display the phenotype, but about a quarter of these contain only patches of alae (Reinhart et al., 2000).

hbl-1 and *lin-41* have been shown to act in parallel. While either single mutant exhibits an impenetrant precocious phenotype, double mutants display a fully penetrant phenotype in which all animals contain wild type like precocious alae (Lin et al., 2003). Moreover, a synergistic enhancement of the *let-7* delayed seam cell suppression was shown

in *hbl-1;lin41* double mutants in the *let-7* mutant background (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). To date, no synergistic interaction of *lin-42* with *lin-41* or *hbl-1* have been reported.

dre-1 acts in parallel to *lin-41* and *lin-42,* because *dre-1* shows synergistic precocious phenotypes with these loci. In both cases *dre-1* enhances the precocious phenotype of either single mutant to a fully penetrant wild type like precocious alae at the L3 molt. Since the *hbl-1(RNAi)* phenotype is already penetrant on its own, it is unclear whether the *hbl-1* phenotype is enhanced in *dre-1* mutants. Aside from precocious seam development, *dre-1* and *hbl-1* mutants share some phenotypes in common including gaps in the adult alae at the L4 molt (Lin et al., 2003) and malformed larvae and larval lethality (Fay et al., 1999; Lin et al., 2003). Moreover, null mutation in *dre-1* yields embryonic lethality, a phenotype observed with *hbl-1* RNAi as well (Abrahante et al., 2003; Fay et al., 1999). However, in *dre-1* mutants embryos develop to the three-fold stage, whereas *hbl-1* embryos arrest development before morphogenesis. Furthermore, HBL-1 and DRE-1 are highly expressed in embryos.

On the other hand, *dre-1* and *hbl-1* differ in several aspects. For example, in contrast to *dre-1*, *hbl-1* is not expressed in or phenotypically affects the somatic gonad, but does affect vulval development (Abrahante et al., 2003; Lin et al., 2003).

Further synergy analysis with *dre-1(dh99)* with *hbl-1, lin-41,* and *lin-42* in sensitized mutant backgrounds should clarify the pathway. For example synergistic suppression of the *let-7* delayed phenotype, (as found for *hbl-1* and *lin-41)* would support the idea of regulators acting in parallel, while no additional suppression would support the idea of regulators working in the same pathway.

Should DRE-1 and HBL-1 function in the same pathway, DRE-1 could negatively regulate LIN-29 indirectly through HBL-1 (Lin et al., 2003). For example, DRE-1(+) could degrade a negative regulator of HBL-1. In this view, the absence of *dre-1* would lead to a phenocopy of *hunchback* loss of function, i.e. precocious development. Further analysis, for example the identification of DRE-1 interacting proteins by yeast two-hybrid screens, should yield insight into the biochemical mechanism of DRE-1, and may reveal additional DRE-1 targets.

5.4.5. *lin-4* may be epistatic to *dre-1*

Epistasis experiments suggest that *dre-1* acts upstream of the microRNA *lin-4.* The *dre-1;lin-4* double mutant resembled the retarded phenotype of the *lin-4* single mutant. Similarly, *lin-4* was found to be epistatic to *lin-41* and *hbl-1* in epistasis experiments (Abrahante et al., 2003; Slack et al., 2000). However, triple mutants of *hbl-1*, *lin-41* and *lin-4* exhibited partial alae formation, revealing that both, *lin-41* and *hbl-1* actually act downstream of *lin-4* (Abrahante et al., 2003), but the epistasis is obscured because of functional

redundancy. Moreover, partial alae formation indicates that *lin-4* may act through other factors in addition to *lin-41* and *hbl-1*. Similarly, *dre-1* was found to act in parallel to *lin-41*. If so, then *dre-1;lin-41* double mutants are predicted to form adult alae in a *lin-4* mutant background. Construction of *dre-1*;*hbl-1;lin-4* triple mutant, should also clarify their functional relationship.

5.4.6. Model of epidermal heterochronic pathway

Based on the current molecular and genetic data we suggest the following heterochronic model. In the L1 stage, components of the early timer, LIN-14 and LIN-28, somehow delay accumulation of *lin-29* message (Figure 5.2.A). Conceivably, LIN-14 nuclear protein could inhibit LIN-29 transcription directly or indirectly, while LIN-28/RNA binding protein could affect mRNA transport, processing or availability (Figure 5.3.). One possibility is that LIN-14 acts together with transcription factors LIN-42 and HBL-1 to antagonize LIN-29 transcription, but this has yet to be explored (Figure 5.3.A.). As *lin-4* microRNA increases during L1, the inhibitory activities of the early timer are downregulated. DAF-12 and presumably other factors now active, then transcribe *lin-29* during the L2 stage (Figure 5.2.A; Figure 5.3.A). Thereafter, LIN-29 transcription increases into L3 and L4 stages perhaps by downregulation of transcriptional repressors. Moreover, regulation by post-transcriptonal mechanisms play a dominant role, since LIN-29 protein accumulation does not occur until the L4 stage (Bettinger et al., 1996; Rougvie and Ambros, 1995). In particular, LIN-41 is a central component of translational inhibition (Figure 5.3.B) (Slack et al., 2000). Stimulation of *let-7* microRNA expression in late larval stages, perhaps by hormonal signals, initializes the late timer (Figure 5.2.B) (Reinhart et al., 2000). As a consequence, LIN-41 and HBL-1 are downregulated, leading to LIN-29 derepression by mid-L4, and thus to promotion of adult programs (Figure 5.2.B) (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). DRE-1 may negatively regulate LIN-29 directly or indirectly through protein degradation (Figure 5.2.B). A simple idea is that translational inhibition by LIN-41 is not completely tight, and DRE-1 plays a role in degrading LIN-29 protein that has escaped translational repression (Figure 5.3.C). Many outstanding questions remain about this circuit. For example, how HBL-1 exerts its function in seam cell development is unclear since HBL-1 expression is absent from seam cells after L1 (Abrahante et al., 2003; Lin et al., 2003). Downregulation of HBL-1 itself in seam cells is independent of microRNAs and may be controlled on the transcriptional level. One attractive hypothesis is that HBL-1 initiates an epigenetic memory of developmental age within the seam. Alternatively, HBL-1 may act from the surrounding hypodermis to influence seam cell temporal fates cell non-autonomously, since HBL-1 expression in the hypodermis is downregulated in L3 (Abrahante et al., 2003; Lin et al., 2003).

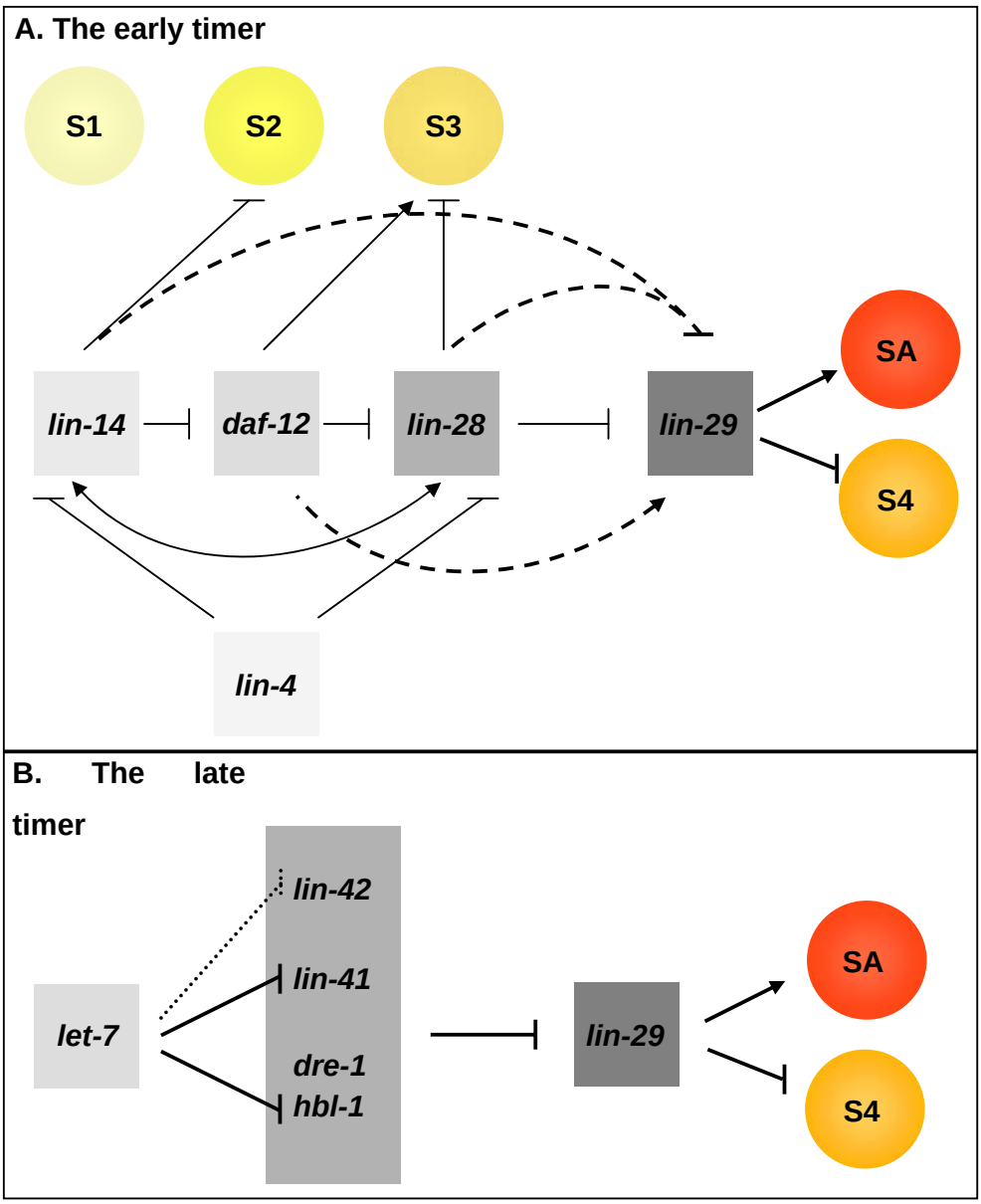

Figure 5.2. Heterochronic epidermal pathway model
(A) The early timer. Activities of the early timer define temporal L1 to L3 development. In L1 *lin-14* specifies S1 programs through repression of S2 and S3 programs thereby inhibiting *daf-12*. Accumulation of *lin-4* in late L1 inhibits *lin-14* and leads to S2 program expression in L2 while S3 programs are repressed by *lin-28*. Downregulation of *lin-28* by *lin-4* in mid L2 results in derepression of S3 programs. Additionally, *lin-14* and *lin-28* may inhibit *lin-29* directly or indirectly in L1. Downregulation of *lin14* and *lin-28* leads to activation of *daf-12* that may promote *lin-29* transcription in the L2 stage. (B) The late timer. Activities of the late timer define L3 to adult development and consist of at least four distinct activities that control specification of S4 fates and precocious prevention of adult fates through combined inhibition of *lin-29.* These activities are defined by *lin-41, lin-42, hbl-1* and *dre-1* (depicted in rectangle shaded in grey). In seam cells *lin-41* and *hbl-1* act in parallel (Abrahante et al., 2003; Lin et al., 2003) and *dre-1* was shown to function in parallel with *lin-41* and *lin-42* (this work). The relationship between *dre-1* and *hbl-1* is unclear and remains to be analyzed further. *dre-1* may act in parallel or in the same pathway with *hbl-1.* Relationships between *lin-42* and *lin-41* or *hbl-1* have not been reported. Inhibition of *lin-41* in L3/L4 by *let-7* leads to derepression of *lin-29* and to SA program specification. *hbl-1* is inhibited by *let-7* in the nervous system. *lin-42* may be inhibited by *let-7*.

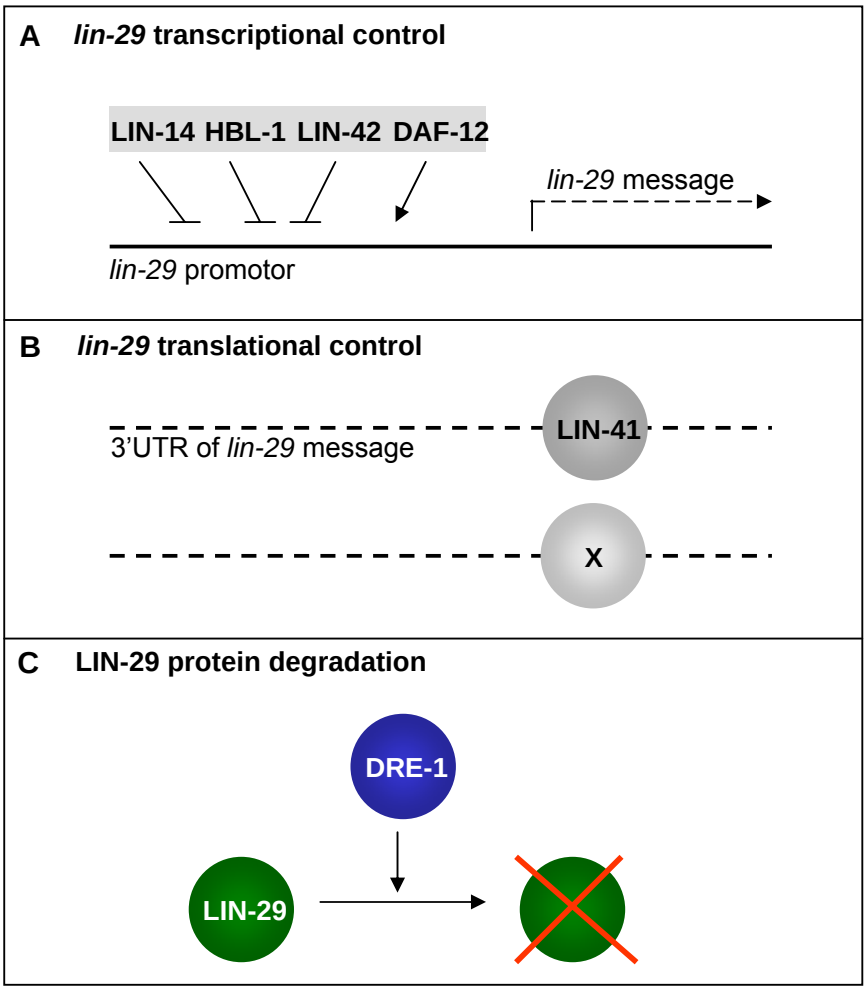

Figure 5.3. Molecular mechanism of *lin-29* control
(A) Transcriptional control. LIN-14, HBL-1 and LIN-42 may inhibit and DAF-12 may promote *lin-29* expression in early larval stages. (B) Translational control. *lin-29* inhibition is thought to be mainly mediated through translational control. LIN-41 and other unknown proteins (X) inhibit precocious accumulation of LIN-29 in late larval stages by negatively regulating *lin-29* translation. (C) Protein degradation. In case of leaky translational control, any LIN-29 protein made, is degraded by DRE-1.

LIN-42 also restricts LIN-29 expression to the L4 stage (Jeon et al., 1999). Although to date no mechanisms have been reported, LIN-42 may be translationally controlled by *let-7,* since it contains *let-7* binding sites in its 3'UTR and partially suppresses *let-7* phenotypes (Figure 5.2.B) (Reinhart et al., 2000). Hence, mechanisms and timing of LIN-42 downregulation may occur similar to LIN-41, although LIN-42 expression is evident into the adult (Jeon et al., 1999).

In summary, the larval to adult switch is tightly controlled through redundant functions that convey different layers of regulation of LIN-29 thereby restricting its expression until the appropriate stage.

5.4.7. *dre-1* promotes gonadal age

In wild type the gonad undergoes stage-specific developmental events that are expressed in strict temporal sequence (Antebi et al., 1998). Most notably, migratory leader cells, the hermaphrodite Distal Tip Cells and the male linker cell, lead outgrowth of the gonad during L2, L3 and L4. In addition, gonadal precursors divide and undergo differentiation, epithelialization and morphogenesis into ovary, spermatheca, and uterus. Moreover, the gonad is tethered to the epidermis, vulva, and sex muscles, and therefore must be coordinated with extragonadal events. Finally, gonadal development must be synchronized with germ cell maturation.

Little is known about the temporal organization of gonadal development. Apparently, it is regulated largely independently from the epidermal seam cell circuit. Notably, most known heterochronic regulators have no known effect on the gonad except *daf-12* (Ambros, 1989; Ambros and Horvitz, 1984; Antebi et al., 1998; Liu and Ambros, 1989), which delays leader cell migration, gonadblast compaction, division and other processes, and *lin-29*, which affects uterine development (Newman et al., 2000). Moreover, only *daf-12*, *lin-29* and *lin-41* are reportedly expressed in the gonad (Antebi et al., 2000; Bettinger et al., 1996; Slack et al., 2000). Finally, none of the identified microRNAs regulate expression of heterochronic genes in the gonad. In this work, we explored the genetic interactions of *dre-1* with other heterochronic regulators and found synergistic interactions in temporal control of gonadal development, providing an entry point for dissecting gonadal heterochrony.

Mutations in *dre-1* leads to variable impenetrant mild gonad migration defects, some of which are interpreted as a failure of adult program execution. *daf-12* null mutation also exhibits impenetrant heterochronic gonadal migration defects. In contrast, the *dre-1;daf-12* double mutant exhibits a strong synergistic gonad migration defect (Mig) interpreted as a repetition of S2/S3 programs. By inference, DRE-1(+) and DAF-12(+) may act redundantly in the promotion of larval S3/S4 programs (Figure 5.4.). This hypothesis is supported by two observations. First, the temperature sensitive period of *daf-12* for the gonad migration phenotype is from mid-L2 to mid-L3 and coincides with the time of S3 program initiation. Second, DAF-12 expression in the DTC accumulates by L2 and remains prominent until L3 and early L4 (Antebi et al., 2000). DRE-1 expression in the DTC is seen from L2 through L4 and is evident into the adult.

Interaction of *dre-1* or *daf-12* with *lin-29* also yielded strong synergistic heterochronic migration phenotypes. Notably, two distinct Mig phenotypes were observed. The first resembled the *dre-1;daf-12* Mig interpreted as a repetition of S2/S3 programs. The second gonad migration phenotype, in which the DTC failed in their second reorientation phase, is interpreted as a failure to execute the second phase of the S4 program. Therefore LIN-29 wild type activity acts in parallel to DRE-1(+) and DAF-12(+) in the promotion of S4 programs

(Figure 5.4.A). The fact that LIN-29 is expressed in the DTC and accumulates during early/mid L3, which is the time of S4 program initiation, supports this hypothesis (Bettinger et al, 1996). In contrast, in the seam cells, where LIN-29 plays a role in SA program specification, expression comes on by mid-L4 (Bettinger et al., 1996). Moreover, LIN-29 has been shown to act cell autonomously.

In addition, *daf-12* mutants fed *lin-41* RNAi give a strong Mig phenotype, in which the second part of the S4 program (migration back towards midbody) was defective. Accordingly, *lin-41* is expressed in the somatic gonad, and comes on in the DTC in late larval stages (Slack et al., 2000). However, this interaction needs to be examined more closely.

The miRNA *lin-4* has mild effects within heterochronic gonadal circuit. Double mutants of *daf-12* or *dre-1* and *lin-4* yielded a weak, but penetrant synergistic gonad migration defect, in which DTC failed to fully migrate back towards mid-body. This defect is interpreted as a failure to completely execute the SA program. By inference, wild type *lin-4* is required for SA program specification and acts redundantly with DRE-1(+) and DAF-12(+).

In sum, we identified that the temporal regulation of gonad development is under control of certain heterochronic activities, namely *dre-1, daf-12, lin-29* and perhaps *lin-41* and *lin-4*, that function somewhat redundantly. In a simple model DRE-1 together with DAF-12 specify S3/S4 programs, LIN-29 is involved in S4 program specification and *lin-4* is needed for the promotion of SA programs (Figure 5.4.A). One caveat to these experiments is that they relied on interactions with a non-null allele of *dre-1*. Another caveat is that these regulators cannot be simply ordered into a pathway because they do not exhibit epistatic interactions. What is required is a mutant that has precocious migration, or which suppresses the Mig phenotype. *lin-42* might be such a candidate since it suppressed the Mig phenotype of *daf-12;dre-1(RNAi)*.

Although the underlying molecular mechanism remains to be identified, two general scenarios for these interactions could be envisioned. In the first model, *dre-1, daf-12* and *lin-29* function in a parallel pathway to somehow act on a common downstream target. Loss of any two functions gives a phenotype.

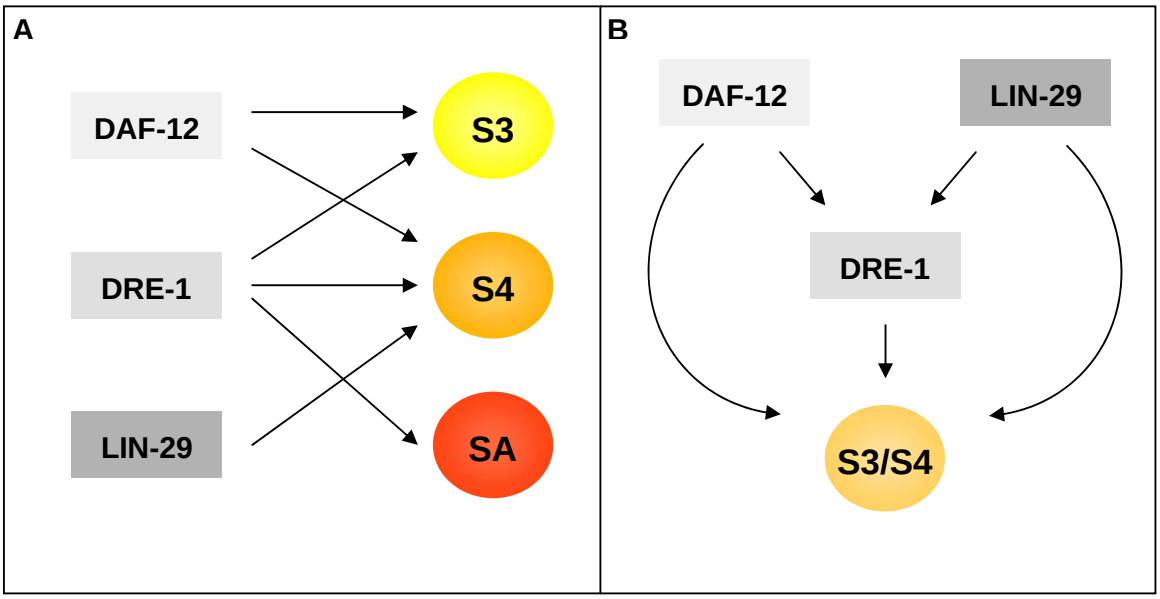

Figure 5.4. Heterochronic gonadal model
(A) DAF-12, DRE-1 and LIN-29 act in parallel to promote gonadal age. DAF-12 promotes S3/S4 programs, DRE-1 S3/S4 and SA programs and LIN-29 S4 programs. (B) DAF-12 and LIN-29 may act through *dre-1* positively regulating its transcription to promote gonadal S3/S4 programs. Alternatively, DAF-12, LIN-29 and DRE-1 promote S3/S4 programs in parallel.

In another model, *dre-1* itself could be a transcriptional target of both DAF-12 and LIN-29 (Figure 5.4.B). In this view, the *dre-1* hypomorphic phenotype (weak Mig) would be further enhanced by *daf-12* or *lin-29* loss of function. Similarly, loss of the two transcription factors might lower *dre-1* expression below some threshold to give gonadal defects. Consistent with this, *dre-1(dh99)* on *dre-1* RNAi displays a stronger Mig defect. Additionally, multiple DAF-12 response elements were found in the *dre-1* promoter (the region included 1 kb uptream of the start codon). However, preliminary expression analysis indicate that *dre-1* may not be a target of DAF-12, since *dre-1::gfp* expression was unaltered in the *daf-12(rh61)* background (data not shown). Further analysis of *dre-1* expression in both mutants backgrounds may help to elucidate this hypothesis.

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5.5. *dre-1* affects chronological age

Landmarks as hatch and ecdysis (molts) are convenient measures of chronological age. While hatching defines the boundary from embryonic to postembryonic development, ecdysis defines the separate larval stages.

Interestingly, *dre-1* null mutants develop to the three fold stage of embryogenesis, but are unable to hatch, suggesting that DRE-1(+) is required for the first critical step of post-embryonic development. Conceivably, DRE-1 could regulate other factors in the hatching process, such as *hch-1*, a tolloid protease that proteolises the eggshell (Hishida et al., 1996).

Remarkably, a partial reduction of *dre-1* reveals defects at all larval molts, in which animals were unable to shed their old cuticle. Moreover, *dre-1* RNAi also lengthens the molt cycle. Other heterochronic regulators also affect molting, but only the L4 to adult molt (Abrahante et al., 2003; Jeon et al., 1999; Slack et al., 2000). Some precocious mutants have one less molt, while delayed mutants can undergo supernumerary molts (Ambros and Horvitz, 1984; Rougvie and Ambros, 1995). Oftentimes animals have difficulty shedding their penultimate cuticle. These activities reflect precocious or delayed expression of *lin-29*, which ultimately controls exit from the molt cycle (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987).

By contrast *dre-1* affects all larval molts and acts at multiple times during *C. elegans* development. Conceivably, *dre-1* may affect the molting process directly or indirectly through other undetermined key regulators of molting. However, little is known about the regulation of ecdysis in *C. elegans*. One hypothesis is that ecdysis is governed hormonally. Accordingly, two members of the nuclear hormone receptor family, *nhr-23* and *nhr-25,* both affect molting (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 1998; Kostrouchova et al., 2001). SCF E3 ligase complexes facilitate fast and irreversible degradation of key regulatory proteins in diverse biological processes, among them major cell cycle transitions (Hershko and Ciechanover, 1998; Passmore and Barford, 2004). Molting may similarly require degradation of key regulators at certain steps to advance the cycle. Hence, DRE-1 as part of a putative E3 ligase complex may mediate this degradation and NHR-23, NHR-25 or other components may be subject to DRE-1 regulation. Moreover, turnover of transcription factors or nuclear receptor coactivators through the ubiquitin pathway is a common scheme in diverse biological processes (Kornitzer et al., 1994; Yan et al., 2003).

Notably, *dre-1* pervasively affects both the heterochronic and molting timers, the first regulator to do so in such a visible way. Two possibilities emerge from this fact. First, *dre-1* could affect both timing circuits independently. Or *dre-1* could act at the intersection of both pathways, coupling chronological timers to heterochronic timers, or vice versa. These two possibilities will be a challenge to explore in the future.

5.6. DRE-1 may function in diverse pathways

dre-1 RNAi and the null mutant displayed various uncharacterized phenotypes seemingly unrelated to developmental timing or molting. The *dre-1* null phenotype is embryonic lethality and early larval arrest. However, hatched larvae exhibited a variety of phenotypes such as body morphology defects (Bmd), smaller body size (Sma), uncoordinated movement (Unc) and constipation. *dre-1* RNAi produced larval lethality. Moreover, animals show rupture of the uterus or rectum, a protruding vulva (Pvul) or an egg laying defect (Egl). Thus DRE-1

may be required for a wide variety of biological processes. Indeed, preliminary two hybrid data suggest that putative substrates of DRE-1 are involved in many pathways. This finding is not surprising, since F-box proteins across species have multiple targets in unrelated pathways. For example, *Drosophila* Slimb is implicated in Hedgehog, Wingless and Dorsal/NFKB signaling, and as well as regulation of circadian rhythm (Grima et al., 2002; Jiang and Struhl, 1998; Ko et al., 2002; Ou et al., 2003; Spencer et al., 1999; Wang et al., 1999).

In summary, *dre-1* plays an important role in *C. elegans* developmental timing of gonadal and extragonadal tissues. In the heterochronic epidermal seam cell circuit it is part of the late timer regulating the larval to adult switch. The fact that *dre-1* mutants have a comparably weak phenotype may reflect parallel pathways. Indeed, the timing of larval to adult development is regulated by at least four genes in parallel. Regulatory mechanisms employed include transcriptional and translational control. The exciting finding that *dre-1* encodes an evolutionary conserved F-box protein seemingly involved in protein degradation adds a new dimension to the regulation of developmental timing in animals, and reveals that multiple regulatory mechanisms are invoked to achieve robust control of developmental timing. Additionally, because genes of the late timer are highly conserved across species, the physiological role of DRE-1 in temporal control may be evolutionarily conserved.

5.7. Future prospects

We will further elucidate the role of *dre-1* within the heterochronic seam cell circuit through three approaches. First, we will analyze the temporal expression pattern of DRE-1 in phenotypically affected tissues (e.g. seam and Distal Tip Cells) in detail. Coincidence of temporal up- or downregulation of *dre-1* with that of other heterochronic regulators (e.g. *lin-29, let-7, lin-42, lin-41* and *hbl-1)* may provide evidence of how *dre-1* exerts its function.

Second, we will test the hypothesis that *dre-1* prevents precocious expression of adult programs in late larval stages through negative regulation of *lin-29.* Hence, we will analyze the LIN-29 expression pattern in the *dre-1* mutant background. Upregulation of LIN-29 in late larval stages will provide evidence that DRE-1 may inhibit LIN-29 possibly through protein degradation.

Third, to clarify whether *dre-1* is functioning in a parallel or in the same pathway with other regulators acting at the same step (e.g. *lin-41, lin-42* and *hbl-1)*, we will perform additional genetic synergy experiments testing for enhancement of precocious phenotypes in sensitized mutant backgrounds (e.g. *lin-4* and *let-7)*. Enhancement will confirm parallel pathway hypothesis whereas no enhancement will place them in the same pathway.

Moreover, we will further characterize *dre-1* seam cell phenotypes. Especially to explore the reasons for the adult alae gap phenotype in adults, we will lineage *dre-1* in the *ajm-1::gfp* background that stains seam cell junctions. Additionally, we will further characterize seam cell phenotypes of *dre-1(dh99)* on *dre-1* RNAi.

Additionally, we will further investigate roles of heterochronic activities in temporal control of gonad development. Therefore, we will screen for functional redundancies of *lin-29* and other heterochronic regulators, analyze missing interactions and explore the role of *lin-42* in gonadal heterochrony.

Finally, we will further analyze the biochemical function of DRE-1 in protein degradation. Hence, we will test for *in vitro* and *in vivo* interactions of candidates of the DRE-1 complex and also search for additional components.

Part II - *dre-2*

5.8. The *dre-2* locus

The *daf-12* Mig enhancer screen yielded two distinct loci, *dre-1* and *dre-2.* Surprisingly, we found only one single allele of *dre-2.* Such low frequencies can have the following reasons: first, *dre-2* may be a particularly small gene, second *dh184* may be an unusual allele, or third the *dre-2* null phenotype may be very severe e.g. lethal.

5.9. *dre-2* may be involved in *C. elegans* developmental timing

dre-2 may play a role in developmental timing. Several lines of evidence suggest this: first, it strongly suppressed *lin-42* precocious adult alae phenotypes. Second, it enhanced the retarded epidermal and gonadal phenotypes of the *daf-12* null mutant. Third, on its own it displayed a semi-penetrant strong delay in gonad migration.

5.9.1. *dre-2* may promote gonadal age

Temporal regulation of gonad development is under control of specific heterochronic activities, namely *dre-1, daf-12* and *lin-29*. These genes function in parallel to ensure proper timing of gonad migration by successive expression of stage-specific programs. Null mutants of these genes have normal gonad development (Ambros, 1989; Ambros and Horvitz, 1984; Antebi et al., 1998; Liu and Ambros, 1989; this work), whereas double mutant combinations result in various synergistic heterochronic gonadal migration phenotypes (this work).

Interestingly, the mutation in *dre-2* leads to a strong gonad migration defect that is interpreted as an erroneous S4 program execution. The gonad migration phenotype is strongly enhanced in severity and penetrance in the *dre-2;daf-12* double mutant, interpreted as a repetition of S2/S3 pathfinding programs. Hence, DAF-12(+) and DRE-2(+) may function in parallel in the promotion of larval S3/S4 programs. Since *daf-12* acts in parallel with other heterochronic regulators, we tested whether *dre-2* functions redundantly as well. We depleted *lin-29* or *dre-1* gene functions by RNAi in the *dre-2* mutant background and screened for SynMig defects. No SynMig phenotypes were observed indicating that *dre-2* solely interacts with *daf-12* in gonad development. However, these results have to be treated with caution, since they are preliminary and further analysis of genetic interactions using double mutants will clarify whether these activities function in parallel.

5.9.2. *dre-2* may function in temporal epidermal development

Mutation in *dre-2* do not result in epidermal phenotypes. However, genetic analysis of double mutants with heterochronic genes suggest that *dre-2* may function in temporal epidermal development. In particular, *dre-2* strongly suppressed the precocious adult alae phenotype of *lin-42*. While all *lin-42(n1089)* mutant animals exhibited precocious adult alae at the L3 molt, only a small fraction of animals displayed the phenotype in the *dre-2* background. Interestingly, *dre-2* enhanced seam cell phenotypes of *daf-12*. *daf-12* null mutants exhibit an impenetrant delayed seam cell phenotype in which occasionally S2 seam cell programs are repeated in L3. However, subsequent programs are expressed on schedule. Remarkably, double mutants of *dre-2* and *daf-12* resulted in an adult alae gap phenotype at the adult stage. This may indicate that *dre-2* enhanced the delayed seam cell phenotype of *daf-12* null resembling the *daf-12(rh61)* phenotype. In *daf-12*(*rh61)* mutants a delay of seam cell programs in L3 is propagated through the lineage. At the adult stage, a fraction of seam cells express larval programs resulting in visible gaps in the adult alae. These adult alae gaps are found in the *dre-2;daf-12* double mutant as well. However, whether the gaps result from repetitions of larval programs remains to be determined. Hence, *dre-2* may be a delayed heterochronic mutant since it suppresses precocious phenotypes, and conversely may enhance delayed phenotypes of heterochronic regulators.

dre-2 may represent a novel class of heterochronic genes that do not affect developmental age *per se*, but enhance or suppress phenotypes of other heterochronic activities. Hence, *dre-2* may exert its function indirectly through direct regulators as *daf-12* and *lin-42* to control temporal development.

Notably, phenotypes of *dre-2* interpreted as a temporal delay could also reflect arrested development. However, suppression of *lin-42* precocious phenotypes suggest that *dre-2* may function within the heterochronic circuit. Further genetic analysis of interactions with other heterochronic genes may proof *dre-2* to be a heterochronic gene, and if so help to place it within the pathway.

5.10. DRE-2 may function in diverse pathways

The *dre-2* mutant exhibited several uncharacterized phenotypes with varying penetrance. Nearly half of mutant animals arrested development as embryos and larvae. Moreover, larvae displayed body morphology defects (Bmd) and uncoordinated movement (Unc). Among adult mutant phenotypes were smaller body sizes (Sma) or ruptures of uterine/seam cell junction (Rup). Therefore, DRE-2 wild type activity may play diverse and essential roles in development.

5.11. Future prospects

A great challenge will be to clarify whether *dre-2* is a heterochronic gene retarded in development or simply arrests development. To address this question, we will analyze genetic interactions of *dre-2* and other heterochronic regulators for enhancement and/or suppression of gonadal and epidermal temporal defects using double mutants. If *dre-2* is a heterochronic gene, these experiments will contribute to the understanding how *dre-2* influences developmental timing and what are its mediators. Furthermore, identification of the *dre-2* gene product, will illuminate how *dre-2* exerts its function. We are currently positionally cloning *dre-2.* Once the *dre-2* locus is identified, we can determine the *dre-2* null phenotype through application of RNAi or creation of a deletion to knock out DRE-2 function. Moreover, DRE-2 expression analysis by *dre-2::gfp* fusion will help to elucidate how *dre-2* functions.