2. Introduction

2.1. Life cycles and life stages

The entire life of an animal, from fertilization through death, is defined as the life cycle, and consists of a sequence of defined life stages, oftentimes including alternative stage options. The life cycle begins with fertilization, followed by embryonic development, birth, post-embryonic development, maturation, adulthood, senescence and death. It is unclear, particularly late in life, to what extent this sequence of events is programmed by the genome and modulated by environment. The steps of embryogenesis, including cleavage, gastrulation, germ layer formation, and organogenesis, are generally autonomous, well defined and under genetic control. After birth, post-embryonic development consists of larval stages, transition from juvenile to adult stage, adulthood and senescence, which are generally more subject to environmental influences.

Various animals develop through a progressive series of changes and have stereotyped, distinct life stages. Life stages in the Ecdysozoa (e.g. Nematoda such as *Caenorhabditis elegans*, Arthropoda such as *Drosophila melanogaster*) are well defined by landmarks such as hatch and molting of the exterior skeleton known as ecdysis. Moreover, some insects undergo dramatic transitions as metamorphosis in which larval tissues are destroyed and adult structures are elaborated. Similarly, in some amphibians such as frogs (e.g. *Rana pipiens*), the entire body plan is remodeled from an aquatic to a terrestrial organism during metamorphosis. On the other hand, mammals such as humans generally develop in linear succession and exhibit gradual, continued growth and maturation. Striking changes or landmarks as metamorphosis or ecdysis are not evident. Nonetheless, the human life cycle contains puberty, the transition from juvenile state to adult sexual maturity (Grumbach, 1998), and the hormonal basis of puberty is thought to be similar to that of metamorphosis. Finally, humans clearly undergo cognitive changes throughout the life cycle. Thus, although stage transitions in such species are not as obvious, they most certainly take place, and may be more numerous than meets the eye.

Given the fact that all animals develop through specified life stages, the question then arises: how are life stages, life stage options and stage transitions regulated and what are the underlying molecular mechanisms? To begin with, scientific research demonstrates that development of an animal from egg to adult is controlled along four axis, three of space (anterior-posterior; dorsal-ventral; proximal-distal) and one of time. In particular, the temporal control of development is of great importance, since all processes of a developing organism need to be synchronized in time. Is there a phyletically conserved core of regulators that specify the life plan of a species, or is the temporal organization of development completely species specific and idiosynchratic? The premise here is that, just as there are conserved

regulators of the body plan, there exist a set of global regulators of temporal development that were invented in antiquity.

2.2. Heterochrony

The term "heterochrony" comes from the Greek word "heteros" meaning other or different and "chronos" meaning time. Already in 1860 Ernst Haeckel used the term heterochrony to refer to differences in the biogenic law saying that "ontogenesis (a whole course of an individual's development, and life-history) is a brief and rapid recapitulation of phylogenesis (evolutionary history)". Hence, heterochrony is characterized by evolutionary changes in ontogenesis and defined as an evolutionary change in rates and timing of developmental processes compared to the phylogenetic sequence (de Beer, 1958; Gould, 1977). In particular, speciation can be achieved by changing the relative timing of developmental events between tissues. By comparing closely related species, heterochronic changes can be deduced.

Historically, heterochrony was categorized into three classes (see examples below), and generally refer to the relative timing of maturation of soma versus gonad. Neoteny is the retardation of somatic development relative to normal gonad development. Progenesis, refers to the acceleration of gonadal development to normal somatic development (sexual maturation in juvenile form). Finally, direct development is acceleration in somatic development resulting in the deletion of larval stages. More recently, heterochrony refers to a change in the relative timing of developmental events within an organism induced by some experimental intervention (e.g. genetic, mechanical), and has also been extended to the cellular level in which gene expression is triggered precociously or retarded relative to wild type. For the most part, I shall be concerned with genetic manipulations that alter the timing of gene expression, cellular and stage specific events.

As a prelude, I will provide examples and discuss mechanisms of heterochrony in various species. Then I will focus on specific cellular programs under temporal control in *C. elegans.* Finally, I will discuss the heterochronic loci and their interactions in a regulatory pathway that specifies post-embryonic developmental timing from the cellular to the organismal level.

2.2.1. Examples of heterochrony

The most prominent example of heterochrony (neotony) is that of the Mexican axolotl *Ambystoma mexicanum.* This salamander fails to undergo metamorphosis and sexual maturation occurs in the larval-like stage. Molecularly, this defect is caused by a failure to release thyrotropin, the thyroid stimulating hormone (Norris et al., 1973; Prahlad and DeLanney, 1965; Taurog et al., 1974). Interestingly, neoteny in salamander species can be

obligate, inducible obligate or facultative and some species undergo metamorphosis upon thyroid induction (Frieden, 1981; Huxely, 1920). This example raises two important issues. First, the importance of hormonal control of temporal development, and second, the importance of single gene heterochronic mutations for evolution, as they can lead to evolutionary variation and speciation (Gould, 1977; Raff, 1987).

One example of progenesis (sexual maturation in juvenile form) is vertebrates that are thought to have evolved from precocious sexual maturity of tunicate tadpole larvae (de Beer, 1958). Direct development is exemplified by some species of sea urchins, which delete larval stages completely and develop directly from embryo to adult (Raff, 1987).

More recently, mutations in specific heterochronic genes have been found that change the course of temporal development in diverse phyla. Dramatic alteration of the onset of human puberty can be interpreted as heterochrony (Huhtaniemi, 2000; Huhtaniemi, 2002). For example, in males puberty initiation can be perturbed by defects in luteinizing hormone signaling pathway. Gain-of-function mutation in the receptor for the luteinizing hormone can result in precocious and loss-of-function in delayed puberty (Weiss et al., 1992; Wu et al., 2000). Furthermore, comparison of chimpanzees to humans revealed that adult humans may have retained juvenile characteristics as large brain size and a flat face that correspond to those of juvenile chimpanzees (Gould, 1977).

In *D. melanogaster,* the *anachronism* gene, which encodes a glycoprotein, controls the correct timing of neuroblasts and neuronal stem cell proliferation. In *anachronism* mutants development proceeds normal, but certain neuroblasts in the larval brain divide precociously relative to wild type (Ebens et al., 1993). Similarly, *hunchback* mutations in fly cause neuroblasts to develop precociously (Isshiki et al., 2001). Notably, *hunchback* is also implicated in the *C. elegans* heterochronic pathway. Mutations in the *ecdysone* receptor and its partner *ultraspiracle* lead to heterochrony (Hodin and Riddiford, 1998; Hodin and Riddiford, 2000). In addition, isoform specific mutations in the E75 nuclear receptor cause animals to skip some L3 stage programs but they nonetheless pupariate (Bialecki et al., 2002).

In the slime mold *Dictyostelium discoideum,* mutations in *rdeC* (*rapidly developing C*) were found that cause precocious premature terminal cell differentiation of the stalk and spore cell (Simon et al., 1992). *rdeC* encodes a regulatory subunit of cAMP-dependent protein kinase A.

Interestingly, heterochronic genes cause temporal defects in various plants. In maize, postembryonic development is regulated by several genes that seem to act in a pathway with hormonal inputs (Berardini et al., 2001; Dudley and Poethig, 1991; Dudley and Poethig, 1993; Evans et al., 1994; Evans and Poethig, 1995; Lawson and Poethig, 1995; Poethig, 1989; Telfer and Poethig, 1998). In *Arabidopsis thaliana,* the genes *CO*, *LD* and *FCA* have

been found to be implicated in control of flowering time and encode a transcription factor, a homeobox protein and RNA binding protein, respectively (Lee et al., 1994; Macknight et al., 1997; Putterill et al., 1995). Moreover, the timing of shoot maturation is controlled by the HASTY locus in *Arabidopsis thaliana* (Telfer and Poethig, 1998). In rice, the *mori1* mutant is defective in the juvenile-adult phase change (Asai et al., 2002).

Notably, the best studied organism for temporal development is *C. elegans.* A heterochronic gene pathway has been identified that controls developmental timing in a variety of tissues (Ambros and Horvitz, 1984). Mutations in these genes result in a temporal transformation of cell fates that cause neoteny (retarded expression of juvenile tissues in a mature adult stage, e.g. *lin-4(lf)*) or acceleration (precocious expression of adult tissues in a juvenile stage, e.g. *lin-14(lf)*). The heterochronic gene pathway is central to this study and is discussed in detail below. First, I briefly introduce *C. elegans.*

2.3. The model organism *C. elegans*

C. elegans is a free-living soil nematode. It is a genetically tractable organism because of its rapid life cycle (3 days), small size (1.5 mm long adult), transparency of the body, large broods (300-350 per animal) and the ease of laboratory cultivation on agar plates or in liquid culture with *Escherichia coli* as a food source. Moreover, *C. elegans* has a defined cell lineage in which the time of birth, position and fate of each of the 959 somatic cells is known (Sulston and Horvitz, 1977). Development can be followed at the cellular level in living preparations by light microscopy. *C. elegans* is a hermaphrodite that breeds by self-fertilization, but can also be crossed with rarely occurring males (1 male/500 animals). Selfing and crossing is essential for genetic analysis, and facilitates the isolation and characterization of mutants (Riddle and Albert, 1997). Furthermore, *C. elegans* was the first metazoan whose genome was completely sequenced (*C. elegans* sequencing Consortium, 1998), with a haploid genome size of 9.7×10^7 nucleotides, about 1/30 of *Homo sapiens* (International Human Genome Sequencing Consortium; Lander et al., 2001). The *C. elegans* genome is organized on five autosomes and one X-chromosome. Sex-determination is achieved by the copy number of the X-chromosome; hermaphrodites have two copies (XX), males only one (X0) (Riddle, 1997).

2.4. *C. elegans* life cycle

C. elegans has six life stages: embryo, four larval stages and adult with optional reversible, developmental arrest at certain larval stages (Figure 2.1.).

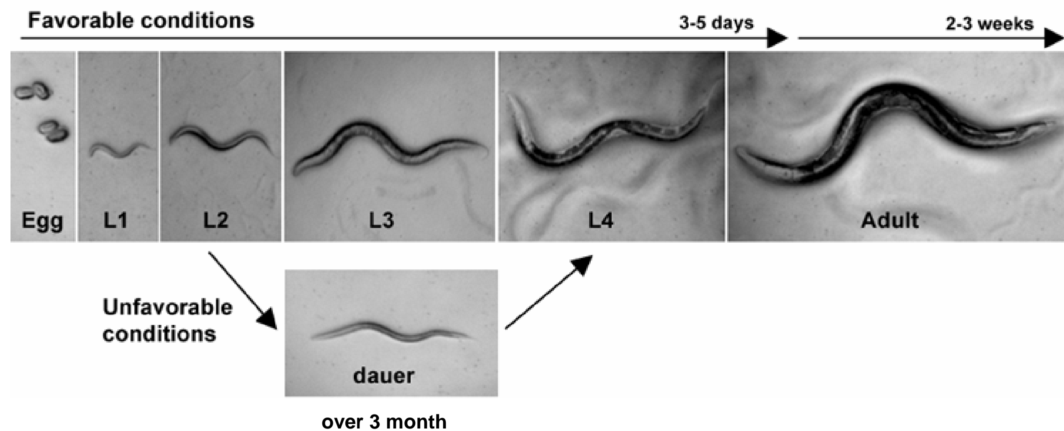

Figure 2.1. *C. elegans* life cycle. *C. elegans* develops through four larval stages (L1-L4) with an alternative L3 choice for dauer diapause (Figure modified from Rottiers, 2003).

2.4.1. Embryonic development

Embryogenesis is divided into two equal phases. The first phase entails cell proliferation and organogenesis, while the second phase entails elongation and morphogenesis. 558 cells are generated in a precise, nearly invariant temporal and spatial pattern until mid-embryo. Subsequently, body elongation, neuronal outgrowth and interconnection occur. Finally, the cuticle is synthesized and the animal hatches into the L1 larva.

2.4.2. Post-embryonic development

After hatch, *C. elegans* develops rapidly through four larval stages (L1 to L4) to adult in three to five days when conditions are favorable (abundant food, low temperature and low population density). 401 cells are added during post-embryonic development (Kimble and Hirsh, 1979; Sulston, 1983; Sulston et al., 1980; Sulston and Horvitz, 1977). These include structures of sexual reproduction (the gonad, vulva, sex muscles) as well as addition of neurons and epidermal cells. Once animals reach mid-L4, all tissues are essentially post-mitotic excepting the germline. Reproduction takes place over a 5 day period, and then animals typically live for another two to three weeks (Figure 2.1.). Each larval stage is marked by ecdysis, but no dramatic remodeling of the organism, such as metamorphosis, occurs.

In unfavorable conditions, such as low food, high temperature and high population density, *C. elegans* can enter an alternative third larval stage, called the dauer diapause. The dauer larva is morphologically, metabolically and behaviorally adapted for long-term survival and dispersal (Cassada and Russell, 1975). The sexually immature, non-feeding and non-aging dauer larvae can live more than three months (Wood, 1988) and resume development when conditions improve. Similarly, in absence of food, L1 larvae can arrest development, go into diapause for several days and resume development in favorable

conditions (Arasu et al., 1991). Importantly, entry into these alternative larval stages is dramatically influenced by environmental factors.

2.5. *C. elegans* stage-specific programs, an overview

Because the *C. elegans* cell lineage is so well defined, stage specific patterns of division, migration, fusion and death can be discerned that would otherwise be obscured in a less cell parsimonious animal. Generally speaking, *C. elegans* larval development is characterized by repetitions of programs that roughly run from one intermolt to the next. These stage-specific programs include stem cell division of epidermal seam cells, endoreplication of intestinal nuclei (Hedgecock and White, 1985; Sulston et al., 1980; Sulston and Horvitz, 1977) and cuticle formation (Singh, 1978), which appear coordinated with one another. Moreover, programs with no repeated pattern occur that are nevertheless coupled to larval stages, such as the migration of the Distal Tip Cells, which are leader cells that mediate outgrowth of the gonad (Figure 2.2.) (Antebi et al., 1998). Other stage-specific programs such as vulval development occur only once within a specific stage (Sulston and Horvitz, 1977). Finally, programs associated with entry and exit of developmental L1 or L3 arrest are coordinated throughout the animal. Below, I highlight some examples of stage-specific programs in certain tissues.

2.5.1. Seam cell programs

The epidermis of the worm, the hypodermis, consists of the hypodermal syncitium and seam cells, which collectively synthesize and secrete the new cuticle before the shedding of the old (Edwards and Wood, 1983; Singh, 1978). The syncitium covers most of the animal and the seam cells are positioned along the left and right lateral midlines (Figure 2.2.A).

During post-embryonic development, some of the most conspicuous temporal programming resides in the seam cells. Seams are blast cells that divide in a stem cell like fashion one or more times during each of the four larval stages (Figure 2.2.B) (Sulston and Horvitz, 1977). They divide according to developmental stage-specific programs, called larval S1, S2, S3, S4 and adult SA programs, that roughly take place during chronological stages L1, L2, L3, L4 larval and A, respectively (Figure 2.2.B) (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). Modifications of larval programs are found in the S2 program, in which seam cells first undergo an equational division followed by stem cell division. Interestingly, larval seam cell programs (S1 to S4) share many common features: seam cells stay in the cell division cycle, synthesize the larval cuticle, and animals continue to molt.

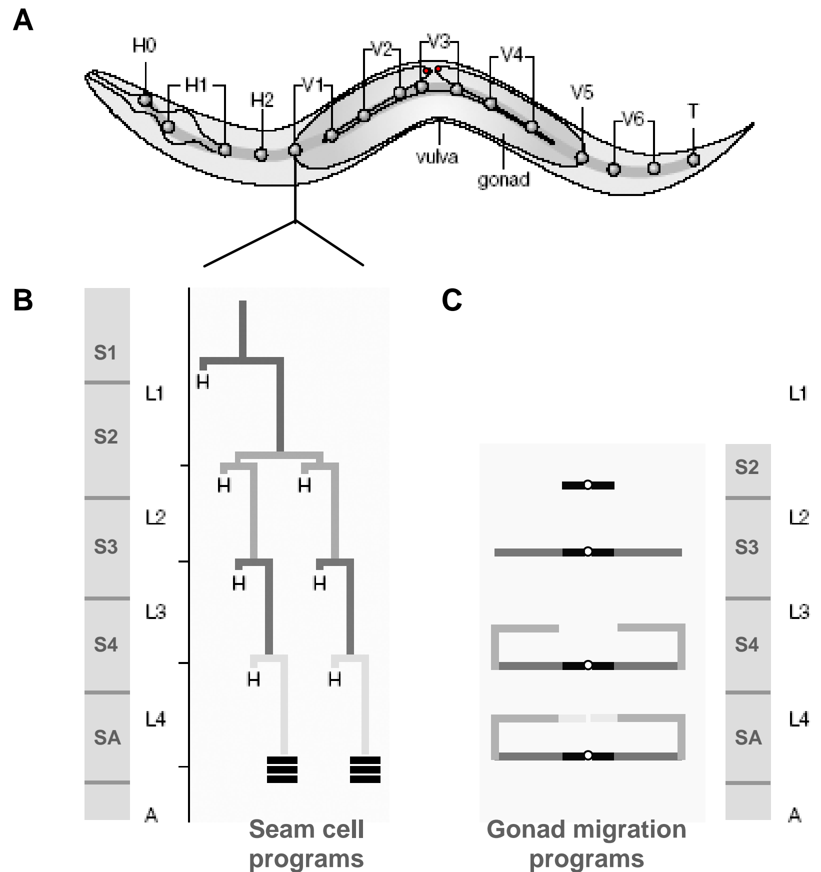

Figure 2.2. *C. elegans* stage-specific programs
Cartoon of an wild type adult worm depicting hypodermal seam cell nuclei and the gonad. Seam cells are arranged as longitudinal rows on either side of the animal. H0-H2, V1-V6 and T are names of blast cell derivatives. The gonad is the U shaped structure indicated in dark grey. The Distal Tip Cells (DTC) are located to the proximal end of the gonad and are shown as red circles. (B) and (C) Chronological stages (larval stage 1 (L1) to adult (A)) are indicated on the y-axis and are counted as hours elapsed; short vertical lines indicate timepoints of molts. Developmental stages are shown in the grey shaded vertical bar (S1-SA). (B) Seam cell programs. In wild type seam cells (V1-V4 and V6) divide once during S1, S3 and S4 stages. The anterior daughter fuses with the hypodermis (indicated by H) and the posterior daughter remains a stem cell. In the S2 program each seam cell undergoes an equational division followed by stem cell divisions. In the SA program seam cells exit the cell cycle, fuse and synthesize adult alae (indicated by triple horizontal lines). (C) Gonad migration programs. The stage-specific migration programs are represented in different grey shadings. DTC remain stationary until mid-L2. Then they express a short S2 program (black line) starting to migrate towards head and tail. In the S3 program they continue to migrate with unchanged direction (dark grey). In the S4 program DTC reflex and migrate centripetally (medium grey). In the SA program DTC complete remaining distance and halt when they have reached mid-body (light grey) (Figure modified from Rougvie, 2001).

However, during the larval to adult switch (SA), a number of dramatic changes take place (Ambros, 1989). First, seam cells exit the cell cycle, fuse to form a syncitium and produce the adult alae, cuticular ridges that extend the length of the lateral midline on each side of the animal (Figure 2.2.A) (Singh, 1978; White, 1988). Therefore, seam cells contribute to the distinct morphological and biochemical character of the adult cuticle (Cox and Hirsh, 1985; Cox et al., 1981; Liu et al., 1995; Singh, 1978; Sulston and Horvitz, 1977). Finally, after the L4 molt, animals exit the molt cycle permanently.

2.5.2. Gonad migration programs

During larval development, the gonad undergoes stereotyped stage-specific migratory programs, which appear coordinated with other stage-specific temporal events and have been termed S2, S3, S4 and SA, respectively (Antebi et al., 1998) in analogy to the seams. Like the seams, programs are proposed to start during intermolt and conclude in the next intermolt, and roughly correspond to chronological L1 to L4 and adult stages (Figure 2.2.C).

In newly hatched L1 larvae, the gonad consists of four cells: the germline precursor cells, Z2 and Z3, and the somatic gonad precursor cells Z1 and Z4. In the hermaphrodite, Z1 and Z4 cells give rise to the Distal Tip Cells (DTC) approximately at the L1 molt (Kimble and Hirsh, 1979). The DTC are located at the anterior and posterior tips of the gonad and are responsible for changing motility and direction of the gonad (Figure 2.2.A).

Although generated at the L1 molt, DTC do not move until mid-L2. By that time, a short S2 program causes the DTC to migrate into anterior and posterior directions (Figure 2.2.C). In the successive S3 program, migration direction is maintained until the mid-L3 stage. Upon S4 program initiation, DTC halt, reorient, move dorsally across the lateral hypodermis, reorient again and migrate centripetally, a process termed reflexion. Within the S4 program, DTC cover 80% of the last migration distance towards mid-body. In the SA program, running from mid-L4 to the L4 molt DTC complete the remaining distance and halt when they have reached mid-body (Figure 2.2.C) (Antebi et al., 1998).

2.6. Chronological age versus developmental age

2.6.1. Chronological age

Hatch and ecdysis (molts) are the conventional landmarks that separate larval stages and are used to clock chronological age in molting species. Ecdysis is perhaps best understood in *D. melanogaster*. The molt cycle is under endocrine control and regulated by the interplay of the ecdysteroid, 20-hydroxyecdysone, and juvenile hormone (Riddiford, 1993; Riddiford et al., 2003). Pulses of ecdysone dictate developmental transitions and regulate molting. The ecdysone signal triggers downstream gene expression through the ecdysone receptor and downstream cascades (Riddiford et al., 2000; Thummel, 1996).

Little is known about the molecular mechanism of ecdysis in *C. elegans*. Recent studies suggest that a cholesterol-derived hormone may act through two members of the nuclear hormone receptor family, *nhr-23* and *nhr-25*, both of which have been shown to play a role in molting. NHR-23 and NHR-25 are homologs of the fly DHR3 and FTZ-F1, respectively (Asahina et al., 2000; Gissendanner and Sluder, 2000; Koelle et al., 1992; Kostrouchova et al., 1998; Kostrouchova et al., 2001; White et al., 1997; Yamada et al., 2000). Interestingly, both are implicated in the ecdysone inducible cascades of the *Drosophila* molt cycle. However, neither ecdysone nor the ecdysone receptor have been found in *C. elegans.*

2.6.2. Developmental age

Although the molt cycle provides convenient temporal boundaries, the question arises if molting *per se* is the relevant developmental boundary. A detailed examination of the molting cycle (synthesis of the new cuticle and shedding of the old one) serves to illuminate this issue. Notably, the molt cycle must begin with the initiation of collagen synthesis, which precedes each molt by several hours (Johnstone and Barry, 1996). For example, the L2 cuticle is already synthesized during L1, and is not shed until the end of L2 (the L2 molt). Similarly, other programs such as seam cell divisions (described above) are initiated during the intermolt, when cells start the cell cycle. At the molt, seams are in the process of mitosis and conclude with fusion of the seam cell daughters to the hypodermal syncitium during the subsequent stage. Hence, developmental programs run from one intermolt to the next and developmentally relevant boundaries fall between chronological boundaries defined by ecdysis. Since chronological and developmental boundaries are not identical, they must be distinguished. Therefore, developmental programs are S1, S2, S3, S4 and SA and correlate to chronological timepoints of late embryo-midL1, midL2-midL3, midL3-midL4 and midL4 to adult, respectively (Ambros and Horvitz, 1984; Antebi et al., 1998). These units have been termed parastages (Antebi, unpublished). Importantly, synchrony of developmental ages of various tissues is achieved by a coordinate expression of stage specific programs throughout the animal (for gene expression, cell cycle commitment, cell migration) that commonly direct development.

Chronological and developmental age have been compared to spatial patterning in *Drosophila* (Ambros and Horvitz, 1984; Banerjee and Slack, 2002; Slack and Ruvkun, 1997). Regulation of spatial patterning involves the establishment of segmental boundaries and parasegmental identities. Segments define convenient boundaries similar to temporal boundaries defined by ecdysis (chronological age), whereas parasegments represent the actual positional developmental unit, just as parastages define a unit of temporal identity.

The molt cycle is one type of timing circuit working in *C. elegans*. The other counter is the heterochronic circuit, which confers temporal identity to the stages. The identities and phenotypes of components of these circuits suggest that they work somewhat independently. Namely, most identified heterochronic genes have little or no effect on the execution of the molt cycle *per se*, while the identified molting mutants do not obviously perturb temporal identity. Nevertheless, these counters have to be somehow coordinated. One example of the possible intersection between them comes from studies in *Drosophila,* in which the expression of genes implied in developmental age (miRNAs as *let-7* and *lin-4* homologs, see below) require the hormone ecdysone (Sempere et al., 2002; Sempere et al., 2003). Conceivably, a *C. elegans* ecdysis hormone may initiate expression of genes that control developmental timing (e.g. *lin-4, let-7).* Another example is *lin-29,* a gene that

specifies adult programs in *C. elegans.* Loss-of-function of *lin-29* results in supernumerary molts, whereas precocious expression results in early cessation of the molting cycle (Ambros and Horvitz, 1984; Bettinger et al., 1996), showing that this gene regulates exit from the molt cycle. These examples provide evidence that chronological and developmental stages are coupled and future research may reveal further links and mechanisms.

2.7. The heterochronic loci control developmental timing in *C. elegans*

The *C. elegans* heterochronic loci alter developmental timing by either causing precocious or delayed development. In precocious mutants, cells of a certain lineage express stage-specific programs one stage too early, and therefore omit a life stage. Conversely, in delayed mutants cells reiterate programs from earlier larval stages and therefore add a life stage (Ambros, 1989). In analogy, homeotic genes (e.g. Hox genes) in fly or other metazoans regulate the development in the dimension of space. When mutant, homeotic genes alter the spatial fates of cells and cause cells to adopt fates normally expressed at another position in the animal (Gellon and McGinnis, 1998). Hence, heterochronic mutations cause transformations of temporal cell fates whereas homeotic mutations cause transformation of positional cell fates.

All identified heterochronic genes (*lin-4, lin-14, daf-12, lin-28, lin-46, let-7, lin-41, lin-42, hbl-1, lin-29*) affect timing events in extragonadal tissues such as the seam cells. *daf-12* and *lin-29* affect the timing of both gonadal and extragonadal tissues (Antebi et al., 1998; Newman et al., 2000). In addition, *lin-41* affects germline age (Slack et al., 2000). Other events impaired by heterochronic loci include neuronal rewiring (Hallam and Jin, 1998), dauer larvae formation (Liu and Ambros, 1989), vulva formation (Bettinger et al., 1997), mail tail formation (Euling et al., 1999), cell cycle progression (Euling and Ambros, 1996), and stage-specific transcriptional activation and repression (Liu et al., 1995).

Although heterochronic genes may affect various tissues, most studies have focused on the seam cell circuit for the following reason. Stem cell divisions of seam cells are repeated at each larval developmental stage in a defined temporal order (S1 through S4). Moreover, the SA seam cell program (cell cycle exit, seam cell fusion and adult alae synthesis) provides visible markers of the adult fate. These stage-specific timing events can be followed through development, providing a convenient model to study heterochrony and to order genes into a regulatory hierarchy.

This regulatory pathway has been built on genetic epistasis and synergy experiments. Also taken into account is the stage specificity of the lineage defect and mRNA and/or protein expression profiles. The pathway has been divided into early and late timing circuits. The early timers guide temporal development from hatch to L3, and the late timing

circuit acts from L3 to adult (Ambros, 2000; Rougvie, 2001). In earlier studies, it was assumed that heterochronic loci affect postembryonic development only (Ambros, 2000), but recent findings suggest roles of certain heterochronic genes in embryonic development as well (Abrahante et al., 2003; Lin et al., 2003).

The next section gives an overview of the *C. elegans* heterochronic activities, their identities, phenotypes, expression and interactions. Since heterochronic studies in *C. elegans* have mainly focused on temporal seam cell development, this will be central to the next section as well. Finally, a summary of regulatory mechanisms at work in the heterochronic gene pathway will be given.

2.7.1. The early timing circuit

The early timing circuit specifies S1, S2 and S3 programs and consists of the heterochronic genes *lin-14, lin-28, lin-4, lin-46* and *daf-12*.

2.7.1.1. *lin-14* specifies S1 to S3 programs

lin-14(+) controls S1-S3 programs. *lin-14* null mutants omit S1 programs and instead express S2 programs precociously in L1. Subsequent programs (S3, S4, SA) are expressed in succession. Consequently, *lin-14(lf)* animals express the SA program precociously and differentiation of the hypodermis occurs one stage early relative to wild type (Table 2.1.).

The *lin-14* locus comprises two genetically separable activities, *lin-14a* and *lin-14b*. Temperature shift experiments have shown that *lin-14a* and *lin-14b* are required at different times during development. Consistent with its requirement during the S1 interval, *lin-14a* is

Table 2.1. Seam cell programs of heterochronic mutants

Genotype	L1	L2	L3	L4	A	AA
Wild type N2	S1	S2	S3	S4	SA	na
lin-4	S1	S1	S1	S1/S2	na	na
lin-14	S2	S3	S4	SA	na	na
lin-28	S1	S3	S4/SA	SA	na	na
daf-12	S1	S2	S2	S3/S4	S4/SA	SA
let-7	S1	S2	S3	S4	S4	SA
lin-42	S1	S2	S3	SA	na	na
lin-41	S1	S2	S3	SA	na	na
hbl1	S1	S2	S3	SA	SA	na
lin-29	S1	S2	S3	S4	S4	S4

L1 to L4: larval stages; A: adult stage; AA: additional adult stage. Seam cell programs are classified as described in 2.5.1. na: not applicable.

needed by hatch to prevent precocious expression of S2 program in the L1 stage. *lin-14b,* however, is needed during late L1 to prevent precocious expression of the S3 program expression in early L2. *lin-14b(lf)* mutants omit the S2 program in L2 resulting to precocious S3 and successive S4 and SA program expression (Ambros and Horvitz, 1987).

lin-14 encodes three different LIN-14 protein isoforms, LIN-14A and LIN-14B1/B2, whereby A and B transcripts are generated from different promoters (Wightman et al., 1991; Wightman et al., 1993). Even though these isoforms act at distinct times of development to specify S1 and S2 programs, a difference in temporal expression between LIN-14A and LIN-14B1/B2 is not responsible for program specification: all isoforms are expressed from mid-embryo to late L1 peaking from late embryo to early L1 stage and are decreased entirely by L2. An explanation may be that LIN-14 isoforms may contribute to a critical overall level of LIN-14, but might not provide distinct functions. For example, upregulation of LIN-14A in *lin-14b* mutants can substitute for LIN-14B function (Reinhart and Ruvkun, 2001; Ruvkun and Giusto, 1989). Consistent with this, the carboxy-terminal half of the protein, which is common to all isoforms, is sufficient for all *in vivo* functions of LIN-14 (Hong et al., 2000). This leads to a model in which the temporal gradient of LIN-14 specifies the sequence of developmental programs. High levels of LIN-14 at early L1 prevent S2, thereby specifying S1 programs; low levels at mid/late L1 prevent S3, thereby specifying S2; absence of LIN-14 at early L2 specifies S3 programs. *lin-14(lf)* animals develop through only three larval stages and cease the molting cycle after the L3 stage. *lin-14* gain-of-function mutants, in which LIN-14 expression maintains inappropriately high in late development, reiterate S1 programs at abnormally late stages leading to retarded phenotypes. Seam cells repeat larval programs, adult alae synthesis never occurs and animals undergo supernumerary molts (Ambros and Horvitz, 1984; Arasu et al., 1991; Ruvkun and Giusto, 1989). Therefore, a key event in the progression of early larval development is the gradual downregulation of LIN-14 isoforms. *lin-14* downregulation occurs on the post-transcriptional level through its 3'UTR. *lin-14* mRNA levels remain constant throughout post-embryonic development, and deletion of the 3'UTR results in the gain-of-function phenotype (Lee et al., 1993; Seggerson et al., 2002; Wightman et al., 1993).

lin-14 encodes a novel nuclear protein that may function as a transcription factor or a transcriptional regulator (Table 2.2.) (Ruvkun et al., 1991). This assumption is supported by the fact that LIN-14 was found to be associated with chromatin (Hong et al., 2000; Ruvkun et al., 1989). LIN-14 is exclusively expressed in the nuclei of extragonadal tissues such as the epidermis, muscle, nervous system and intestine (Ruvkun and Giusto, 1989).

lin-14 also affects temporal fates of early larval programs in various extragonadal cellular processes including intestinal cell lineages, cell cycle progression, vulva formation, dauer entry, and synaptic remodeling (Ambros and Horvitz, 1984; Euling and Ambros, 1996;

Hallam and Jin, 1998; Liu and Ambros, 1989). However, gonadal development appears to be normal in *lin-14* mutants (Ambros and Horvitz, 1984; Liu and Ambros, 1989).

In summary, *lin-14* is a key component of the early timing circuit. It is a global temporal control gene affecting the program schedule of various extragonadal cell lineages. Moreover, it is necessary for the timing of developmental transitions as dauer entry. Gradual temporal downregulation of LIN-14 is responsible for the sequential progression from S1 to S3 developmental stages and is mediated post-transcriptional.

2.7.1.2. *lin-28* regulates the S2/S3 transition

lin-28(+) specifies S2 to S3 temporal fates. *lin-28(lf)* results in precocious phenotypes. Null mutants omit S2 programs and resemble *lin-14b(lf)* mutants. A program schedule of S1, S3, S4, SA leads to precocious SA program expression, including adult alae synthesis and exit from the molt cycle, one stage early at the L3 molt (Table 2.1.) (Ambros and Horvitz, 1984). *lin-28* mutants exhibit precocious development in other tissues such as vulva and male rays (Ambros and Horvitz, 1984; Euling and Ambros, 1996). In addition, sex determination is impaired (Ambros and Horvitz, 1984). By contrast, gonadal development appears normal in *lin-28* mutants (Ambros and Horvitz, 1984; Liu and Ambros, 1989). *lin-28(lf)* is suppressed by *lin-46(lf)*, another component of the early timer suggesting that *lin-28* specifies S2 programs by delaying the activity of *lin-46(lf)* (Ambros and Moss, 1994; Moss et al., 1997).

The *lin-28* gene encodes three different isoforms that result from alternative splicing and post-translational modification (Seggerson et al., 2002). The LIN-28 protein is expressed in the cytoplasm of extragonadal tissues as the hypodermis, muscle and neurons. Expression peaks in late embryo and L1, decreases in L2 and disappears in L3 and L4 stages (Moss et al., 1997). When LIN-28 protein levels remain inappropriately high at late stages, as in *lin-28* gain-of-function mutants, reiteration of S2 programs at L3 and later larval stages occurs (Moss et al., 1997) resulting in a retarded phenotype where hypodermal terminal differentiation does not occur on schedule (Ambros and Horvitz, 1984; Moss et al., 1997). *lin-28* is regulated post-transcriptionally, since *lin-28* mRNA levels remain high throughout development (Moss et al., 1997; Seggerson et al., 2002) while LIN-28 protein levels decrease.

lin-28 encodes a protein with two potential RNA-binding motifs: a cold-shock domain (CDS) and a pair of retroviral-type CCHC zinc fingers (Moss et al., 1997). These motives suggest a role in RNA binding or post-transcriptional regulation of target genes (Hong et al., 2000; Moss et al., 1997). Interestingly, LIN-28 homologues are found in diverse animals (Table 2.2.) (Moss and Tang, 2003).

Table 2.2. Conservation of *C. elegans* heterochronic gene products

***C. elegans* gene product**	***Drosophila melanogaster***		***Homo sapiens***	
	Name/Accession number	Identities	Name/Accession number	Identities
lin-4	*dme-mir-125*/MI0000417[1]	86%/21bp	*hsa-mir-125b*/MI0000446[1]	86%/21bp
LIN-14	none	none	CAA44373	25%/101aa
LIN-28	CG17334-PA	39%/143	NP 078950	36%/146aa
LIN-46	CG2945-PA	25%/389	Q9NQX3	30%/148aa
DAF-12	DHR96	64%/85aa (DBD) 25%/260aa (LBD)	orphan nuclear receptor PXR.2/ AF364606.1	44%/129aa
let-7	*let-7a*/AE003659	100%/21bp	*let-7*/AL049853	100%/22aa
LIN-42	Period/ CG2647-PA	33%/109aa	Period circadian protein 3 (HPER3)/AAH26102	25%/210aa
LIN-41	CG1624-PA	41%/323aa	Vertebrate Tripartite Motif protein/ XP067369	36%/610aa
HBL-1	CG9786-PA	63%/119aa	FLJ38144/XP 371132	31%/135aa
LIN-29	CG32466-PA	75%/180aa	AAL55863	60%/145aa

C. elegans heterochronic gene products have been blasted to *D. melanogaster* and *H. sapiens* genomes using the NCBI database (http://www3.ncbi.nlm.nih.gov). Accession numbers were obtained from the NCBI database unless noted otherwise. [1]Accession number obtained from http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml. BLAST homologies are given in percentages of identical amino acids over a given range of amino acids. DBD, DNA Binding Domain; LBD, Ligand Binding Domain.

2.7.1.3. *lin-4* translationally represses *lin-14* and *lin-28*

lin-4 regulates the transition from S1 to S2 and later fates. The *lin-4(lf)* mutant is delayed and null mutants indefinitely repeat S1 programs at late stages. Animals never produce adult alae and undergo supernumerary molts (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987; Chalfie et al., 1981). *lin-4* exhibits developmental timing defects that mostly resemble *lin-14(gf)* phenotypes. For example *lin-4(lf)* mutants, like *lin-14(gf)* mutants, prevent vulva differentiation (Euling and Ambros, 1996) and alter the stage of dauer entry (Liu and Ambros, 1989). In contrast, gonad development is normal in *lin-4* mutants (Ambros and Horvitz, 1984; Liu and Ambros, 1989).

Interestingly, *lin-4* is the founding member of a new class of small non-coding regulatory RNAs, termed micro RNAs (miRNAs). *lin-4* encodes two small RNAs, 22 nt and 61 nt in size (Feinbaum and Ambros, 1999; Lee et al., 1993). The mature 22 nt *lin-4* RNA, derived from the longer precursor form, binds to partly complementary anti-sense sites in

3'UTRs of target mRNAs and subsequently represses their translation (Lee et al., 1993; Wightman et al., 1993). Translation repression occurs at a post-initialization step of translation (Olsen and Ambros, 1999; Seggerson et al., 2002). Recently, *lin-4* homologs have been found in humans and fly (Lagos-Quintana et al., 2002; Sempere et al., 2004) (Table 2.2.).

lin-4 is weakly expressed at mid-L1 and increases towards the L2 stage, where its levels are highest, and continues into adult. Expression is initiated by a food signal. Animals that hatch in the absence of food and enter L1 diapause, fail to express *lin-4.* Development and *lin-4* expression commence upon return of food (Arasu et al., 1991).

lin-4 complementary elements (LCEs) are found in the 3'UTRs of *lin-14* (seven) and *lin-28* (one) suggesting that *lin-4* negatively regulates *lin-14* and *lin-28* (Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). Indeed, epistasis experiments place *lin-14(lf)* and *lin-28(lf)* downstream of *lin-4(lf)* (Ambros, 1989; Ambros and Horvitz, 1987; Moss et al., 1997). Moreover, the time of *lin-4* accumulation corresponds to the time where LIN-14 and LIN-28 protein levels are downregulated. This drop in protein levels is dependent on *lin-4.* Mutations in the 3'UTRs of *lin-14* and *lin-28* resulting in disruption of the LCEs lead to maintenance of high LIN-14 or LIN-28 protein levels and causes retarded gain-of-function phenotypes (Ambros and Horvitz, 1987; Arasu et al., 1991; Lee et al., 1993; Moss et al., 1997; Wightman et al., 1991; Wightman et al., 1993). Conceivably, the differences in the number of LCEs in *lin-28* to *lin-14* 3'UTR may be responsible for sharper downregulation of *lin-14* (Feinbaum and Ambros, 1999).

Since *lin-4* downregulates the almost ubiquitously expressed LIN-14, *lin-4* expression may be hormonally regulated (Slack and Ruvkun, 1997). Interestingly, upregulation of *mir-125,* a putative *lin-4* homologue in fly, requires ecdysone (Sempere et al., 2003).

In summary, *lin-4* represents a new class of small, non-coding, regulatory RNAs. *lin-4* represses *lin-14* and *lin-28* by binding to their 3'UTRs by a complementary antisense mechanism to inhibit their translation. Accumulation of *lin-4* by the late-L1 stage and subsequent downregulation of LIN-14 and LIN-28 protein levels allows the advance from S1 to S2 and S3 programs and therefore progression of early larval development.

2.7.1.4. *lin-46*

lin-46(+) specifies S3 programs. *lin-46* null mutants have a delayed phenotype in which seam cell impenetrantly repeat S2 programs in L3 instead of the appropriate S3 program. *lin-46(lf)* suppresses the precocious *lin-28(lf)* phenotype (Ambros and Moss, 1994) and the precocious *lin-14(lf)* phenotype (Moss, personal communication). Furthermore, it enhances the *daf-12(lf)* L3 seam cell phenotype (Fielenbach and Antebi, unpublished) suggesting that *lin-46* together with *daf-12* specifies S3 seam cell programs. *lin-46* encodes a protein related

to the moeA family and the E-domain of vertebrate gephrin (Ambros, personal communication).

2.7.1.5. The nuclear hormone receptor *daf-12* acts at the convergence of dauer and heterochronic pathways

daf-12 is an important gene within the heterochronic pathway for several reasons. First, it plays an instructive role in L3 development by regulating the choice between reproductive (S3 programs) and dauer development (S3d programs). In particular, environmental signals from upstream dauer pathways (insulin, TGFβ and cGMP) converge on *daf-12* to control the switch between reproductive and dauer development. Hence, *daf-12* acts at the convergence of dauer and heterochronic pathways. Second, this choice is controlled by a hormonal mechanism. Finally, *daf-12* acts in the progression from S2 to S3 programs by simultaneous activation of S3 reproductive and inhibition of S2 programs. Importantly, *daf-12* is unusual within the heterochronic pathway because it conspicuously affects developmental age of both, extragonadal and gonadal tissues, suggesting it may coordinate programs throughout the body.

Some *daf-12* mutants have delayed heterochronic defects starting in L2-L3 stages. For example, allele *rh61* repeats S2 seam programs inappropriately at L3 (Antebi et al., 1998). Mutants such as *rh61* behave as a recessive gain-of-function, and actually have more severe phenotypes than null mutants. For seam phenotypes, *daf-12* mutants resemble *lin-14b(gf)* or *lin-28(gf)*, although these gain-of-function mutants repeat S2 program indefinitely (Ambros and Horvitz, 1987; Moss et al., 1997). The single S2 reiteration of *daf-12* mutants results in a program schedule of S1, S2, S2, S3, S4, SA, respectively (Table 2.1.). As a consequence, cells often fail to express the SA program on schedule, and on occasion adult alae formation occurs at an additional fifth molt, one stage later than wild type. Developmental timing delays in extragonadal tissues other than seams are also observed in *daf-12* mutants, including sex myoblast and intestinal cell divisions.

daf-12 mutants also affect gonadal developmental age. In this case, strong delayed mutants are thought to cause the repetition of S2/S3 programs at L3/L4, as in the seam (above). Notably, Distal Tip Cell behavior is normal until mid-L2, expressing the S2 program of longitudinal migration. At L3, however, DTC fail to advance to S3/S4 programs and reiterate the S2/S3 programs instead. Specifically, gonad arms fail to undergo the L3 specific reflexion and instead migrate with unchanged direction into head and tail of the animal.

Genetic dissection of *daf-12* reveals great complexity. *daf-12* alleles fall into six classes based on separable heterochronic and dauer formation phenotypes (Antebi et al., 1998). The phenotypic complexity reveals two genetically separable activities, *daf-12a* and *daf-12b*. *daf-12a* promotes S3 programs of reproductive development (and short life span) in

favorable environment. In contrast, *daf-12b* promotes S3d programs of dauer development (and a long life-span) in unfavorable conditions (Antebi et al., 1998; Antebi et al., 2000). Genetic evidence reveals that *daf-12b* is essential for dauer diapause, while *daf-12a* activity is only partly required in the advance from S2 to S3 programs. Hence, in addition to *daf-12a,* a redundant function is involved in the promotion of reproductive growth under replete conditions. Temperature-shift experiments indicate that *daf-12a* is mainly required at the mid-L2 stage to specify S3 programs for seam cell development. Additionally, *daf-12* is needed at later stages than mid-L3, a period at which S4 programs are initiated. Consistent with these data is the *daf-12* expression analysis.

DAF-12 is predominately expressed in the nucleus of most cell types. Notably, it is expressed in tissues that exhibit heterochronic and dauer phenotypes like epidermis, vulva, somatic gonad, intestine, pharynx and sex myoblasts. Additionally, DAF-12 is expressed in the nervous system and body wall muscle. Expression appears in late embryo stages and is evident into the adult, but decreases in dauer larvae. In reproductive growing larvae, expression peaks at mid-L2 (Antebi et al., 2000). Consistent with temperature-shift experiments (see above), DAF-12 accumulates in the seam cells at mid-L2, which is the time window of S3 program initiation. Expression in seams diminishes by late L3. The Distal Tip Cells, that lead gonad migration, express *daf-12* most strongly at L2, but expression maintains until early L4, consistent with a role of *daf-12* in later development.

Epistasis experiments place *daf-12* between *lin-14* and *lin-28. daf-12* acts downstream of *lin-14* and upstream of *lin-28* (Antebi et al., 1998). Specifically, *lin-14b* inhibits both, *daf-12a* and *daf-12b* in the promotion of S3/S3d and later programs. Moreover, *daf-12* acts downstream of *lin-42* since it suppresses the precocious phenotypes of *lin-42.* To advance to S3 programs *daf-12* feedback inhibits *lin-28* (Antebi et al., 1998). Moreover, *lin-4* is epistatic to *daf-12. lin-4* null mutants cause high levels of LIN-14 protein at abnormally late stages that in turn inhibit *daf-12* indefinitely. At the same time, *lin-4* enhances the *daf-12* phenotype in a *lin-14* mutant background. These and other data suggest that *daf-12* acts redundantly with *lin-4*, but through a different mechanism, to inhibit *lin-28* (Antebi et al., 1998; Seggerson et al., 2002).

daf-12 encodes a Nuclear Hormone Receptor (NHR). NHRs are transcription factors that are regulated by lipophilic hormones. *daf-12* contains all the hallmarks of NHRs, including an amino-terminal DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD) (Antebi et al., 2000). Vertebrate homologues include vitamin D, LXR pregnane, and androstane nuclear receptors (Table 2.2.) (Antebi et al., 2000). Its ortholog in *Drosophila*, DHR96 is of unknown function. Finally, two paralogs, *nhr-8* and *nhr-48* are found in the *C. elegans* genome. DAF-12, NHR-8, NHR-48 and DHR96 comprise a unique branch

of nuclear hormone receptor family, called the ESCKA family, because they share an identical DNA recognition helix containing these amino acids.

The identification of DAF-12 as a nuclear hormone receptor suggests that lipophilic hormones could regulate heterochronic and dauer pathways. Indeed, *daf-12* was found to be the recipient of hormonal control. Two genes, *daf-9*, a cytochrome P450 and *daf-36*, a terminal dioxygenase, are implicated in hormone biosynthesis of a *daf-12* ligand (Gerisch et al., 2001; Rottiers, 2003). Genetic evidence suggests that the *daf-9* produced hormone regulates *daf-12* action (Gerisch and Antebi, 2004; Gerisch et al., 2001; Jia et al., 2002). In the presence of hormone, *daf-12* promotes reproductive growth selecting S3 programs, whereas in the absence of hormone, *daf-12* together with its co-repressor DIN-1 commits to dauer development selecting S3d programs. DIN-1 is a homolog of the SHARP corepressor, a coregulator of nuclear receptors and other transcription factors (Ludewig, 2004). *din-1* suppresses all the phenotypes resulting from unliganded *daf-12*, including heterochronic defects. This regulatory mechanism does not exclude the possibility that a second hormone regulates *daf-12* in the activation of S3d programs.

The *daf-12* locus encodes 3 protein isoforms, DAF-12A1 (A1), DAF-12A3 (A3) and DAF-12B (B), respectively (Antebi et al., 2000). While both DAF-12A isoforms contain DBD and LBD, the B isoform contains the LBD only. Mutation analysis suggests that the A isoforms are essential for *daf-12* function. Furthermore, *daf-12a* and *daf-12b* activities do not correlate to these isoforms (Antebi et al., 2000). Instead, it is proposed that a and b activities arise from liganded and unliganded receptor. All 3 *daf-12* isoforms contain the same 1.4 kb long 3'UTR. Interestingly, *let-7* complementary sites are present in this 3'UTR (Reinhart et al., 2000), but DAF-12 translational regulation remains to be determined.

In summary, *daf-12* mediates the choice between two alternative programs of reproductive development (S3) and dauer diapause (S3d) in the L3 stage and regulates the progression from the S2 to the S3 stage. Importantly, *daf-12* as nuclear hormone receptor coordinates global developmental decisions through a hormonal mechanism. Hormonal control of developmental timing is ancient. Alterations in the thyroid hormone or ecdysone hormone level cause heterochrony in amphibia and insects (Bender et al., 1997; Frieden, 1981). Mutations in the Ecdysone receptor and its partner ultraspiracle, lead to heterochrony (Hodin and Riddiford, 1998; Hodin and Riddiford, 2000).

2.7.2. The late timing circuit

The late timing circuit regulates the progression from L3 to adult development by expressing S3, S4 and SA programs in succession. The late timer consists of the heterochronic genes *lin-41, lin-42, hbl-1, let-7* and *lin-29.*

2.7.2.1. *lin-42,* a circadian rhythm component acts at several stages in development

lin-42(+) regulates programs at several stages, but most conspicuously prevents early expression of the SA program. *lin-42(lf)* mutants have precocious phenotypes. In null mutants seam cell development appears essentially normal until the L3 stage: S1, S2 and S3 seam cell programs are expressed in sequence. However, at the L3 molt *lin-42* mutant animals express the SA program precociously deleting the S4 program (Table 2.1.). Consequently, terminal differentiation of the hypodermis, including seam cell fusion and adult alae synthesis, occurs one stage early compared to wild type (Abrahante et al., 1998; Jeon et al., 1999). Moreover, *lin-42* animals have difficulties in executing the final molt being unable to shed the old cuticle (Abrahante et al., 1998).

Interestingly, *lin-42* encodes a protein most similar to members of the PERIOD (PER) family of circadian rhythm proteins (Table 2.2.) (Jeon et al., 1999). PER proteins contain a PAS domain that mediates protein-protein interactions with other PAS or non-PAS containing proteins. In fly and humans, PER proteins control the circadian rhythm defining day and night cycles (King and Takahashi, 2000; Young, 2000). Oscillation of mRNA and protein levels of PER occurs with the circadian cycle. In fly, PER also influences developmental time: *per*^{l} or *per*^{s} mutations result in long or short circadian periods and therefore animal development is lengthened (delayed) or shortened (precocious) compared to wild type, respectively (Kyriacou et al., 1990). Moreover, in fly a coupling of circadian rhythm and ecdysone release has been observed. Ecdysone is known to trigger developmental transitions in fly (Riddiford, 1993; Siegmund and Korge, 2001). Interestingly, *lin-42* mRNA levels oscillate with a 6 hour period synchronized with the molting cycles and peak during intermolt periods (Jeon et al., 1999).

LIN-42 is expressed from late embryo to adult. Expression is seen in the cytoplasm as well as in the nucleus of lateral hypodermal cells, in the hyp7 syncitium and head and tail hypodermal cells. The expression is enhanced in seam cell cytoplasm during all molts. In addition, LIN-42 is expressed in vulval cells, pharyngeal cells, a few head neurons, and the intestine (Jeon et al., 1999).

In the heterochronic pathway, *lin-42* acts downstream of *lin-29* either promoting S4 seam cells fates or inhibiting SA programs until the L4 molt. Surprisingly, it also enhances the precocious S3 seam cell defect of weak *lin-14* mutants, and thus plays a role in early larval development. Moreover, *lin-4;lin-42* mutants mutually suppress their seam cell timing defects. Double mutants are wild type in seam cell development (Abrahante et al., 1998). Consistent with this data is that *lin-42* is not regulated by *lin-4* as the *lin-42* 3'UTR does not contain *lin-4* binding sites (Jeon et al., 1999).

In summary, two lines of evidence suggest that LIN-42 may serve as a link between two different biological time-keeping circuits, circadian rhythm and the heterochronic pathway.

First, oscillation of the mRNA of both *per* and *lin-42* occurs: PER mRNA fluctuates with the circadian cycle; *lin-42* mRNA with the molting cycle. Second, LIN-42 activity is needed repeatedly at several stages during post-embryonic development.

2.7.2.2. *lin-41* specifies S4 fates by inhibiting LIN-29 and is regulated by *let-7*

lin-41(+) specifies S4 and prevents SA programs. In *lin-41(lf)* mutants, seam cell development is normal up to the L3 stage. Thereafter, seams omit the S4 program and instead express the SA program precociously. Consequently, they terminally differentiate and form the adult alae one stage early (Table 2.1.). Conversely, overexpression of LIN-41 leads to retarded phenotypes where S4 programs are reiterated at the adult stage. At low frequency, *lin-41(lf)* animals execute the SA program at the L2 molt, deleting S3 and S4 programs altogether (Lin et al., 2003). Moreover, *lin-41* has trouble executing the last molt; oftentimes animals are unable to shed the L4 cuticle. By inference, *lin-41(+)* is required to repress SA programs in late larval stages. In *lin-41(lf)* mutants germline age is also impaired. A severe defect in oocyte production accounts for the sterility phenotype found in *lin-41(lf)* animals.

lin-41 encodes a RBCC protein. It contains a Ring finger, a B box, a Coiled Coil domain and a NHL domain, that are all predicted to mediate protein-protein interactions (Slack et al., 2000). Additionally, the ring finger is thought to be involved in protein-nucleic acid interactions. Orthologs of LIN-41 are found from *Drosophila* to human with highest conversation in the NHL domain (Table 2.2.). As a member of the RBCC family LIN-41 could function as a RNA binding protein as well as a transcriptional regulator or even in protein degradation as a subunit of an E3 ubiquitin ligase. *lin-41* encodes two very similar isoforms.

LIN-41 is expressed from late embryo until adult predominantly in the cytoplasm (Slack et al., 2000). Expression is seen in hypodermal seam cells, Distal Tip Cells, spermatheca, uterine cells, somatic gonad, neurons, body wall and pharyngeal muscle cells. In most cell types expression is seen throughout development. However, in certain cells and tissues LIN-41 is temporally regulated. For example, expression in seam cells is decreased during the L4 stage, while expression in the spermatheca, Distal Tip Cells, and certain uterine cells is limited to late larval and adult stages.

The *lin-41* mRNA contains two complementary sites for the miRNA *let-7* and one for *lin-4*. LIN-41 downregulation in the seam cells coincides with the time of *let-7* upregulation. Indeed, *let-7* was found to negatively regulate *lin-41* post-transcriptionally (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004). First, *lin-41(lf)* was found to suppress the *let-7(lf)* retarded and lethal phenotype, implying that *lin-41* acts downstream of *let-7*. Moreover, overexpression of LIN-41 leads to retarded phenotypes similar to those of *let-7(lf)*. Additionally, the *lin-41* 3´UTR was sufficient for temporal downregulation of a reporter gene

expression and deletion of *let-7* complementary sites abolished this downregulation. Consistent with these data, *let-7* temporal expression is unaffected in *lin-41(lf)* mutants (Johnson et al., 2003). The miRNA *lin-4* also acts upstream of *lin-41* (Abrahante et al., 2003). However, *lin-4* was not involved in *lin-41* regulation (Slack et al., 2000).

The transcription factor *lin-29* is the latest acting gene in the heterochronic pathway. It promotes SA programs at the late L4 stage. Therefore, its expression is limited to L4 and later stages. Epistasis analysis placed *lin-41* upstream of *lin-29* as *lin-29(lf)* retarded phenotypes were epistatic to the *lin-41(lf)* precocious phenotypes. Furthermore, *lin-41(lf)* mutants expressed LIN-29 precociously at the L3 stage (Slack et al., 2000). By inference, *lin-41(+)* inhibits *lin-29(+)*, restricting its expression to the L4 stage. Therefore, LIN-41 is thought to interfere with *lin-29* translation through binding to its mRNA at earlier larval stages (Slack et al., 2000).

In summary, the precocious mutant *lin-41* acts between *let-7* and *lin-29* in the heterochronic seam cell circuit. *let-7* upregulation leads to decline in LIN-41 protein levels during the L4 stage. LIN-29 expression is thought to be controlled by LIN-41 posttranscriptionally. *let-7* mediated decrease in LIN-41 relieves LIN-29 and leads to adult fate specification. *lin-41(lf)* causes precocious adult seam cell fates whereas overexpression results in a delay of adult fates and therefore reiteration of larval fates.

2.7.2.3. *hbl-1* acts at multiple times during *C. elegans* development and is regulated by microRNAs

hbl-1 also specifies S4 and prevents SA programs. The mutant has precocious phenotypes. Development is nearly normal until the L3 stage. However, at the L3 molt, seams express the SA program, including seam cell fusion and adult alae formation, one stage earlier than wild type (Table 2.1.) (Abrahante et al., 2003; Lin et al., 2003). Occasionally, the SA program occurs two stages early at the L2 molt (Abrahante et al., 2003). By inference, *hbl-1(+)* is required to repress adult fates in late larval stages.

hbl-1 encodes the *C. elegans hunchback* homolog (Table 2.2.). The transcription factor HBL-1 contains nine C2-H2 type zinc fingers, six of which are highly conserved among Hunchback orthologs (Abrahante et al., 2003; Lin et al., 2003). Interestingly, fly hunchback (*hb)* plays a role in temporal patterning of the fly nervous system (Isshiki et al., 2001) and spatial patterning of the embryo (Lehmann and Nusslein-Volhard, 1987). Therefore, sequence and function of *hunchback* is conserved across phyla. Moreover, fly *hb* appears to be translationally controlled through its 3'UTR by the protein complex of Pumilio, Nanos and Brat (Sonoda and Wharton, 1999; Sonoda and Wharton, 2001).

Overall HBL-1 expression is highest in embryo, decreases in subsequent stages (Fay et al., 1999) and is temporally regulated (Abrahante et al., 2003; Lin et al., 2003).

Paradoxically, HBL-1 expression in seam cells diminishes after mid-L1. In the hypodermis (hyp7) high HBL-1 expression in young L1 declines until L3. In ventral cord and anterior nerve ring neurons expression is high in young L1, evident until mid-L3 and decreases towards the adult stage (Abrahante et al., 2003). In the Hermaphrodite-Specific Neuron (HSN) expression is high during L1 and L2, but ceases in L3 (Abrahante et al., 2003).

The *hbl-1* 3'UTR contains as many as eight complementary sites for *let-7* and the *let-7* family (*mir-48* and *mir-84*), six complementary sites for *lin-4*, and two for *mir-69* indicating that *hbl-1* is translationally controlled by miRNAs (Abrahante et al., 2003; Lin et al., 2003). Indeed, epistasis experiments place *hbl-1* downstream of *let-7. hbl-1* strongly suppresses *let-7* lethality and partially suppresses the retarded phenotype in the seam cells suggesting that *let-7* may inhibit *hbl-1* translationally (Abrahante et al., 2003; Lin et al., 2003). Additionally, overexpression of HBL-1 partially results in retarded and vulval bursting phenotypes resembling *let-7(lf)* mutant phenotypes (Abrahante et al., 2003). Remarkably, *let-7* dependent downregulation of HBL-1 expression occurs solely in the nervous system (Abrahante et al., 2003; Lin et al., 2003). Temporal downregulation in other tissues as seam cells and hypodermis are controlled by other factors. While decline in hypodermal expression is mediated by the *hbl-1* 3'UTR, downregulation in seam cells is independent of the *hbl-1* 3'UTR implying underlying transcriptional regulation (Abrahante et al., 2003; Lin et al., 2003). By inference, *let-7* regulates temporal HBL-1 expression in the nervous system, but not in seams and the hypodermis (Abrahante et al., 2003).

Although epistasis experiments position *lin-4* downstream of *hbl-1* as *lin-4* suppresses the *hbl-1* precocious seam cell phenotype, *lin-4* acts upstream of *hbl-1* by other criteria (Abrahante et al., 2003). Consistent with these data is that *lin-4* is required to downregulate HBL-1 expression in the ventral cord neurons in addition to *let-7* (Abrahante et al., 2003; Lin et al., 2003).

hbl-1 acts upstream of the transcription factor *lin-29. lin-29(lf)* suppresses the *hbl-1(lf)* precocious seam cell phenotype (Abrahante et al., 2003; Lin et al., 2003). Additionally, *hbl-1(lf)* animals express LIN-29 precociously at the L3 stage (Abrahante et al., 2003). By inference, *hbl-1(+)* inhibits *lin-29(+)*, restricting LIN-29 accumulation to the L4 stage.

hbl-1 mutants have additional defects in seam cells and other tissues. Surprisingly, *hbl-1(lf)* animals exhibit an adult seam cell phenotype displaying gaps in the adult alae. Consequently, *hbl-1(+)* may promote SA programs in the L4 stage or maintain them in adult (Lin et al., 2003). In addition, seam cell nuclei fail to exit the division cycle and reiterate their division during the L4 stage (Abrahante et al., 2003; Lin et al., 2003). Furthermore, *hbl-1* is required for S2 program specification in seam cells. In *hbl-1(RNAi)* animals some seam cells omit the S2 program resulting in a decrease of seam cell number (Abrahante et al., 2003).

Moreover, *hbl-1(lf)* mutants cause precocious divisions of the Vulva Precursor Cells (VPC) leading to a protruding vulva (Pvul) phenotype and an egg-laying defect (Abrahante et al., 2003). *hbl-1(lf)* animals have trouble shedding the L4 cuticle (Abrahante et al., 2003). Evidently, *hbl-1* also functions during embryogenesis since the null phenotype is embryonic lethality as shown by RNAi (Abrahante et al., 2003). In addition, *hbl-1* RNAi produces larval lethality, and malformed larva with a semi-Dpy appearance (Abrahante et al., 2003; Lin et al., 2003).

In summary, the precocious mutant *hbl-1* acts between *let-7* and *lin-29* whereby *hbl-1* negatively regulates LIN-29 expression. In the nervous system *let-7* upregulation leads to decline in HBL-1 protein levels during the L4 stage. Other factors, possibly other miRNAs regulate the decline of HBL-1 in the hypodermis, whereas HBL-1 decrease in the seam cell may be regulated on the transcriptional level. *hunchback* sequence and function appears to be conserved across phyla.

2.7.2.4. *let-7* regulates the transition from larval to adult stage

let-7(+) is required for the transition from larval to adult stage. The delayed mutant *let-7(lf)* has an adult lethal phenotype that is associated with vulva bursting. In null mutants seam cell development appears essentially normal until the L4 stage. However, at the L4 molt *let-7(lf)* animals fail to express the SA program and instead reiterate the S4 program. Execution of the SA programs such as adult alae formation occurs at an additional fifth molt. Consequently, terminal differentiation of the hypodermis occurs one stage later than wild type and mutants add one life stage (Table 2.1.) (Reinhart et al., 2000). In contrast, overexpression of *let-7* results in precocious execution of the SA program and adult alae formation at the L3 molt.

Interestingly, *let-7* encodes a microRNA that is different in sequence, but similar in function to *lin-4* (Reinhart et al., 2000). The mature *let-7* RNA, 21 nucleotides in size, is processed from a longer (73 nt) stem-loop precursor transcript (Pasquinelli et al., 2000). The *let-7* precursor is subsequently processed by the RNAse DICER to the mature and active 21 nt RNA (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

Expression of *let-7* is temporally regulated. Therefore, *let-7* is referred to as a small temporal RNA (stRNA). Expression starts by the early L3 stage and is highest at early L4 to adult stages. Consistent with this, the temperature sensitive period for let-7 mutants is from mid-L3 to mid-L4 (Reinhart et al., 2000). Remarkably, the *let-7* sequence is conserved from nematode to human, including vertebrate, ascidian, hemichordate, mollusc, annelid and arthropod, but not found outside bilaterian animals (Table 2.2.) (Pasquinelli et al., 2000). Interestingly, temporal expression of *let-7* is also conserved in other species like fly and

zebrafish (Pasquinelli et al., 2000). Furthermore, several *let-7* targets and target sites are conserved in insects and vertebrates (Pasquinelli et al., 2000).

Like *lin-4, let-7* is assumed to repress translation of targets by complementary base pairing with their 3'UTRs. *let-7* complementary sites are found in the 3'UTRs of *lin-41* and *hbl-1* suggesting that *let-7* directly regulates these genes translationally (Lin et al., 2003; Reinhart et al., 2000).

Indeed, *lin-41* and *hbl-1*, which act redundantly in the late timer themselves, are found to be negatively regulated by *let-7*. Several lines of evidence suggest this regulation. First, epistasis analysis showed that both *lin-41* and *hbl-1* act downstream of *let-7* (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000). Second, the time of decline in LIN-41 (in the seams) and HBL-1 (in the nervous system) coincidences with that of *let-7* upregulation. Third, the *lin-41* and *hbl-1* 3'UTRs are sufficient to downregulate reporter gene expression at the appropriate time (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). Moreover, deletion of *let-7* complementary sites from the *lin-41* 3'UTR abolishes downregulation of reporter gene expression (Slack et al., 2000). Fourth, while *let-7* overexpression resembles *lin-41(lf)* mutant phenotypes, *lin-41* overexpression resembles *let-7(lf)* phenotypes (Reinhart et al., 2000). These data strongly suggest that *let-7* mediates the temporal downregulation of *lin-41* and *hbl-1* from L4 to adult stages by a translational control mechanism through complementary base pairing of *let-7* with the 3'UTR of *lin-41* and *hbl-1.*

Within the late timing circuit of the heterochronic pathway, *let-7* acts upstream of *lin-29.* In *let-7* mutants LIN-29 expression is delayed by one stage relative to wild type implying that *let-7* functions to positively regulate *lin-29.* This interaction is likely indirect through *lin-41* and *hbl-1* (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000). However, *let-7* may not work alone as the *let-7(lf)* phenotypes only partly resemble those of *lin-29* mutants (Reinhart et al., 2000).

The 3'UTRs of *lin-14, lin-28, lin-42* and *daf-12* also contain *let-7* complementary sites suggesting that *let-7* may directly regulate these genes as well (Reinhart et al., 2000). Consistent with this finding is that all precocious heterochronic mutants (*lin-14*, *lin-28*, *lin-41*, *lin-42)* suppress the retarded seam cell and the vulva lethal phenotype (Reinhart et al., 2000). However, timing of the stage-specific decline in LIN-14 and LIN-28 protein level is not perturbed in *let-7(lf)* mutants implying that *let-7* is not involved in *lin-14* and *lin-28* regulation (Reinhart et al., 2000). Whether *let-7* functions directly in regulating *lin-42* or *daf-12* has not been determined.

Temporal regulation of *let-7* in seam cells is controlled on the transcriptional level through the *let-7* promotor (Johnson et al., 2003). Factors bind to the inverted repeat of an Temporal Regulatory Element (TRE) enhancer element to regulate *let-7* expression. Two

early acting heterochronic genes, *lin-28* and *daf-12* are required for transcriptional control of the stage-specific timing of *let-7* expression (Johnson et al., 2003). In particular, the requirement for the nuclear hormone receptor *daf-12* implies that hormonal signaling plays a role in the transition from early to late larval development. Interestingly, in fly *let-7* expression may be regulated by ecdysone or the ecdysone pathway (Sempere et al., 2002; Sempere et al., 2003; Thummel, 2001). However, redundant temporal regulation of *let-7* could also occur on the level of *let-7* processing, as it is the case in sea urchins (Pasquinelli et al., 2000).

In summary, the microRNA *let-7* represses *lin-41* and *hbl-1* through complementary base pairing with *lin-41* and *hbl-1* 3'UTRs inhibiting their translation. Accumulation of *let-7* at the L4 stage leads to downregulation of LIN-41 and HBL-1 and thus to expression of LIN-29 to trigger the switch from larval to adult development. *let-7(lf)* mutants have retarded seam cell development, expressing S4 programs at the adult stage. Hence, they delay the initiation of LIN-29 expression. *let-7* is a small temporal RNA with conservation in sequence and function across phyla. Hence, the mode of *let-7* action may represent an ancient mechanism in the control of developmental timing.

2.7.2.5. *lin-29* controls the switch from larval to adult development

lin-29 is the most downstream gene in the heterochronic pathway and the ultimate target of regulation in the seam cell circuit. Mutations in *lin-29* result in retarded development. Seam cell development of *lin-29(lf)* mutants is wild type until the L4 stage; S1 to S4 programs are expressed in sequence. In L4, however, a failure in SA program initiation results in indefinite repetition of S4 programs at the adult stage (Table 2.1.). Seam cells stay in the stem cell division mode, and consequently all aspects of adult development fail to occur: cell cycle exit, seam cell fusion and cessation of the molting cycle. Therefore, *lin-29(lf)* animals completely lack adult alae and undergo supernumerary molts. By inference, *lin-29(+)* is essential for all the processes listed above, and thus for a switch from larval to adult development (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987; Bettinger et al., 1996; Rougvie and Ambros, 1995).

lin-29 encodes a C2H2 type transcription factor of the Krüppel family containing five zinc fingers (Rougvie and Ambros, 1995). LIN-29 has orthologs in *Sacchromyces cerevisiae, Rattus norvegicus, Oryzias latipes* and human that show high conservation (Table 2.2.). *lin-29* encodes two different transcripts that are absent in embryo, increase from L1 to L4 and decrease dramatically in young adults (Rougvie and Ambros, 1995).

LIN-29 is localized in the nucleus of various gonadal and extragonadal tissues and appears to be temporally regulated. LIN-29 accumulation in seam and other hypodermal cells during the L4 stage coincides with the time of SA program initiation. However, in the Distal Cip and anchor Cells LIN-29 accumulates during the L3 stage (Bettinger et al., 1996).

LIN-29 is also expressed in the somatic gonad, uterine and vulval muscle cells, vulval cells, pharynx, sex-myoblasts and their progeny, cells in the head and tail, neurons of the ventral cord and preanal ganglion (Bettinger et al., 1996). Consistent with its expression pattern, *lin-29* mutation also affects the development of the vulva and the uterus-vulva connection, the egg laying system, and the mail tail (Ambros and Horvitz, 1984; Bettinger et al., 1997; Euling et al., 1999; Newman et al., 2000).

As the most downstream gene known, *lin-29* is epistatic to all heterochronic genes (Abrahante et al., 2003; Ambros, 1989; Jeon et al., 1999; Lin et al., 2003; Slack et al., 2000). Consequently, all genes must act through *lin-29* to time seam cell development, and thus affect *lin-29* expression. Therefore, precocious mutants (e.g. *lin-14)* cause precocious LIN-29 expression whereas retarded mutants delay (e.g. *let-7*) or abolish it (e.g. *lin-4*) (Bettinger et al., 1996; Reinhart et al., 2000). LIN-29 expression is thought to be controlled by HBL-1 and LIN-41, both of which restrict LIN-29 expression to the L4 stage for SA program initiation (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). HBL-1 may control *lin-29* expression transcriptionally, since in flies Krüppel is a downstream target of Hunchback and LIN-29 belongs to the Krüppel family of transcription factors (Abrahante et al., 2003; Lin et al., 2003). In contrast, LIN-41 RNA binding protein is proposed to repress *lin-29* translationally for several reasons: first, LIN-29 expression is restricted to the L4 stage, but *lin-29* mRNA accumulates as early as the L2 stage (Bettinger et al., 1996; Rougvie and Ambros, 1995). Second, the decline of LIN-41 expression coincidences with that of LIN-29 accumulation at the L4 stage (Rougvie and Ambros, 1995; Slack et al., 2000).

LIN-29 controls the promotion of SA programs and the repression of larval programs on the transcriptional level. Thus, it stage-specifically activates expression of adult hypodermal collagens like *col-19* and represses larval hypodermal *col-17* (Liu et al., 1995; Rougvie and Ambros, 1995). Other aspects of SA program execution that are likely to be mediated by LIN-29 are expression of genes involved in cell cycle exit, seam cell fusion, seam cell morphology and exit from the molting cycle.

In summary, *lin-29* is responsible for the larval to adult switch promoting SA programs from mid-L4. *lin-29(+)* is negatively regulated by *hbl-1(+)* and *lin-41(+)* for S4 program specification in L3/L4. Release of LIN-29 repression by mid-L4 leads to LIN-29 expression and adult fate specification. LIN-29 itself represses larval and activates adult gene expression transcriptionally.

2.7.2.6. Parallel functions in the late timer

Several lines of evidence suggest that *hbl-1* and *lin-41* act together to specify seam cell development in late larval stages. First, the precocious seam cell phenotypes of neither *hbl-1* nor *lin-41* are penetrant. In *hbl-1(lf)* mutants, all animals exhibit the precocious alae at the L3

molt, but only some seam cells express precocious alae (Lin et al., 2003). Additionally, the alae quality is weak compared to wild type adult alae (Abrahante et al., 2003). Similarly, in *lin-41(lf)* animals the precocious seam cell phenotype is impenetrant. In particular, only half of *lin-41(lf)* animals exhibit the phenotype, and moreover only one third of the seam cells show the precocious defect in an affected animal (Slack et al., 2000). Second, *hbl-1;lin-41* double mutants show an enhanced fully penetrant precocious phenotype; all animals contain a full-length wild type alae at the L3 molt (Lin et al., 2003). Moreover, precocious alae at the L2 molt observed occasionally in either single mutant is enhanced in the double mutant (Abrahante et al., 2003). Third, both *hbl-1* and *lin-41* act downstream of *let-7,* but each single gene suppresses the *let-7* retarded phenotype only partially (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). Notably, in double mutants of *hbl-1* and *lin-41,* the *let-7* suppression is enhanced. Triple mutants exhibit a more penetrant precocious adult alae formation at the L3 molt and even the L2 molt (Abrahante et al., 2003; Lin et al., 2003). Finally, by epistasis experiments *lin-4* was found to be epistatic to *hbl-1* and *lin-41* (Abrahante et al., 2003; Slack et al., 2000). Double mutants of *hbl-1* or *lin-41* and *lin-4* resembled the retarded *lin-4* phenotype. However, *hbl-1;lin-41* double mutants in a *lin-4* mutant background lead to partial adult alae formation indicating that *lin-41* and *hbl-1* act in parallel and downstream of *lin-4* (Abrahante et al., 2003).

These data imply that parallel action of *hbl-1* and *lin-41* is sufficient for S4 program specification and that *let-7* inhibits both *hbl-1* and *lin-41* to promote adult programs. However, epistasis experiments with *hbl-1, lin-41* and *lin-4* argue that *hbl-1* and *lin-41* are insufficient for SA program specification implying that *lin-4* may additionally act through other unknown redundant factors.

2.7.3. The heterochronic epidermal pathway model

This model highlights and summarizes actions and interactions of heterochronic loci described in the epidermal circuit with focus on temporal fate specification in the seams. How do heterochronic loci specify developmental age? First, the diversity of heterochronic gene products and the complexity of genetic interactions indicate that the pathway is not linear, but rather contains a complex network. However, a number of simplifying themes emerge from the analysis. For example, cells always adopt the highest temporal fate available. These temporal fates are defined by the heterochronic activities that mainly act on a inhibitory basis. An increase in one gene activity (e.g. *lin-4)* represses downstream gene products (i.e *lin-14)* that in turn represses subsequent developmental programs. This way prevention of precocious expression of successive stages is inhibited. Advance to the subsequent stages, results from derepression of gene activities of the next stage. This way sequential stage-appropriate program expression is reinsured.

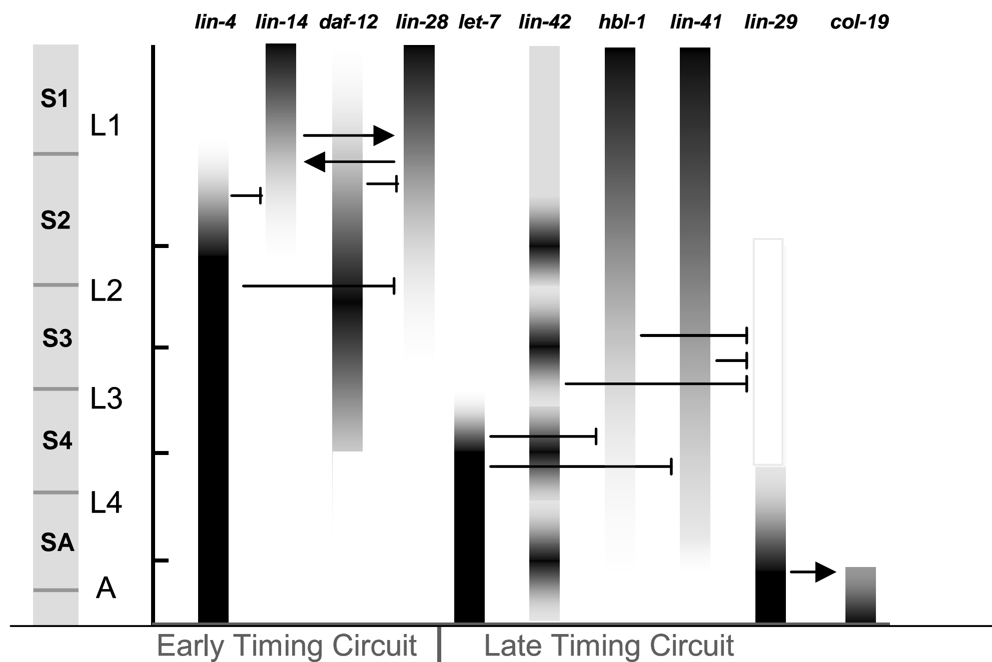

Figure 2.2. Model of the heterochronic epidermal pathway.
(A). Model summarizing genetic interactions of heterochronic gene products. Positive interactions are represented by an arrow, negative interactions by lines with horizontal bars(⊣). Developmental stages L1 to L4/A are shown on the Y axis. Assignment of genes to the early and late timer is indicated on the X axis. Temporal expression of proteins is indicated by shaded boxes whereby dark shading indicates accumulation. Expression patterns are based on expression analysis of GFP fusion protein, immunolocalization or RNA studies (*lin-4* and *let-7).* Expression patterns indicated are shown for epidermal seam cells and can be different for other tissues. For HBL-1, expression in the nervous system was used, in seam cells expression is limited to the L1 stage. (Figure modified from Rougvie, 2001).

2.7.3.1. The early timer

In wild type, LIN-14 and LIN-28 expression start by late embryo. At L1 high levels of LIN-14A repress S2 programs. LIN-14B, LIN-28 and LIN-42 repress S3 programs. Presumably, LIN-14B does so by inhibiting *daf-12*. Since S2 and later programs are repressed, cells by default select S1 programs. After hatching, feeding of L1 larvae results in the synthesis of the miRNA *lin-4*, a translational repressor of *lin-14* and *lin-28* (Arasu et al., 1991; Feinbaum and Ambros, 1999). During mid-to-late L1 *lin-4* has reached a level sufficient to repress *lin-14a* translationally. *lin-4* mediated downregulation of LIN-14A (and overall LIN-14 protein decline to a medium level) leads to derepression of S2 programs, while S3 programs are still repressed by the activities LIN-14B and LIN-28. Consequently, at mid-L1 to mid-L2 cells adopt S2 programs. A positive feedback loop between LIN-14 and LIN-28, acting in opposition to *lin-4,* keeps both proteins at a certain level reinsuring S2 program specification (Ambros, 1989; Arasu et al., 1991; Moss et al., 1997). In mid-L2 a *lin-4* mediated decrease of LIN-14B (resulting in complete absence of all LIN-14 isoforms) and LIN-28 levels derepress

S3 programs. Additionally, another *lin-4* independent mechanism, also declines LIN-14 and LIN-28 levels at this time (Arasu et al., 1991; Moss et al., 1997). Furthermore, DAF-12 simultaneously feedback inhibits *lin-28* to advance from the S2 to the S3 programs.

2.7.3.2. Connection of the early to the late timer

Heterochronic genes acting in the early timer also have an effect on later larval development. For example mutation in *lin-28* results in developmental programming defects in early larval stages. However, *lin-28(lf)* alters the timing of the larval to adult switch revealing a role in late larval development. Hence, the early timer must be somehow coupled to the late timer. Recently, Slack and co-workers (Johnson et al., 2003) showed that LIN-28 and DAF-12, both of which belong to the early timer, are required for transcriptional control of the stage-specific timing of *let-7* expression. In particular DAF-12 is a good candidate for transcriptional control of *let-7* as it is expressed throughout development (peaking in mid-L2) and is the late acting gene in the early timer promoting the progression from S2 to S3 programs (Antebi et al., 1998). DAF-12 could activate *let-7*, leading to initiation of the late timer. Alternately, DAF-12 could repress *let-7*, and *daf-12* downregulation could lead to the derepression of *let-7*. In any case, DAF-12 must work together with some other function, e.g. LIN-46, to drive expression of the late timer, since alone null mutants have weak heterochronic defects that are not as strong as *let-7* phenotypes.

2.7.3.3. The late timer

At mid L3 LIN-41 and HBL-1 negatively regulate the expression of the transcription factor LIN-29. Since SA programs are repressed at mid-L3, cells adopt S4 programs. The stRNA *let-7* acts as a translational repressor of both, *lin-41* and *hbl-1.* Accumulation of *let-7* expression by the L4 stage leads to translational inhibition of *lin-41* and *hbl-1.* Decline in LIN-41 and HBL-1 protein levels results in release of *lin-29* repression by mid-L4. Derepression of LIN-29 leads to promotion of adult and repression of larval programs. Consequently, cells adopt SA programs and terminal differentiation of the hypodermis occurs at the L4 molt. Additionally, LIN-42 plays a role in the inhibition of *lin-29* at L3 and therefore specification of S4 programs.

2.7.4. Conservation of heterochronic genes

In general, components of the early timer are not as evolutionarily conserved as components of the late timer. There are several possible explanations for the weaker conservation of early components. Either genes like *lin-14* may be nematode specific or alternatively they may have evolved more quickly. Table 2.2. gives an overview of all heterochronic loci with homologues in *D. melanogaster* and human.

2.8. Cell autonomous versus cell non-autonomous regulation of developmental age

Developmental age could be regulated cell non-autonomously by an organism-wide mechanism, e.g. including hormonal control. Alternatively, it could be determined cell autonomously, or a combination of the two. Several lines of evidence suggest that developmental age is in large parts regulated cell autonomously. First, the heterochronic genes act within the affected tissues as revealed by expression studies (e.g. *lin-14, lin-28)* (Moss et al., 1997; Ruvkun and Giusto, 1989). LIN-29 for example was found to act cell autonomously (Bettinger et al., 1997). Second, affected tissues of a heterochronic mutants can consist of a mosaic of cells expressing larval fates (e.g. dividing seam cells) mixed with adult fates (fused seams that express adult alae) as seen in many mutants e.g. *daf-12* or *lin-41* (Antebi et al., 1998; Slack et al., 2000). Another example is that of partial precocious dauer larva formed by *lin-28* mutants. Certain cells express the dauer program precociously, while others express the wild type program (Liu and Ambros, 1989). Further evidence provides *lin-14* expression studies. Expression of LIN-14(+) in neurons of *lin-14(lf)* animals rescues only precocious synaptic remodeling, but no other heterochronic phenotypes (Hallam and Jin, 1998).

Hormonal control achieves coordinate, synchronous development and is a common scheme to developmental timing (see above). Several lines of evidence suggest that the heterochronic circuit has non-autonomous (i.e. hormonal) input. The identification of *daf-12* as a nuclear hormone receptor, implicates hormonal input into the heterochronic pathway. Consistent with this, DAF-12 is regulated by DAF-9, a hormone biosynthetic gene. In turn, DAF-12 regulates *let-7* expression. In addition, initiation and exit from dauer diapause requires synchronous programming of tissues throughout the animal. Interestingly, regulation of *Drosophila mir-125* (a putative *lin-4* homologue) and *let-7* are regulated by ecdysone and the ecdysone pathway in (Sempere et al., 2002; Sempere et al., 2003). A simplifying notion is that hormonal input at one or more stages initializes the heterochronic circuit in distinct tissues, coordinating events throughout the organism. These circuits then work cell autonomously to specify the developmental age of cells.

2.9. Regulatory mechanisms involved in the control of developmental timing

This section will give an overview of mechanisms that are used in the control of developmental timing.

2.9.1. Transcriptional control

Transcriptional control of developmental timing is conferred by the heterochronic genes *lin-14,* which is a transcriptional regulator, the nuclear hormone receptor *daf-12*, the Per homolog *lin-42* and the transcription factor *lin-29.* Very little is known about transcriptional targets and their regulation. Among these factors, *lin-29* was shown to regulate the transcription of stage-specific collagen genes (Liu et al., 1995; Rougvie and Ambros, 1995). Presumably some of the targets should include other heterochronic regulators themselves. Consistent with this, *let-7* is thought to be a target of DAF-12 but it is unknown if this is direct or indirect.

2.9.2. Translational control

One prominent theme in heterochronic regulation is that of translational control. Many heterochronic genes have near constant mRNA levels throughout development, but protein levels vary. Thus regulation must also occur on the translational level (Ambros, 2000; Rougvie, 2001). At present, at least three cases of negative posttranscriptional regulation have been reported. Two of these involve the microRNAs *lin-4* and *let-7* and one involves the RNA binding protein LIN-41.

LIN-41 may translationally inhibit *lin-29* by binding to its 3'UTR, thereby limiting its expression temporally (Slack et al., 2000). LIN-28 is another protein that is implemented in RNA binding (Moss et al., 1997).

let-7 and *lin-4* represent a remarkable class of regulatory microRNAs that are essential components of the *C. elegans* heterochronic pathway. Both timing circuits, the early and the late timer are initiated by these stRNAs: accumulation of *lin-4* downregulates LIN-14 and LIN-28 protein levels to advance the early timer (Feinbaum and Ambros, 1999; Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). Similarly, accumulation of *let-7* downregulates LIN-41 and HBL-1 to advance from the larval to the adult stage. In addition, both stRNAs govern developmental timing by inhibiting downstream regulators that are controlling transcription (LIN-14, HBL-1) and translation (LIN-28, LIN-41) simultaneously. It is thought that imperfect mismatches within the 3`UTR of genes leads to translational inhibition. Consistent with this idea, they are found associated with large ribonucleoprotein complexes (Olsen and Ambros, 1999). Since these RNAs play such important roles, their mechanism needs to be further illuminated.

2.9.3. MicroRNAs and non-coding RNAs

let-7 and *lin-4* are the founding members of a large family of evolutionary conserved, small, non-coding RNAs, called micro or miRNAs (Ambros, 2001; Ambros et al., 2003; Carrington and Ambros, 2003; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001;

Lee et al., 1993; Reinhart et al., 2000; Sempere et al., 2003). Approximately 30% of the *C. elegans* miRNAs are conserved in insects and/or vertebrates (Ambros, 2003), about 100-120 miRNAs are thought to exist in *C. elegans,* and about 200-250 in vertebrates, which is 0.2-0.5 % of the genes in the genome, respectively (Ambros et al., 2003; Lim et al., 2003).

miRNAs play roles in diverse developmental processes: the *C. elegans* miRNAs *lin-4* and *let-7* function in developmental timing (Lee et al., 1993; Reinhart et al., 2000); in *Drosophila* miRNA *bantam* is implemented in the regulation of cell proliferation and cell death (Brennecke et al., 2003) and *mir-14* in cell death and fat metabolism (Xu et al., 2003b); human miRNAs may interfere with translation in neurons and may be involved in the molecular basis of learning and memory (Kim et al., 2004).

miRNAs are transcribed into a larger mostly 60 nt to 70 nt precursor form that folds into a stem-loop or hairpin-like structure. From this precursor the approximately 22 nt mature miRNA is cleaved out from one strand of the stem-loop structure by the RNAse DICER (DCR-1) (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). miRNAs are proposed to inhibit translation of target genes by imprecise base-paring with the target mRNA. This RNA/RNA hybrid contains loops and bulges (Ambros, 2001; Pasquinelli and Ruvkun, 2002).

miRNAs are similar in size to small interfering RNAs (siRNAs) of the RNA interference pathway (RNAi). RNAi is an evolutionary conserved defense mechanism against exogenous double stranded RNA. Perfect base pair formation of exogenously supplied siRNAs with target mRNAs, triggers degradation and results in silencing of the target gene (Fire et al., 1998; Hutvagner et al., 2001; Hutvagner and Zamore, 2002; Kennerdell and Carthew, 2000).

Both, siRNAs and miRNAs require DICER activity at the initial step of processing. However, siRNAs are processed from longer double stranded RNAs into short 21-25 nt long double stranded siRNAs. Subsequent incorporation of the siRNA into the RISC complex triggers mRNA degradation (Martinez et al., 2002). In contrast, miRNAs are processed from a hairpin precursor. This processing also requires DICER activity and leads to the mature, single stranded approximately 22 nt miRNA that imperfectly hybridizes with the target 3'UTRs to inhibit translation (Hutvagner et al., 2001; Ketting et al., 2001). Therefore, the RNAi machinery seems to require about 22 nt long double stranded RNA. Interestingly, depletion of DICER activity results in *lin-4* and *let-7* like phenotypes as the procession machinery is perturbed (Grishok et al., 2001). Moreover, DICER function is conserved across taxa. It is deployed in *let-7* processing in *Drosophila* and human (Hutvagner et al., 2001) and was found to be involved in the RNAi pathway in the fly (Bernstein et al., 2001).

Recently, a class of endogenous siRNAs was found in *C. elegans* (Ambros et al., 2003). About 20 nt in size, complementary to the 3'UTR of genes, these siRNAs are thought act through the RNAi pathway to trigger target degradation (Ambros et al., 2003). Similarly, in plants some miRNAs act like siRNAs and lead to target degradation (Llave et al., 2002; Rhoades et al., 2002; Tang et al., 2003). Furthermore, endogenous siRNAs are implicated in heterochromatic silencing in *Sacchromyces pombe* (Reinhart and Bartel, 2002; Volpe et al., 2002).

In *C. elegans* another class of small RNAs, the tiny noncoding RNA (tncRNAs), are similar in size to micro and siRNAs, but in contrast to miRNAs are not transcribed from a longer stem-loop precursor and are not phylogenetically conserved. Interestingly, some of them are temporally regulated (Ambros et al., 2003).

In general, miRNAs may unify features of transcriptional regulators and protein degradation regulators. miRNAs are somewhat comparable to transcriptional regulators in the way that they may cooperatively regulate target gene expression as found for *hbl-1.* The 3'UTR of *hbl-1* contains several miRNA binding sites for *lin-4*, *let-7* family as well as *mir-69* (Abrahante et al., 2003; Lin et al., 2003). Since *let-7* and family members (*mir-48, mir-84, mir-204*) and *mir-69* exhibit a similar temporal expression profile and share targets, they might act in concert to tighten translational control of targets. Also the number of binding sites may play a regulatory role.

2.10. Thesis question

Hormonally regulated *daf-12* plays multiple roles during *C. elegans* development (Antebi et al., 1998; Antebi et al., 2000; Gerisch and Antebi, 2004; Gerisch et al., 2001; Jia et al., 2002).

In postembryonic larval development it plays two roles. First, it mediates the choice between reproductive growth (S3) and dauer diapause (S3d) of the third larval stage. Second, *daf-12* regulates the progression from S2 to S3 and subsequent programs in both gonadal and extragonadal tissues. Hence, *daf-12* acts at the convergence of dauer and heterochronic pathways. In the adult stage, *daf-12* influences *C. elegans* life span in a *daf-2* dependent manner.

The diverse roles of *daf-12* in development seem to be reflected in the complexity of mutant alleles. Six classes of *daf-12* mutants exist that correspond to separable heterochronic and/or dauer phenotypes. In general, null or DBD mutants are Daf-d and exhibit impenetrant heterochronic phenotypes. LBD mutants are Daf-d (or Daf-c) and exhibit strong heterochronic phenotypes. For further description two representatives, the *daf-12* null allele *rh61rh411* and the *daf-12* gain-of-function allele *rh61* that carries a stop codon in the LBD are chosen.

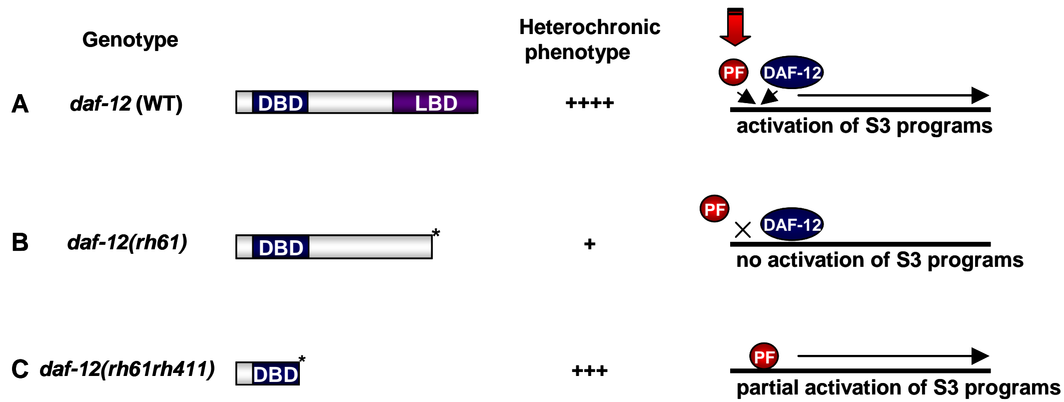

Figure 2.3. Model for *daf-12* redundant functions.
(++++) wild type; (+++) weak heterochrony; (+) strong heterochrony. (A) Wild type situation. DAF-12A(+) together with a parallel function (PF) promotes S3 programs of reproductive growth, ensuring the proper advance from the S2 to the S3 stage. (B) Receptors of recessive gain-of-function alleles inhibit target gene expression (of S3 programs). The receptor binds to DNA, but lesions in the LBD lead to ligand insensitivity and loss-of-activation. Consequently, gain-of-function alleles interfere with a parallel function. The failure in S3 program promotion results in strong heterochronic phenotypes. (C) *daf-12* null mutant products have lost both, DNA binding and activation capability due to complete loss-of-function. Consequently, neither activation nor repression of target genes occurs. In turn, a parallel function partially activates S3 reproductive programs resulting in impenetrant heterochrony. On the functional level, the parallel activity could act as a transcription factor or could be a component in a transcription factor pathway (indicated by an red arrow).

daf-12 function comprises two genetically separable activities, *daf-12a* and *daf-12b. daf-12a* promotes reproductive growth (activation of S3 programs and inhibition of dauer S3d programs) in favorable environments. In contrast, *daf-12b* promotes dauer development (activation of S3d programs and inhibition of S3 programs) in unfavorable conditions (Antebi et al., 1998; Antebi et al., 2000). Furthermore, genetic evidence implies that *daf-12b* is essential for dauer diapause specification whereas *daf-12a* is only partly required for reproductive growth promotion: *daf-12* null alleles (e.g. *rh61rh411*) and recessive gain-of-function alleles (e.g. *rh61*) are defective in dauer formation. In contrast, *daf-12* null alleles have impenetrant, whereras recessive gain-of-function mutants have penetrant gonadal and extragonadal heterochrony.

These data lead to a simple model in that receptors from *daf-12* gain-of-function alleles interfere with a parallel function (Figure 2.3.).

The aim of this study is to identify postulated *daf-12* redundant or parallel activities. Such genes can be identified by screens for mutants that enhance the weak heterochronic phenotypes of *daf-12* null alleles to a strong *daf-12 rh61*-like phenotype. Among the proposed redundant function, we expect to identify additional components of the heterochronic gene pathway that specify third and later stage options of reproductive growth.