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Orthologs of the C. elegans Heterochronic Genes Have Divergent Functions in C. briggsae

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Background

- The heterochronic pathway of *Caenorhabditis elegans* is exemplary as a mechanism of developmental timing: mutations in genes of this pathway alter the relative timing of certain developmental events independent of spatial or cell type specific regulation. It is the most thoroughly characterized developmental timing pathway known. Most of the heterochronic genes are conserved across great evolutionary time, and a few homologs seem to have developmental timing roles in certain contexts. The degree to which other organisms have explicit developmental timing mechanisms, and what factors comprise those mechanisms, isn't generally known.

- Developmental pathways evolve even if the resulting morphology remains the same, a phenomenon called Developmental Systems Drift. Components of developmental pathways and their roles may stay the same over time, their relationships to each other may change, or new factors can join or leave a pathway as it evolves, while still producing the same developmental result. It is known, for example, that the components and relationships of certain spatial patterning pathways are mostly conserved. But in sex determination, for example, the pathways evolve rapidly and those of even closely related organisms can differ dramatically.

- We set out to explore the evolution of this well-characterized developmental timing pathway by characterizing the phenotypes of the heterochronic gene orthologs in a closely related nematode *C. briggsae*. They share the same ecological niche and are nearly identical in development and morphology. Both worms develop after hatching through four larval stages (L1-L4) before reaching adulthood. Both develop the egg laying system, including the vulva, during the L3 and L4. Lateral hypodermal cells divide and add nuclei to a syncytium at each larval stage and differentiate at adulthood producing a cuticle structure called alae, a feature we use to characterize heterochronic mutants.

- We do not know at the outset of this project how much of the pathway is the same as in *C. elegans*. But we do know that *C. briggsae* possesses orthologs of all the heterochronic genes. We used the CRISPR/Cas9 gene editing system to introduce mutations into these orthologs, creating more than **40** new mutant alleles of *C. briggsae*. All are believed to be null alleles unless otherwise indicated. For genes that have severe pleiotropies in *C. elegans*, we employed the auxin-inducible degron system (AID) which allows us to propagate a strain as wildtype and then study its mutant phenotype when grown on the plant hormone auxin. We believe we are the first to employ this system in *C. briggsae*.

Studying the evolutionary conservation of the heterochronic genes orthologs in *C. elegans* and *C. briggsae* is the first step in addressing the question of how this pathway changes in evolution.

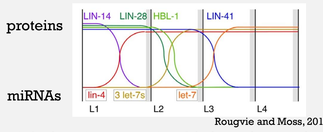

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Figure 1. A schematic representation of the heterochronic pathway showing when certain genes are active. miRNAs silence protein expression through 3'-UTRs of their target genes.

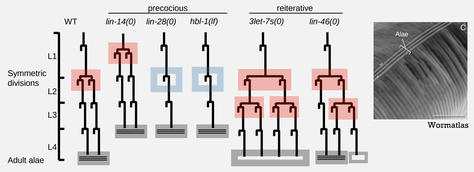

Figure 2. The patterns of seam cell divisions in WT and heterochronic mutants of *C. elegans*. Red boxes indicate symmetric seam cell divisions at L2 when their number doubles. Blue squares indicate skipped symmetric divisions.

Figure 3. "Arrested L4" phenotype of *cbr-lin-28(0)* mutant.

cel-lin-4(-)

Reiterate L1 stages
Lack adult alae
Lack vulva
cel-lin-4(-); cel-lin-14(0) – precocious phenotype is epistatic

cbr-lin-4(-)

Reiterate L2 stages
Gapped adult alae
Lack vulva
cbr-lin-4(-); cbr-lin-14(0) – L1 stages skip but no precocious alae

cel-lin-28(0)

Skip L2 stages
Develop precocious alae
Develop protruding vulva

cbr-lin-28(0)

Do not skip L2
Develop gapped precocious alae
Sometimes develop protruding vulva

Arrest or delay their development at the mid-L4 stage

cel-lin-46(0)

25% of mutants:
Partly reiterate L2
Develop gapped adult alae

cel-lin-46(0) suppresses *cel-lin-28(0)* phenotype nearly completely

cel-lin-46 5'-UTR del

Skip L2
Develop precocious alae
Develop protruding vulva

cbr-lin-46(0)

75% of mutants:
Partly reiterate L2
Develop gapped adult alae

cbr-lin-46(0) suppresses precocious alae in *cbr-lin-28(0)* mutant and weakly – L4 developmental arrest

cbr-lin-46 5'-UTR del

Do not skip L2
Develop precocious alae
Develop protruding vulva

cel-let-7(-)

Lack adult alae
Have an extra molt
Burst at vulva at the end of the development

cbr-let-7(-)

Develop adult alae
Have an extra molt
Do not burst

cel-lin-14(0)

Skip L1 stages
Precocious alae
Protruding vulva

cbr-lin-14(0)

Skip L1 stages
Precocious alae
Protruding vulva

miRNA
Proteins:
Transcription factor
RNA-binding
Protein-protein interactions

lin-4 ⊣ lin-14
lin-14 ⊣ mir-241 mir-48 mir-84
mir-241 mir-48 mir-84 ⊣ lin-28
lin-28 ⊣ let-7
let-7 ⊣ lin-41
let-7 ⊣ lin-46
lin-28 ⊣ lin-46
lin-46 ⊣ hbl-1
hbl-1 ⊣ lin-41
lin-41 ⊣ lin-29
lin-14 → L1
hbl-1 → L2
lin-41 → L3
? → L4
lin-29 → Adult cell fates

cel-let-7(-); cel-lin-28(0) – precocious alae suppressed
cel-let-7(-); cel-lin-41(0) – precocious phenotype is epistatic
cel-let-7(-); cel-hbl-1(lf) – precocious phenotype is epistatic

cbr-let-7(-); cbr-lin-28(0) – precocious alae not suppressed, arrested L4 phenotype suppressed
cbr-let-7(-); cbr-lin-41(lf) – L4 developmental arrest/delay is epistatic
cbr-let-7(-); cbr-hbl-1::aid – precocious phenotype is epistatic

cel-lin-29(0)

Do not develop adult alae
Retarded vulval development
cel-lin-29 is a larval/adult cell fates switch
cel-lin-29(0) suppresses precocious alae in *cel-lin-41(0)* and *cel-hbl-1(lf)*

cbr-lin-29(0)

Do not develop adult alae
Retarded vulval development
cbr-lin-29 is a larval/adult cell fates switch
cbr-lin-29(0) suppresses precocious alae and L4 developmental arrest in *cbr-lin-28(0)* and *cbr-lin-41(0)*

cel-lin-14 strong gain-of-function *cel-lin-14 weak gain-of-function*

Reiterate L1 stages (strong) or L1 then L2 (weak)
Lack adult alae (strong) or develop gapped adult alae (weak)
Lack vulva

cbr-lin-14 strong gain-of-function

Reiterate L1 then L2 stages.
Develop gapped adult alae
Lack vulva

cel-hbl-1 loss-of-function

Null alleles – embryonic lethal
Weak loss of function alleles – skip L2 stages, develop protruding vulva and precocious alae

cel-hbl-1(lf) phenotype is epistatic to *cel-mir-241 mir-48; mir-84(-)* phenotype

cbr-hbl-1::aid

RNAi – L1 lethality
Auxin-induced degradation – develop protruding vulva and precocious alae; sometimes skip L2

cbr-hbl-1(lf) phenotype is epistatic to *cbr-mir-241 mir-48; mir-84(-)* phenotype

cel-hbl-1(lf); cel-lin-41(0) – stronger precocious phenotype – might develop precocious alae at L2 molt, full alae at L3 molt

This indicates that *cel-lin-41* acts redundantly with *cel-hbl-1* to promote L3 cell fates and possibly L4

cbr-hbl-1(lf); cbr-lin-41(0) – stronger precocious phenotype – might develop precocious alae at L2 molt, gapped alae at L3 molt

This indicates that *cbr-lin-41* acts redundantly with *cbr-hbl-1* to promote L3 cell fates and possibly L4

cel-lin-41(0) or cel-lin-41::aid

Precocious alae at L3 molt
Null alleles are sterile
Arrest or delay their development at the mid-L4 stage (not reported previously)

cbr-lin-41(0) or loss-of-function

No precocious alae
Null alleles are sterile
Arrest or delay their development at the mid-L4 stage

cel-mir-241 mir-48; mir-84(-)

Reiterate L2 stages
Lack adult alae
Burst at vulva at the end of the development

Single mutants of each miRNA have weak WT-like phenotypes

cbr-mir-241 mir-48; mir-84(-)

Reiterate L2 stages
Lack adult alae
Burst at vulva at the end of the development

cbr-mir-48 has a reiterative phenotype
cbr-mir-241, *cbr-mir-84* – nearly WT

cel-lin-28(0); cel-hbl-1(lf)

Skip L2 stages
Vulva arrests its development at L4
Develop precocious alae at L3
Abnormal gonad migration

cbr-lin-28::aid; cbr-hbl-1::aid

Skip L2 stages
Vulva arrests its development at L4 or protruding
Develop precocious alae at L3
Abnormal gonad migration

Conclusions

1. There was a significant evolutionary drift in the heterochronic pathway between *C. elegans*, *C. briggsae* and their common ancestor.
2. Relative positions of the genes are largely conserved.
3. Strengths of their regulatory relationships, particularly those that are parallel and not strictly linear, appear to differ the most.
4. Some heterochronic genes play roles in other aspects of the animal's growth and development, including gonad development.