Research Proposals

Goal of the Research Proposal

Sharpen your skills in
a) Critical evaluation of the literature to identify and understand major unanswered questions in a field.
b) Formulate research hypotheses, which address one or more questions or gaps in knowledge in a field.
c) Devise experimental strategies to answer these questions, with controls and unbiased data analysis so that solid conclusions can be made.
And
d) Familiarize you with the practical use of the Research Proposal format
Research Proposals

Requires in depth knowledge of a research area.

Articulate major unanswered questions or gaps in knowledge in a research area.

Describe experimental approaches & data analysis that test specific hypotheses and/or address unanswered questions in a research area.

Proposal organizational format is directed at telling the reader what questions/hypotheses you are addressing, why the findings/answers are important and what experimental approaches will be employed to obtain the findings.

Three broad classes of research proposals

1. Hypothesis-driven
2. Hypothesis-generating
3. Methods development
Specific Aims

- A stand-alone description of the problems/hypotheses that will be examined, and usually includes a discussion of how the findings would advance the field.

- A listing of what lines of investigation will be used in the study and what will be learned.

Experimental Design and Methods

Three essential sections
Three essential sections

Specific Aims

Background and Significance
- Description of the current state of the field, critically evaluating existing knowledge and gaps that the proposed Aims will fill.
- Address the broader significance of the field and the findings that will arise from your proposed work. Building a case for why the proposed studies should be done.

Experimental Design and Methods
- Description of the experimental approaches that will be used to execute each Aim.
The logic behind the experiments, controls and interpretations is more important than details.
- Briefly describe, if relevant, alternative outcomes and/or approaches.
- At the end of each section, summarize the possible results in relation to advancing the Aim.
The Specific Aims has three components

1. Background narrative (like an abstract) that provides a context for the questions that will be addressed.

2. List of the questions /how the questions will be addressed.

3. Discussion of the significance of the results that will be obtained.

1. SPECIFIC AIMS

   Appropriate temporal patterning is essential for embryogenesis and post-embryonic development. Progression through the four larval stages in D. melanogaster is regulated by homotic genes (reviewed in Slack and Mercola 1997; Authes 2000). Homotic genes encode a diverse set of proteins, mutarrests in which result in the alteration or cessation of stage-specific programs of cell division, migration and/or differentiation, thereby timing development.

   Identification of a stage-specific protein cascade in a delayed transition to adulthood is essential for phenotype where cessation of a stage-specific program results in an early transition to adulthood (a premature phenotype).

   Transcriptional repression of the early-acting homotic genes, tro-24 and tro-22, is essential for appropriate progression through early larval stages (Barlow et al. 1993; Wilkins et al. 1993). This repression is mediated by a temporal gradient of L2N-14 and L2N-18 protein, high, intermediate and low levels of these proteins promote the activation of L1, L2 and L3 stage-specific programs, respectively. One mechanism to downregulate tro-24 and tro-22 involves the small temporal RNAi, and the product of the tro-4 gene (Authes 1999; Lee et al. 1994; Wilkins et al. 1993; Moso et al. 1997). However, recent evidence demonstrates the presence of a second mechanism to repress tro-22 translation, independent of tro-4. The role of tro-4 independent repression (t1R1) of tro-22 in developmental timing is unknown but may act to modulate L2N-18 protein levels at the L1 to L2 transition. Genetic studies using a GFP-tagged tro-22 transgene indicate that this novel repression of tro-22 involves the nuclear hormone receptor, drl-1 (T.Y. Authes, personal communication). A working model is that tro-4 dependent transcriptional repression initiates the downregulation of tro-22 in L1 and the L1 RNAi induces downregulation in order to repress at a low level of L2N-18 protein in L1 and L3. Regulations of L1R by drl-1 may act to coordinate the transcription of tro-22 downregulation with other events of larval development, such as the molting cycle. The name of this proposal is to determine the molecular mechanisms of L1R and to identify genes that are essential for this timing mechanism.
Listing of the Aims
(Here as a list of declarative statements.)

Aim 1. In order to test the hypothesis that gap-12 translationally represses lin-28, the temporal profile of endogenous lin-28 mRNA and protein levels in wildtype and gap-12 mutant animals will be determined.

Aim 2. Determine if lin-4-independent repressor elements (LIREs) in the 3' UTR of lin-28 mRNA are necessary and sufficient for the downregulation of lin-28 by the LIR pathway.

Aim 3. Determine if LIREs in the lin-28 3'UTR are required for developmental timing of the L2-L3 transition during larval development.

Aim 4. Perform genetic screen to identify genes that are required for LIR.

The listing of Aims or subAims can be either as declarative statements or as a question or a hypothesis.

Aim 3 (SubAim 3)
Determine if lin-4-independent repressor elements (LIREs) in the lin-28 3'UTR are required for developmental timing of the L2-L3 transition during larval development.

Are the LIREs in the lin-28 3'UTR required for developmental timing of the L2-L3 transition during larval development?

I hypothesize that the lin-28 3'UTR is required for developmental timing of the L2-L3 transition during larval development.
At least some experimental approaches should have a logical progression

Aim 1: In order to test the hypothesis that def-17 translationally represses lin-35, the temporal profile of endogenous lin-35 mRNA and protein levels in wildtype and def-17 mutant animals will be determined.

Aim 2: Determine if lin-4-independent repression elements (LIREs) in the 3' UTR of lin-35 mRNA are necessary and sufficient for the downregulation of lin-35 by the LIR pathway.

Aim 3: Determine if LIREs in the lin-35 3' UTR are required for developmental timing of the L3-L4 transition during larval development.

Aim 4: Perform genetic screens to identify genes that are required for LIR.

However, current organization result in dependent Aims. Instead, they should be SubAims of a single Aim

Aim 1

SubAim 1

In order to test the hypothesis that def-17 translationally represses lin-35, the temporal profile of endogenous lin-35 mRNA and protein levels in wildtype and def-17 mutant animals will be determined.

SubAim 2

Determine if lin-4-independent repression elements (LIREs) in the 3' UTR of lin-35 mRNA are necessary and sufficient for the downregulation of lin-35 by the LIR pathway.

SubAim 3

Determine if LIREs in the lin-35 3' UTR are required for developmental timing of the L3-L4 transition during larval development.

Aim 4: Perform genetic screens to identify genes that are required for LIR.
Aims should be integrated/highly related to the other Aims in the proposal.

Aim 1. In order to test the hypothesis that daf-12 translationally represses lin-28, the temporal profile of endogenous lin-28 mRNA and protein levels in wildtype and daf-12 mutant animals will be determined.

Aim 2. Determine if lin-4-independent repression elements (LIREs) in the 3' UTR of lin-28 mRNA are necessary and sufficient for the downregulation of lin-28 by the LIR pathway.

Aim 3. Determine if LIREs in the lin-28 3' UTR are required for developmental timing of the L3-L4 transition during larval development.

Aim 4. Perform genetic screens to identify genes that are required for LIR.

Description of the significance of the findings, if the proposed studies are successfully completed. (Need not be a separate section, can be imbedded into the narrative.)

Overall Significance of Aims. First identified in the *C. elegans* heterochronic pathway, translational repression by miRNAs is emerging as a common mode of regulation in development. Regulation of gene expression by miRNAs may be shared from worms to mammals as indicated by the conservation across phyla of the lin-7 miRNA (Pasquinelli et al. 2000). Although, it has been demonstrated that the miRNA lin-4 regulates lin-28 in early larval stages, it remains to be tested whether a second miRNA pathway is involved in the LIR pathway. This proposal will examine this hypothesis and has the potential to identify novel miRNA regulators.
Background and Significance

- Set reader up for the Aims and experiments in the Aims.
- Convince the reader why the results obtained will move a field forward --- why we should care?

- Move from general to specific.
- Use headings to divide sections.
- Employ figures & tables to facilitate explanation.
- Avoid presenting extraneous information.
- Length constrained.

**General context**

Regulation of embryonic and post-embryonic development requires the coordinated specification of cell fates in time and space. Due to its relatively simple and invariant cell lineages, C. elegans post-embryonic development provides an excellent model for the study of developmental timing. In other organisms, temporal control regulates developmental timing events such as the regulation of the insertional mutagenesis cycle by endonucleases (Homan et al. 1996). The identification of dfl-2 as a nuclear hormone receptor with effects on developmental timing has led to the hypothesis that the dfl-2 mRNA is a diffusible signal that coordinates temporal patterning throughout the worm (Johnson et al. 1996; Johnson et al. 2000).

In C. elegans, Hox gene expression is regulated by four stages of larval development distinguished morphologically by nuclei and the subsequent formation of the fully motile adult. During larval development stages of larval development (L1-L4) the appropriate expression of stage-specific genes is controlled by heterochromatin genes, among which the most well described are lin-4, lin-29, lin-39 and lin-41 (Shelton and Horvitz 1984). In contrast, genes of the lin-28 gene family are expressed in a sequence-specific manner and are essential for the expression of later developmental genes. A significant role of the lin-28 gene family is to maintain repression of later developmental genes in a stage-specific manner (Shelton and Horvitz 1997; Shelton et al. 1998).

**More specific background, essential info**

**Regulation of Developmental Timing in the L2-L3 Transition**

The phenotypes of mutants with mutant lin-28 alleles are consistent with a model that lin-28 represses the activities of the L3 program. Loss of function in lin-29 mutant animals shows the L3 program and associated repression of the L3 program to L2 (Andrews and Horvitz 1984). For example, the L2-L3 transition is normally repressed by the L2-specific cell division in L2 leading to an abnormally reduced number of cells with (Andrews and Horvitz 1984). In contrast, gains of function in lin-28 mutants repress the L3 program and consequently have elevated levels of L2-L3 transition of the L2 stage (Shelton et al. 1997). A positive RNA binding protein (lin-39), the L2-L3 protein likely controls L3-specific events by binding to and regulating downstream target RNA. L2-L3 protein levels are dynamic during larval development, with the highest expression at late embryonic and early larval stages, reduced expression in L2 larvae and undetectable L2-L3 levels in L2 larvae (Shelton et al. 1997).
Importance of lin-28 3'UTR regulation

What proposal will focus on

Another pathway that needs to be explored

The LRR Pathway is Regulated by lin-28.

The following evidence supports the model that lin-28 is upstream of the LRR pathway and regulates expression of the LRR-specific genes. 1. Genetic analysis demonstrates the presence of the LRR pathway is determined by lin-28 and indicates the involvement of lin-28 in this pathway (Table 1).

Table 1. Evidence for lin-28 regulation of LRR gene expression.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>LRR Gene</th>
<th>Transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin-28(p)</td>
<td>null</td>
<td>LRR</td>
<td>repressed</td>
</tr>
<tr>
<td>lin-28(p)</td>
<td>null</td>
<td>LRR</td>
<td>induced</td>
</tr>
<tr>
<td>lin-28(p)</td>
<td>null</td>
<td>LRR</td>
<td>normal</td>
</tr>
</tbody>
</table>

However, this LRR-dependent demethylation of lin-28 does not occur in triple mutant animals which

...
In the Experimental Section, each Aim or subAim should have four components

1. Rationale for the experiment
2. Experimental plan and controls
3. Interpretations
4. Alternative approaches and limitations
In the Experimental Section, each Aim or subAim should have four components

1. Rationale for the experiment  
2. Experimental plan and controls  
3. Interpretations  
4. Alternative approaches and limitations

Ok to use these as heading. But more useful to have additional headings telling the reader what is the experiment, question or hypotheses that is being examined in the section.

**Aim 1:** Test the hypothesis that dpp-27 transcriptionally represses ftz.

**Rationale:** The first point is to determine if mutations in dpp-27 alter ftz-28 mRNA and protein levels during larval development. In animals carrying the meth, allele of dpp-27, ftz-28 mRNA levels are observed, indicating dpp-27 involvement in the LRR pathway. However, this has not yet been demonstrated that dpp-27 mutants have elevated levels of ftz-28 mRNA or upregulated ftz-28 protein.

This Aim will test whether the expression of the ftz-28 GTPase accurately reflects the regulation of the ftz-28 gene. Furthermore, these experiments will determine if dpp-27 affects ftz-28 transcription or translation. The strategy to determine both ftz-28 mRNA levels and protein

**Experimental plan and controls:** To determine if ftz-28 protein levels are affected in dpp-27 mutant larvae, northern blot and western blot analysis will be performed on lysates from wild-type and dpp-27 mutant (mutant) larvae. Additionally, effects of the LRR pathway on development ftz-28 mRNA and protein levels will be examined using a strain in which both the ftz-4 and ftz-28 genes are ablated (D. Line). In this genetic, the absence of function between ftz-28 and ftz-28, GTP depends on the LRR, and is affected by dpp-27 mutants. (C. Line, personal communication). additional tests will be performed by knockout of the absence of function 500 genes.

**Stages of larval will be determined with Northern hybridization to the dsRNA of ftz-28 affected by dpp-27 mutants. (C. Line, personal communication). Northern analyses will be performed as described in Polakow and Darnell, 1990. Multiple time points within each larval stage will be taken.

**Northern analysis:** Northern analysis will be performed as described in Polakow and Darnell, 1990. RNA will be prepared from staged larvae, repaired by electrophoresis, and transferred to Zetablot membrane (BioRad). Membranes will be crosslinked and immunoprecipitated with a ribosomal ribonuclease (RNA-probe). Hybridizations will be quantified using a phosphorimage scanner (Molecular Dynamics). To control for the amount of RNA loaded per lane, membranes will be stripped and re-probed with an ammonia UV probe. Lysates from women which lead to the N-1 U1 will be used as a negative control.
Experiment: The first point is to determine if mutations in dpl-2 alter lin-30 mRNA and protein levels during larval development. In mutants carrying the wild-type allele of dpl-2, lin-30 mRNA and protein levels are observed, indicating dpl-2 involvement in the LR pathway. However, a role for dpl-2 has not been demonstrated in the regulation of lin-30 mRNA or protein. This essay will not therefore whether the expression of the lin-20 CEP transgene accurately reflects the regulation of the linen gene. Furthermore, these experiments will determine if dpl-2 effects lin-20 transcription or translation. The strategy is to determine both lin-30 mRNA levels and endogenous LIN-20 protein levels at various time points during larval development to deduce dpl-2 effects.

Experimental plan and Controls: To determine if LIN-30 protein levels are affected in dpl-2 mutant animals, northern blot and western blot analysis will be performed on lysates from wildtype and dpl-2 (plasmid) injected animals. Additionally, effects of the LR pathway on endogenous lin-20 mRNA and protein levels will be determined using a strain in which the t-46-dependent expression and the lin-20 protein feedback are absent (i.e., lin-30[ts], lin-20). In this genotype, the down-regulation of lin-20-30 GFP depends on the LR and is affected by dpl-2 mutations. Controls, random combinations, and stage-specific combinations will be performed to determine the absence of translation. Following the addition of food to ced-r1, larvae will be collected at increasing times during larval development. Stages of larvae will be determined with Nomarski differential interference contrast (DIC) microscopy as described in Eisen and Austin, 1999. Multiple time points within each larval stage will be used. Northern analysis will be performed as described in Frankfurter and Austin, 1999. RNA will be prepared from staged lysates, separated by electrophoresis, and transferred to Zetablot membranes (Biorad). Samples will be cross-linked and incubated with a radiolabeled antisense lin-20 probe. Following hybridizations and washing, membranes will be exposed to film and quantitated with a Phosphorimager ( Molecular Dynamics). To control for the amount of RNA loaded per lane, membranes will be stripped and re-probed with an exonuclease VII probe. Lysates from worms which lack lin-20 (i.e., null alleles) will be used as a negative control.

Logic for what will be done

General outline of experiment (limit the detail)

Controls

Typos!!
Should be lin-20 (not lin-14)
For western analysis, proteins will be separated by SDS-PAGE and transferred to PVDF membranes. Blots will be probed with anti-LIN-22 (to be obtained from E. Moss). Bands will be detected and quantified using enhanced chemiluminescence and the Molecular Dynamics imaging system with Image Quant software. To control for the amount of protein loaded per lane, these will also be probed with anti-6-his which will allow for normalization and comparison of bands between

*Expected finding* which lead to experiments in *Aim 2.*

**Aim 2.** Identify elements in the 3' UTR of lin-22 that are necessary and sufficient for *dpd-22* translational repression.

**Rationale:** The 3' UTR of lin-22 has been determined to be necessary to direct the temporal downregulation of *lin-22.* Deletion of 3' UTR elements in the 3' UTR results in a gain-of-function lin-22 allele that remains elevated at the L4 stage (Moss et al. 1987). Furthermore, *lin-22*::GFP under the regulation of the 3' UTR of the ace-54 gene is not temporally downregulated (Moss and Anfinsen, personal communication). These results suggest that all the known negative regulatory signals in Lin-22 act via the 3' UTR, but this hypothesis has not been directly tested for the L1 pathway. Therefore, it will be determined whether the 3' UTR of lin-22 is sufficient to direct the downregulation of a GFP reporter gene by the L1 pathway, and what elements within the 3' UTR are required.

**Relevant**

**Question to be addressed**

**How, in general,** it will be addressed
Experimented plan and Control:

Determine if the Lin-28 3' UTR is sufficient for dysregulated expression in the LIN pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a rol-10 promoter (Hou et al. 2000) driven GFP coding region under the regulation of either the lin-28 3' UTR (Moss et al. 1997) or control (see below) 3' UTR sequences. The rol-10 promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germline transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., rol-6 (m3150)), into wildtype animals. Animals will be crossed onto lin-28[let-745] and let-6[let-745(14A)[let-745(14A)+]] genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIN pathway. As outlined in Table 1, a LIR dependent construct is expected to be downregulated by the L4 stage in wildtype and let-6[let-745(14A)] genetic backgrounds. However, in a lin-28[let-745]let-6[let-745(14A)][let-745(14A)+] genetic background, as which the LIN pathway is absent, a LIR-dependent construct is expected to remain elevated at the L4 stage. Southern blotting of DNA from transgenic strains will be performed to confirm the presence and abundance of the transgenes.

To analyze the reporter gene constructs in wildtype and the different genetic backgrounds, larvae at the L1 and L4 stages (see Aim I) will be collected and fluorescent microscopy will be used to visualize GFP expression patterns. Relative GFP levels of reporter constructs will be normalized between stages from CCD images taken with equivalent exposure times (Hou et al. 2000). To obtain a pattern in the larval stage of detection, multiple exposures will be taken. Image analysis will be.

Healing indicates question to be addressed in this section

Presents overall approach, but not lost in experimental details

Experimented plan and Control:

Determine if the Lin-28 3' UTR is sufficient for dysregulated expression in the LIN pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a rol-10 promoter (Hou et al. 2000) driven GFP coding region under the regulation of either the lin-28 3' UTR (Moss et al. 1997) or control (see below) 3' UTR sequences. The rol-10 promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germline transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., rol-6 (m3150)), into wildtype animals. Animals will be crossed onto lin-28[let-745] and let-6[let-745(14A)[let-745(14A)+]] genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIN pathway. As outlined in Table 1, a LIR dependent construct is expected to be downregulated by the L4 stage in wildtype and let-6[let-745(14A)] genetic backgrounds. However, in a lin-28[let-745]let-6[let-745(14A)][let-745(14A)+] genetic background, as which the LIN pathway is absent, a LIR-dependent construct is expected to remain elevated at the L4 stage. Southern blotting of DNA from transgenic strains will be performed to confirm the presence and abundance of the transgenes.

To analyze the reporter gene constructs in wildtype and the different genetic backgrounds, larvae at the L1 and L4 stages (see Aim I) will be collected and fluorescent microscopy will be used to visualize GFP expression patterns. Relative GFP levels of reporter constructs will be normalized between stages from CCD images taken with equivalent exposure times (Hou et al. 2000). To obtain a pattern in the larval stage of detection, multiple exposures will be taken. Image analysis will be.
Expected result, given the model

Reasonable possibility if expected result not found

Experiments and Comments

Determination of the lin-35 3' UTR is sufficient for downregulation by the LR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a lin-35 promoter (Wong et al. 2000) driving GFP coding region under the regulation of either the lin-35 3' UTR (Moss et al. 1997) or control (see below) 3' UTR sequences. The lin-35 promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germline translocations will be performed by microinjection of reporter constructs along with a marker, which allows for the selection of transfectants in z. red-6 (a red-6 allele), into wildtype animals. Acetab will be crossed into lin-6(tg[ lin-35::gfp]) and lin-6(tg[ lin-35::gfp]) genetic backgrounds in order to analyse the sensitivity of reporter constructs to the LR pathway. As outlined in Table 1, a LR dependent construct is expected to be downregulated by the LR stage in wildtype and lin-6(tg[ lin-35::gfp]) genetic backgrounds. However, in a lin-6(tg[ lin-35::gfp]) genetic background, in which the LR pathway is absent, a LR-dependent construct is expected to remain elevated at the LR stage. Southern blotting with DNA from transgenic strains will be performed to confirm the presence and abundance of the transgenes.

To analyse the reporter gene constructs in wildtype and the different genetic backgrounds, larvae at the L1 and L4 stages (see Aim 1) will be collected and fluorescence microscopy will be used to visualize GFP expression patterns. Relative GFP levels of reporter constructs will be analyzed between stages from CCD images taken with equivalent exposure times (Wong et al. 2000). To obtain a pattern in the linear range of detection, multiple exposures will be taken. Image analysis will be
In first three hypotheses driven Aims (SubAims)
- Show that daf-12 & LIR translationally regulated lin-28 (Aim 1)(SubAim 1)
- Identify sequences, acting in cis, in the lin-28 3'UTR responsible
  for the regulation (Aim 2) (SubAim 2)
- Show that 3'UTR sequences regulate lin-28 level and activity
  in vivo (Aim 3)(SubAim 3)
- The next logical question is what genes/gene products are acting
  in trans for LIR regulation of lin-28? (Aim 4)
  (this is a discovery Aim)

Aim 4. Perform genetic screen to identify genes that are required for LIR.

Rationale: To identify genes involved in the LIR pathway for lin-28 down-regulation, I will perform a
screen for heterochronic mutants in a lin-28[507]; lin-4[ak126] genetic background where normal
developmental timing is governed by the LIR pathway. A strain with a col-19::gfp transgene
specifically expressed in the hypodermis of adult animals and not in larvae or embryos will be used.
This transgene is regulated by the activity of heterochronic gene pathway and therefore is a useful tool
to efficiently identify precocious or retarded mutants (Ahringer et al. 1999). This screen may
identify known translational repressors or novel genes, including miRNA-encoding genes, and/or
novel alleles of lin-28 that confer a gain-of-function phenotype may be identified.
Aim 4. Perform genetic screen to identify genes that are required for LIR.

Rationale: To identify genes involved in the LIR pathway for lin-28 down-regulation, I will perform a screen for heterochronic mutants in a lin-68; lin-14(n1008) genetic background where normal developmental timing is governed by the LIR pathway. A strain with a col-10::GFP transgene specifically expressed in the hypodermis of adult animals and not in larval or embryos will be used. This transgene is regulated by the activity of heterochronic gene pathway and therefore is a useful tool to efficiently identify precocious or retarded mutants (Abrahante et al. 1998). This screen may identify known translational repressors or novel genes, including mRNA encoding genes, and/or novel alleles of lin-28 that confer a gain-of-function phenotype may be identified.

Proposed experimental approach

Types of genes that might be identified
Should provide the logic for the approach, even if it is obvious to you.

DAF-12 is a nuclear hormone receptor, therefore it is unlikely to be a direct translational regulator of lin-28. Thus, there must be other genes controlled by DAF-12 that act in trans to repress lin-28.

Aim 4. Perform genetic screen to identify genes that are required for LIR.

Rationale: To identify genes involved in the LIR pathway for lin-28 downregulation, I will perform a screen for heterochronic mutants in a lin-28(n1474) genetic background where normal developmental timing is governed by the LIR pathway. A strain with a rol-6(su100) transgene specifically expressed in the hypodermis of adult animals and not in larvae or embryos will be used. This transgene is regulated by the activity of heterochronic gene pathway and therefore is a useful tool to efficiently identify precocious or retarded mutants (Abraham et al. 1998). This screen may identify known translational repressors or novel genes, including miRNA encoding genes, and/or novel alleles of lin-28 that confer a gain-of-function phenotype may be identified.

Genetic Analysis of Newly Isolated Mutants: To facilitate the mapping of mutant genes, mutants will be crossed with C. elegans isolate from Hawaii, CG6166, as described by Wicks et al. (Wicks et al. 2001). This approach takes advantage of single nucleotide polymorphisms which alter restriction enzyme sites (“snip-SNPs”) in C. elegans relative to the wildtype parent strain used for all other experiments herein. C. elegans var. Bristol N2. Larvae will be prepared from F1 or F2 animals that display the mutant phenotype (homozygous mutants) or that are wildtype (homozygous wildtype or heterozygous). Multiple PCR reactions with the two samples will perform using primers designed to amplify sequences which flank snip-SNPs—one for each arm of the chromosome and one for the center region (Wicks et al. 2001). The relative levels of the C. elegans-derived product and the N2-derived product will allow for the determination of map position. For example, a SNP allele from C. elegans that is closely linked to the wildtype allele of a mutation being mapped will be underrepresented in (or absent from) the PCR products from mutant animals. Once linkage to a chromosomal region is established, more precise mapping will be performed using primers corresponding to additional snip-SNPs in the region.

After snip-SNP mapping, has narrowed down the location of a mutation a chromosomal region, complementation analysis will be performed to test for new alleles of known genes. New gene will be identified by two approaches: covalent rescue will be performed using covalent DNA from the gene, and for regions where a battery of RNAi feeding strains are available (Frazier et al. 2000) these will be used. The mutant phenotype can be replicated by RNAi with a candidate gene.
Interpretations: It is predicted that additional genes involved in the LIR pathway will be identified. To support this prediction, the effects on developmental timing and lin-28 expression of the null allele of dop-12 have been proposed to be due to the interference with the activity of dop-12-interacting proteins (Arnosti et al. 2000). Such interacting proteins may be identified in this screen. Potential candidate genes are novel mRNAs and translational repressors containing putative RNA binding domains, such as members of the STAR subfamily (Petran and Honzík 1999). If newly isolated candidate genes lack an RNA binding motif, then it is expected that multi-protein complexes may be required, as in the case for Nanos and Pumilio-dependent repression of the hunchback mRNA in Drosophila. Because both lin-28 and dop-12 are expressed in phenotypically affected lineages, it is predicted that putative lin-28 regulators will similarly expressed (e.g., hypodermal somatic cells, muscle and neurons). Further, it is expected that expression of putative lin-28 regulators will be detected after the mid to late L1 stage with strong expression in the L2 stage. If expression is detected in late embryos or early L1s, then that would suggest the presence of factors which prevent the early downregulation of lin-28.

Alternate Methods and Limitations: A inherent limitation of this approach is that redundant pathways may prevent the identification of genes by forward genetic screens. Another potential pitfall is the possibility that alleles that result in a desired phenotype could be rare. For example, if a particular phenotype requires a misexpression or a special kind of regulatory mutation then it may be infeasible to screen enough animals required for the identification of such a gene. However, one advantage of this screen is that dominant mutations can be identified. The ability to screen more F1s for dominant mutations increases the probability that a rare mutation will be found. If no new genes are identified in the LIR pathway, then that would raise the possibility that dop-12 may regulate lin-28 translation directly. To address this, direct binding of DAF-12 to elements in the 3' UTR of the lin-28 mRNA would be tested. The effect on lin-28 3'UTR binding of the presence or absence of the dop-12 ligand binding domain would also be analyzed. However, these experiments are beyond the scope of this proposal.
“Discovery” Aims are viewed as problematic for grants as they are risky – uncertain if they will be successfully completed.

Nevertheless, in a number of cases, what is identified in a discovery Aim will significantly advance a field.

For Advanced Genetics, a discovery Aim is possible. We want to expand your skill in coming up with a logical experimental approach to fill a gap in the field of interest. (But needs to be justified & well explained.)
Where do Aims come from?

Three essential elements in coming up with an Aim.

1. Information from the literature and/or preliminary experimental findings.

2. A hypothesis that derives from the literature findings and/or preliminary results.

3. Experiment or set of experiments that will test the hypothesis.
Example

**Literature/experimental findings:**

- Sugar transporter Glut8 co-localizes with the autophagy protein AT6x in mouse liver cells, based on immunofluorescence.

- Glut8 (-/-) knockout mouse displays an increased autophagy phenotype in liver cells.

> Based on the mutant phenotype, what is the wild type function of Glut8?

>>> Negative regulator of autophagy
Example

**Literature/experimental findings:**
- Sugar transporter Glut8 co-localizes with the autophagy protein ATGx in mouse liver cells, based on immunofluorescence.
- Glut8 (-/-) knockout mouse displays an increased autophagy phenotype in liver cells.

**Hypothesis:**
- Glut8 is a negative regulator of ATGx activity in the autophagy Pathway, acting as an inhibitor through binding ATGx and making it unavailable to stimulate the autophagy pathway.

*Prediction*: Blocking Glut8 – ATGx binding, but not other Glut8 protein functions, will lead to constitutive autophagy.

Example continued

**An experimental approach for testing the hypothesis:**

a) Determine if the cytoplasmic domain of Glut8 binds to ATGx, using the yeast two-hybrid screen.

b) Identify small regions of Glut8 and ATGx that are responsible for binding, using the reverse yeast two-hybrid system.

c) From the regions/amino acids of Glut8 and of ATGx responsible for binding, generate knock-ins (or transgenes) that contain alanine mutations in these sites, which should block binding but retain other functions (these are called alanine-scanning, separation of function mutations).

Test each mutant in liver cells, under conditions where the corresponding endogenous gene product is absent, for constitutive autophagy.
While the aims should be hypothesis driven (not just data collection) don't be hypotheses limited or paradigm blinded.

The best experiment is if either outcome is informative in addressing the goal of the Aim.

For your class proposal

1) From the literature, find an area of interest where there are open questions or gaps in knowledge.

2) Derive one or more testable hypotheses related to the open questions/ gaps in knowledge.

3) Assemble an experimental approach that addresses the hypotheses and any predictions that might arise from the hypotheses.
For your class proposal

1) From the literature, find an area of interest where there are open questions or gaps in knowledge.

2) Derive one or more testable hypotheses related to the open questions or gaps in knowledge.

3) Assemble an experimental approach that addresses the hypotheses and any predictions that might arise from the hypotheses.

Advanced Genetics students: Approach must include at least some genetic analysis.
**General Tips**

1. Look at successful proposals.
2. Have a good idea.
3. Know the literature, issues, questions/controversies in the area.
4. Instead of just feedback, try feed forward, where you discuss your ideas with others before beginning the writing process.
5. Place the work in a broader perspective, indicating significance.
6. Use clear and concise writing style.
7. Proofread – zero tolerance for typos, formatting & citation errors
8. Critique your own proposal.
9. Have others critique your proposal.

---

**Plagiarism**

Two useful websites that define plagiarism and provide tips on how to avoid it in your writing.

https://wts.indiana.edu/writing-guides/plagiarism.html

http://writingcenter.unc.edu/handouts/plagiarism/
Week of Jan 29, Small Group Discussion Sections

Long Chain FA Proposal
ADAR Editing Proposal

- What is the hypotheses?
- For each component of the proposals, what are the positives and negatives in the authors execution of the section.
- Is the writing clear as to what the author is proposing?
- Are you convinced it is a significant problem?
- Do the experiments address the issues/questions?
- Are you convinced that the author can execute the proposed studies?