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

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

Three essential sections

Specific Aims

Background and Significance

Experimental Design and Methods
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.

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.
Three essential sections

Specific Aims

Background and Significance

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.
(4) SPECIFIC AIMS

Appropriate temporal patterning is essential for embryogenesis and post-embryonic development. Progression through the four larval stages in C. elegans is regulated by heterochronic genes (reviewed in Slack and Riddle 1997; Ambros 2000). Heterochronic genes encode a diverse set of proteins, mutations in which result in the initiation or omission of stage-specific programs of cell division, migration and/or differentiation, thereby altering developmental timing. Retraction of a stage-specific program results in a delayed transition to adulthood (a recessive phenotype) whereas omission of a stage-specific program results in an early transition to adulthood (a dominant phenotype).

Translational repression of two early-acting heterochronic genes, lin-14 and lin-28, is essential for appropriate progression through early larval stages (Karakus et al. 1989; Wrightman et al. 1991; Mosk et al. 1997). This repression generates a temporal gradient of LIN-14 and LIN-28 protein. High, intermediate and low levels of these proteins promote the activation of L1, L2 and L3 stage-specific programs, respectively. One mechanism to de-repress lin-14 and lin-28 involves the small temporal RNA (stRNA) product of the lin-4 gene (Hauri et al. 1993; Lee et al. 1993; Wrightman et al. 1993; Mosk et al. 1997). However, recent evidence demonstrates the presence of a second mechanism to repress lin-28 translation, independent of lin-4. The role of lin-4 independent repression (LIR) of lin-28 in developmental timing is unknown but may act to modulate LIN-28 protein levels at the L2 to L3 transition. Genetic studies using a GFP-tagged lin-28 transgene indicate that this novel repression of lin-28 involves the nuclear hormone receptor, daf-12 (C. elegans, personal communication). A working model is that (i) lin-4 dependent translational repression initiates the downregulation of lin-28 in L1 and the LIN-28 protein in L2 and L3. Regulation of LIR by daf-12 may act to coordinate the transition of lin-28 downregulation with other events of larval development, such as the molting cycle. The aims of this proposal are to determine the molecular mechanism of LIR and to identify genes that are essential for this timing mechanism.

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Listing of the Aims
(Here as a list of declarative statements.)

Aim 1. In order to test the hypothesis that daf-12 translationaly 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 repressors (LIREs) in the 3' UTR of lin-28 mRNA are necessary and sufficient for the down-regulation 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. Determine if 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 Aims should have a logical progression

Aim 1. In order to test the hypothesis that daf-12 translationally represses lin-38, the temporal profile of endogenous lin-38 mRNA and protein levels in wildtype and daf-12 mutant animals will be determined.

Aim 2. Determine if lin-4 independent repressor elements (LIREs) in the 3’ UTR of lin-38 mRNA are necessary and sufficient for the downregulation of lin-38 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.
An Aim can also stand on its own, but should still 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 LIR-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 LIN-14 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.

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 let-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.

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 lineage, C. elegans post-embryonic development provides an excellent model for the study of developmental timing. In other organisms, hormonal control regulates developmental timing events such as the regulation of the insect mating cycle by ecdysone (Thevenet 1996). The identification of daf-12 as a nuclear hormone receptor with effects on developmental timing has led to the hypothesis that the daf-12 ligand may be a diffusible signal to coordinate temporal patterning throughout the worm (Ambrose et al. 1998; Amesh et al. 2000; Snow and Larsen 2000).

In C. elegans, embryogenesis is followed by four stages of larval development distinguished morphologically by molt and the subsequent formation of the fully mature adult. During larval development (stages L1-L4), the appropriate activation of stage-specific programs (L1-L4) is controlled by heterochronic genes, among which the most well-described are lin-4, lin-14, lin-15 and lin-29 (Ambrose and Horvitz 1984; Ambrose 1989; Slack and Ruvkun 1997; Ambrose 2000). Mutations in heterochronic genes result in the deletion or alteration of stage-specific programs of cell division, differentiation or migration.

lin-29 Regulates Developmental Timing at the L2-L3 Transition

The phenotypes of animals with mutant lin-29 alleles are consistent with a model that lin-29 regulates the activation of the L3 program. Loss-of-function lin-29 mutant animals skip the S2 program and prematurely express the S3 program in L2 (Ambrose and Horvitz 1984). For example, in lin-29(k0) animals, lateral hypodermal seam cells omit the S2-specific cell division in L3 leading to an abnormal number of seam cells (Ambrose and Horvitz 1984). In contrast, gain-of-function (GOF) lin-29 mutants repeat the S2 program in L3 and correspondingly have elevated levels of LIN-28 protein at the L4 stage (Moss et al. 1997). A putative RNA-binding protein (Moss et al. 1997), the LIN-28 protein likely impairs L3-specific events by binding to and regulating downstream target mRNAs. LIN-28 protein levels are dynamic during larval development, with the strongest expression in late embryos and L1 larvae, reduced expression at L2 larva and undetectable LIN-28 levels in L3 larvae (Moss et
Mechanisms to Downregulate lin-28 During Early Larval Development.

There are three inputs which contribute to the downregulation mechanism of lin-28: lin-4 dependent repression, positive feedback by lin-14, and the LR pathway. Northern analysis demonstrates no change in lin-28 mRNA levels during larval development (Y. Moss, personal communication), indicating the dependence on translational control to regulate LIN-28 protein levels. Specifically, the 3' UTR of the lin-28 mRNA is emerging as a critical regulatory region. The repression of a lin-28::GFP transgene under the control of the 3' UTR of the lin-28 gene is not expressed by lin-4 or the LIR pathway, and does not depend on lin-14. This demonstrates that the 3' UTR of lin-28 is required for all three regulatory mechanisms. Moreover, regulation of lin-28 by the mRNA is mediated by specific elements in the lin-28 3' UTR. A lin-20 allele in which a lin-4 complementary 3' UTR sequence was deleted confers a gain-of-function phenotype. This proposal will focus on the LIR pathway for lin-28 translational repression.

The LIR Pathway is Regulated by daf-12.

The following evidence supports that daf-12 is upstream of lin-28 and regulates execution of the ES specific program. 1. Genetic analysis demonstrates the presence of the LIR pathway to downregulate lin-28, and indicates the involvement of daf-12 in this pathway (Table 1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>LIR-28 Protein</th>
<th>Daf-12</th>
<th>Lin-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin-28::GFP</td>
<td>normal</td>
<td>HES1 HIGH, Mos, 1997</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>lin-28::GFP; lin-28::GFP lin-4 large</td>
<td>viable</td>
<td>HES1 HIGH, Mos, 1997</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>lin-28::GFP; lin-28::GFP lin-4 small</td>
<td>viable</td>
<td>HES1 HIGH, Mos, 1997</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Developmental timing is governed by the LIR pathway in animals lacking lin-4 and with a temperature-sensitive allele of lin-14 (lin-4(lf); lin-14(tsl)). In this genotype, lin-28 is downregulated normally, presumably by the LIR pathway alone (Aimoto et al. 1997).

However, this LIR-dependent downregulation of lin-28 does not occur in triple mutant animals which also have a mutation in daf-12, i.e., lin-4(lf); lin-14(tsl); daf-2(md), thus demonstrating a daf-12 action in the LIR pathway (Y. Aimoto, personal communication). 1. daf-12(lf)lin-28/md double mutant animals have a precocious phenotype equivalent to lin-28/md alone. Thus, lin-28 is epistatic to daf-12 in the heterochronic gene pathway (Aimoto et al. 1998), consistent with daf-12 playing a role in the regulation of lin-28 levels. 2. Mutations in lin-4 and lin-14 enhance the heterochronic phenotype in daf-12 mutants (Aimoto et al. 1998). This suggests daf-12 acts in a separate pathway independent of lin-4 and lin-14 to control developmental timing, potentially through lin-28 regulation. 3. Further, the retarded phenotype caused by a class I allele of daf-12 (Aimoto et al. 2000) is similar to that of lin-28/md mutants. 4. Because lin-4 is involved in lin-28 repression, it could be hypothesized that the miRNA hsl-7 acts in the LIR pathway. However, this is unlikely: hsl-7 is not expressed in the L1 to L3 stages when the LIR mechanism acts (Kemkhent et al. 2000). Also, hsl-7 mutant animals do not have elevated lin-28 expression in a wildtype or a lin-4(lf); lin-28/md (Aim et al.) genetic background, in which developmental timing depends on LIR (Y. Aimoto, personal communication).

Model for LIR pathways.

As stated in the Specific Aim, a working model is that lin-4-dependent translational repression initiates lin-28 downregulation resulting in a switch from high to intermediate levels of LIN-28 protein in L1. Thus in L2 and L3, the LIR pathway maintains downregulation resulting in a switch from an intermediate to a low level of LIN-28 protein (Figure 3). The aim in this proposal will test the function and molecular mechanism of the LIR pathway to repress Lin-28 in the early larval stages.

![Figure 3: Working model of translational repression of lin-28 by lin-4 and daf-12.](image)
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 informative to have headings telling the reader what is the experiment, question or hypotheses that is being examined in that section.
Aim 1. Test the hypothesis that df22 transcriptionally represses lre-26.

Rationale: The first goal is to determine if mutations in df22 alter lre-26 mRNA and protein levels during larval development. In animals carrying the rvl+ allele of df22, lre-26-GFP levels are elevated, indicating df22 involvement in the LIR pathway. However, it has not yet been demonstrated that df22 mutant have elevated levels of lre-26 mRNA or endogenous LIR-26 protein. This Aim will test whether the expression of the lre-26-GFP transgene accurately reflects the regulation of the lre-26 gene. Furthermore, these experiments will determine if df22 affects lre-26 transcription or translation. The strategy is to determine both lre-26 mRNA levels and endogenous LIR-26 protein levels at various time points during larval development in wildtype and df22 (rvl+) mutants.

Experimental plan and Considerations: To determine if LIR-26 protein levels are affected in df22 mutant animals, northern blot and western blot analysis will be performed on lysates from wildtype and df22 (rvl+) staged larvae. Additionally, effects of the LIR pathway on endogenous lre-26 mRNA and protein levels will be examined using a strain in which both the lre-26 dependent repression and the lre-26 positive feedback are absent (i.e., lre-26 (lre-14(lf))). In this genotype, the down-regulation of lre-26-GFP depends on the LIR, and is affected by Adf22 mutations (N. Ambros, personal communication). L1-stage larvae will be synchronized by batching in the absence of food. Following the addition of food (E. coli), 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 Osborn and Ambros, 1999. Multiple time points within each larval stage will be taken.

Northern analysis will be performed as described in Fleishman and Ambros, 1999. RNA will be prepared from staged lysates, separated by electrophoresis, and transferred to Zetablot membrane (BioRad). Membranes will be cross-linked and treated with a radiolabeled antisense lre-26 probe. Following hybridization and washing, membranes will be exposed to film and quantified with a Phosphorimag (Molecular Dynamics). To control for the amount of RNA loaded per lane, membranes will be stripped and re-probed with an antisense U6 probe. Lysates from worms which lack lre-26 (i.e., null allele) will be used as a negative control.
AIM 1. Test the hypothesis that def-17 translationally represses lin-38.

**Rationale:** The first goal is to determine if mutations in def-17 alter lin-38 mRNA and protein levels during larval development. In animals carrying the 1007 allele of def-17, lin-38::GFP levels are elevated, indicating def-17 involvement in the LRR pathway. However, it has not yet been demonstrated that def-17 mutants have elevated levels of lin-38 mRNA or endogenous LIN-38 protein. This Aim will test whether the expression of the lin-38::GFP transgene accurately reflects the regulation of the lin-38 gene. Furthermore, these experiments will determine if def-17 affects lin-38 transcription or translation. The strategy is to determine both lin-38 mRNA levels and endogenous LIN-38 protein levels at various time points during larval development in wildtype and def-17(1007) animals.

**Experimental plan and Consider:** To determine if LIN-38 protein levels are affected in def-17 mutant animals, northern blot and western blot analysis will be performed on lysates from wildtype and def-17(1007)/stripe larvae. Additionally, effects of the LRR pathway on endogenous lin-38 mRNA and protein levels will be examined using a strain in which both the lin-38::GFP transgene and the lin-38 promoter are driven by the LRR. In this genotype, the dose-dependence of lin-38::GFP depends on the LRR, and is affected by def-17 mutations (N. Anello; personal communication). L1-stage larvae will be synchronized by bathing in the absence of food. Following the addition of food (E. coli), larvae will be collected at increasing times during larval development. Stages of larvae will be determined using Nomarski differential interference contrast (DIC) microscopy as described in Ohara and Anello, 1996. Multiple time points within each larval stage will be taken.

Northern analysis will be performed as described in Flaherty and Anello, 1999. RNA will be prepared from staged lysates, separated by electrophoreses, and transferred to Zetabond membrane (Bio-Rad). Membranes will be cross-linked and hybridized with a radiolabelled antisense lin-38 probe. Following hybridization and washing, membranes will be exposed to film and quantified with a PhosphorImager (Molecular Dynamics). To control for the amount of RNA loaded per lane, membranes will be stripped and re-probed with an antisense U6 probe. Lysates from worms which lack lin-38 can be used as a negative control.

For western analysis, proteins will be separated by SDS-PAGE and transferred to PVDF membrane. Blots will be probed with anti-LIN-38 (to be obtained from E. Moss). Bands will be detected and quantified using enhanced chemiluminescence and the Molecular Dynamics imaging system with ImageQuant software. To control for the amount of protein loaded per lane, blots will also be probed with anti-β-tubulin which will allow for normalization and comparison of bands between larval stages. Non-specific IgG (Sigma) will be used as a negative control antibody. Lysates from worms which lack lin-38 (i.e., null alleles) will be used as an additional negative control for anti-LIN-38 specificity.

**Interpretation:** These experiments will determine if def-17 regulates, directly or indirectly, lin-38 mRNA or protein levels. It is important to note that because lin-38 mRNA levels remain constant through larval development (E. Moss; personal communication), def-17 regulation of lin-38 transcription is not expected. Northern analysis will serve as a control to verify that potential changes in LIN-38 protein levels between wildtype and def-17(1007) animals are due to translational control. If LIN-38 protein levels remain elevated in L1 and L3 in def-17(1007)/toe larvae as compared to wildtype animals, then this will support a two-step translational repression model for regulation of the LRR in which both bcl-10-dependent repression and LRR are required. Elements in lin-38 required for regulation by the LRR pathway will be examined in Aim 2. If no misexpression is observed in lysates from def-17(1007)/toe relative to wildtype lysates, it is possible that functional redundancy with the lin-38 pathway is sufficient to appropriately downregulate lin-38. Analysis of lysates from lin-38(10); lin-38(10)/toe animals will address this possibility.

**Alternate Methods and Limitations:** Due to some heterogeneity of developmental timing, each lysate will represent an average of protein levels in pooled individual animals at slightly different stages. This heterogeneity may prevent the detection of LIN-38 protein levels in lysate from def-17(1007) or lin-38(10); lin-38(10)/toe animals, if such changes are subtle relative to wildtype. To address this possibility, the sensitivity of the detection protocol will be optimized in order to use as few individual animals as possible. In addition, shorter time intervals within the L3 stage can be examined.

**Typos!! Should be lin-4(10); lin-14(10)**

**Expected finding leads to experiments in Aim 2.**

**Approach if alternative result**
AIM 2. Identify elements in the 3' UTR of lin-28 that are necessary and sufficient for daf-12 translational repression.

Rationale: The 3' UTR of lin-28 has been determined to be necessary to direct the temporal downregulation of lin-28. Deletion of lin-4 complementary elements in the 3' UTR results in a gain-of-function lin-28 allele that remains elevated at the L4 stage (Moss et al. 1997). Furthermore, lin-28::GFP under the regulation of the 3' UTR of the uve-54 gene is not temporally downregulated (Moss and Ambros, personal communication). These results suggest that all the known negative regulatory inputs to lin-28 act via the 3' UTR, but this supposition has not been directly tested for the LIR pathway. Therefore, it will be determined whether the lin-28 3' UTR is sufficient to direct the downregulation of a GFP reporter gene by the LIR pathway, and what elements within the 3' UTR are required.

Experimental plan and Controls:

Determine if the lin-28 3' UTR is sufficient for downregulation by the LIR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a col-10 promoter (Hong 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 col-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. col-6 (sul-6096)), into wildtype animals. Animals will be crossed into lin-4(f2)lin-1(lx1) and lin-4(f2)lin-1(lx1);dep-1/hr611 genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIR pathway. As outlined in Table 1, a LIR-dependent construct is expected to be downregulated by the L4 stage in wildtype and lin-4(f2);lin-1(lx1) genetic backgrounds. However, in a lin-4(f2);lin-1(lx1);dep-1/hr611 genetic background, in which the LIR pathway is absent, a LIR-dependent construct is expected to remain elevated in the L4 stage. Southern blotting with 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 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 (Hong et al. 2000). To obtain a picture in the linear range of detection, multiple exposures will be taken. Image analysis will be
Experimental plan and Concepts:

Determine if the lin-28 3' UTR is sufficient for downregulation by the LIR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a col-10 promoter (Hong 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 col-10 promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germplasm transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., col-6 (su1006)), into wildtype animals. Arrays will be crossed into lin-4(flt:lin-1-4ts) and lin-4(flt:lin-1-4ts) daf-12(rh61) genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIR pathway. As outlined in Table 1, a LIR-dependent construct is expected to be downregulated by the L4 stage in wildtype and lin-4(flt:lin-1-4ts) genetic backgrounds. However, in a lin-4(flt:lin-1-4ts) daf-12(rh61) genetic background, in which the LIR pathway is absent, a LIR-dependent construct is expected to remain elevated in the L4 stage. Southern blotting with 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 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 (Hong et al. 2000). To obtain a picture in the linear range of detection, multiple exposures will be taken. Image analysis will be

Interpretations: The working model predicts that the 3' UTR is sufficient for both the lin-4-dependent repression and LIR mechanisms. Therefore, according to the model, the temporal pattern of expression of the col-10-GFP-lin-28 3' UTR reporter construct will recapitulate the expression patterns observed for lin-28 in wildtype and in mutant strains (see Table I). In particular, the col-10-GFP-lin-28 3' UTR construct is expected to be downregulated in a wildtype and (lin-4(flt:lin-1-4ts) genetic backgrounds in which the LIR pathway is functional, but not in the LIR-deficient genotype, lin-4(flt:lin-1-4ts) daf-12(rh61). If, on the other hand, the 3' UTR is not sufficient to faithfully reproduce lin-28 expression, then these data would indicate that other elements in either the 5' UTR or in the LIN-28 protein are responsible for LIR. For example, downregulation of lin-28 may require access of the affected nascent polypeptide to protein degradation machinery, and hence may require motifs akin to the "destruction box" for ubiquitin-mediated proteolysis for targeted LIN-28 protein degradation. If

Presents overall approach, but not
lost in experimental
details

Expected result, given
the model

Reasonable possibility if
expected result not
found
Experimental plan and Controls:

Determine if the lin-28 3' UTR is sufficient for downregulation by the LIR pathway. Standard molecular biology techniques will be used to generate reporter constructs, consisting of a col-10 promoter (Hong 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 col-10 promoter directs specific expression in the lateral hypodermal cells, thus aiding in the analysis of expression levels. Constructs will be confirmed by sequencing. Germ line transformation will be performed by microinjecting reporter constructs along with a marker, which allows for the selection of transformants (e.g., col-6 (su1006)), into wildtype animals. Arrays will be crossed into lin-4(fla-1-4ts) and lin-4(fla-1-4ts);def-2(r661) genetic backgrounds in order to analyze the sensitivity of reporter constructs to the LIR pathway. As outlined in Table 1, a LIR-dependent construct is expected to be downregulated by the L4 stage in wildtype and lin-4(fla-1-4ts) genetic backgrounds. However, in a lin-4(fla-1-4ts);def-2(r661) genetic background, in which the LIR pathway is absent, a LIR-dependent construct is expected to remain elevated in the L4 stage. Southern blotting with 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 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 (Hong et al. 2000). To obtain a picture in the linear range of detection, multiple exposures will be taken. Image analysis will be

Anticipates potential pitfall

Illustrates what is necessary to obtain reliable data
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 ATGx in mouse liver cells, based on immunofluorescence.

- Glut8 (−/−) knockout mouse displays an increased autophagy phenotype in liver cells (and also suppresses fatty liver disease in mice fed a high fructose diet).

**Hypothesis:**

- Glut8 is a negative regulator of ATGx activity in the autophagy pathway and acts as an inhibitor through binding ATGx and making it unavailable to stimulate the autophagy pathway.

**Prediction:** Blocking Glut8 – ATGx binding, but not other 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 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.

Advanced Genetics students:
Approach should 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.

http://www.indiana.edu/~wts/pamphlets/plagiarism.shtml
http://www.unc.edu/depts/wcweb/handouts/plagiarism.html
Next Monday, in Small Group Discussion Sections

Long Chain FA Proposal
ADAR Editing Proposal

- 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?