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

Three essential sections

Specific Aims

Background and Significance

Experimental Design and Methods
Three essential sections

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.

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.
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.
5

General background

Specific background

Novel finding

Model/hypothesis to explain finding

Goal & what will be learned

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

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 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.
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-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 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.
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 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.

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, temporal control regulates developmental timing events such as the regulation of the insect metamorphosis cycle by ecdysone (Thummel, 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 (Amitai et al., 1998; Amred et al., 2000; Thummel and Larsson, 2000).

In C. elegans, embryogenesis is followed by four stages of larval development distinguished morphologically by most and the subsequent formation of the fully mature adult. During larval development (stages L1-L4), the appropriate execution of stage-specific programs (L1-L4) is controlled by heterochronic genes, among which the most well-described are lin-4, lin-14, lin-29 and lin-39 (Ambros and Horvitz 1984; Ambros 1989; Slack and Horvitz 1997; Ambros 2000). Mutations in heterochronic genes result in the deletion or activation of stage-specific programs of cell division, differentiation, or migration.

lin-29:Regulates developmental timing at the L3-L4 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 mutants exhibit the S2 program and precociously express the S3 program in L2 (Ambros and Horvitz, 1984). For example, in lin-29(ek1) animals, lateral hypodermal seam cells exit the S2-specific cell division in L3 leading to an abnormal number of seam cells (Ambros and Horvitz 1984). In contrast, gain-of-function (Gf) lin-29 mutants retain the S2 program in L3 and correspondingly have elevated levels of LIN-29 protein at the L4 stage (Moss et al. 1997). A putative DNA-binding protein (Moss et al., 1997), the LIN-29 protein likely represses L3-specific events by binding to and repressing downstream target mRNAs. LIN-29 protein levels are dynamic during larval development, with the strongest expression in late embryos and L1 larvae, reduced expression in L2 larvae and undetectable LIN-29 levels in L3 larvae (Moss et
Importance of *lin-28* 3'UTR

What proposal will focus on

Importance of *daf-12*

Argue against alternative possibilities

Model to be tested in this proposal

Mechanisms that Downregulate *lin-28* During Early Larval Development.

There are three inputs which contribute to the downregulation mechanism of *lin-28* in *iwm-4* dependent repression, positive feedback by *lin-14*, and the LIR pathway. Northern analysis demonstrates no change in *lin-28* mRNA levels during larval development (E. Mess, 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 *iwm-4* gene is not repressed 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 of *iwm-4* is mediated by specific elements in the *lin-28* 3' UTR. A *lin-20* allele in which a *lin-4* complimentary 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 the model that *daf-12* is upstream of *lin-28* and regulates execution of the L3 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 1: Evidence for LIR pathway in *lin-28* Translational Repression.](image)

**Genotype** | Phenotype | *lin-28* Protein | Function
--- | --- | --- | ---
*daf-12* | *daf-12* | *lin-28* downregulated | governed by the LIR pathway in animals lacking *iwm-4* and with a temperature sensitive allele of *lin-14 (iwm-4), lin-20(f2201); lin-14(001) In this genotype, *lin-28* is downregulated normally, presumably by the LIR pathway alone (Ahn et al. 1997).
*HDL* | *HDL* | *lin-28* upregulated | by the LIR pathway

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(iw1), lin-14(eu1), daf-22(ken1)*, thus demonstrating *daf-12* action in the LIR pathway (E. Ahn et al., personal communication). 1. *daf-12*(+/hd1) *lin-28*(*f2201*) double mutant animals have a progression phenotype equivalent to *lin-28*(*f2201*) alone. Thus, *lin-28* is sensitive to *daf-12* in the heterochronic gene pathway (Ahn et al. 1997), consistent with *daf-12* playing a role in the regulation of *lin-28* levels. 3. Mutations in *iwm-4* and *lin-14* enhance the heterochronic phenotype in *daf-12* mutants (Ahn et al. 1997). 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. 4. Further, the enhanced phenotype caused by a class 1 allele of *daf-12* (Ahn et al. 2003) is similar to that of *lin-28* mutants.

Because *lin-4* is involved in *lin-28* repression, it could be hypothesized that the mRNA, *let-7*, acts in the LIR pathway. However, this is unlikely: *let-7* is not expressed in the L1 to L3 stages when the LIR mechanism acts (Kemphort et al. 2000). Also, *let-7* mutant animals do not have elevated *lin-28* expression in a wildtype or a *lin-4(iw1), lin-14(eu1)* genetic background, in which developmental timing depends on LIR (V. Ahn et al., 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. Then 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 1). 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 1: 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 dfj-12 transcriptionally represses lin-28.

**Rationale:** The first goal is to determine if mutations in dfj-12 alter lin-28 mRNA and protein levels during larval development. In animals carrying the rde-2 allele of dfj-12, lin-28::GFP levels are elevated, indicating dfj-12 involvement in the LIR pathway. However, it has not yet been demonstrated that dfj-12 mutants have elevated levels of lin-28 mRNA or endogenous LIN-28 protein. This Aim will test whether the expression of the lin-28::GFP transgene accurately reflects the regulation of the lin-28 gene. Furthermore, these experiments will determine if dfj-12 affects lin-28 transcription or translation. The strategy is to determine both lin-28 mRNA levels and endogenous LIN-28 protein levels at various time points during larval development in wildtype and dfj-12(rde-2) animals.

**Experimental plan and controls:** To determine if LIN-28 protein levels are affected in dfj-12 mutant animals, northern blot and western blot analysis will be performed on lysates from wildtype and dfj-12(rde-2) staged larvae. Additionally, effects of the LIR pathway on endogenous lin-28 mRNA and protein levels will be examined using a strain in which both the lin-28::GFP transgene and the lin-28 positive feedback are absent (i.e., lin-14(0); lin-14(0))). In this genotype, the down-regulation of lin-28::GFP depends on the LIR, and is affected by dfj-12 mutations (N. Ambros, personal communication). L1-stage larvae will be synchronized by hatching 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 Olsen and Ambros, 1999. Multiple time points within each larval stage will be taken.

**Northern analysis** will be performed as described in Finkelstein and Ambros 1999. RNA will be prepared from staged lysates, separated by electrophoresis, and transferred to Zetaprobe membrane (BioRad). Membranes will be cross-linked and incubated with a radiolabeled antisense lin-28 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-28 (i.e., null alleles) will be used as a negative control.
AIM 1. Test the hypothesis that def-17 translationally represses lin-36.

**Rationale:** The first goal is to determine if mutations in def-17 alter lin-36 mRNA and protein levels during larval development. In animals carrying the rde-2 allele of def-17, lin-36::GFP levels are elevated, indicating def-17 involvement in the LIR pathway. However, it has not yet been demonstrated that def-17 mutants have elevated levels of lin-36 mRNA or endogenous LIN-36 protein. This Aim will test whether the expression of the lin-36::GFP transgene accurately reflects the regulation of the lin-36 gene. Furthermore, these experiments will determine if def-17 affects lin-36 transcription or translation. The strategy is to determine both lin-36 mRNA levels and endogenous LIN-36 protein levels at various time points during larval development in wildtype and def-17 mutant backgrounds.

**Experimental plan and Considerations:** To determine if LIN-36 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(rde-2) staged larvae. Additionally, effects of the LIR pathway on endogenous lin-36 mRNA and protein levels will be examined using a strain in which both the lin-36-dependent repression and the lin-36 positive feedback are absent (i.e., lin-14(fu); lin-14(0)). In this genotype, the down-regulation of lin-36::GFP depends on the LIR, and it is affected by lin-36 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 Ohan and Ambros, 1999. Multiple time points within each larval stage will be taken.

Northern analysis will be performed as described in Feinbaum and Ambros 1999. RNA will be prepared from staged lysates, separated by electrophoreses, and transferred to Zetaprobe membrane (BioRad). Membranes will be cross-linked and incubated with a radiolabeled antisense lin-36 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-36 (i.e., null alleles) will be used as a negative control.

 Typos!! Should be lin-4(0); lin-14(0)
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 def-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 unc-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 (Moos 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. Germinal 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(f[d:m-14ts]) and lin-4(f[d:m-14ts];del.1[her6]) 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(f[d:m-14ts]) genetic backgrounds. However, in a lin-4(f[d:m-14ts];del.1[her6]) 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...
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(3UTR) reporter construct will recapitulate the expression patterns observed for lin-28 in wildtype and in mutant strains (see Table 1). In particular, the col-10-GFP-lin-28(3UTR) construct is expected to be downregulated in a wildtype and lin-4(0);lin-14(ts) genetic backgrounds in which the LIR pathway is functional, but not in the LIR-deficient genotype, lin-4(0);lin-14(ts);daf-12(hd2). 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 mauer polyprotein to protein degradation machinery, and hence may require mauer skin to the "destruction box" for ubiquitin-mediated proteolysis for targeted LIN-28 protein degradation. If

Expected result, given the model

Reasonable possibility if expected result not found

Experimental plan and Controls: To 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 isolation of transformants (e.g., col-6 (sa1096)), into wildtype animals. Arrays will be crossed into lin-4(0);lin-14(ts) and lin-4(0);lin-14(ts);daf-12(hd2) 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(0);lin-14(ts) genetic backgrounds. However, in a lin-4(0);lin-14(ts);daf-12(hd2) 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.

Anticipates potential pitfall

Illustrates what is necessary to obtain reliable data

To analyze the reporter gene constructs in wildtype and the different genetic backgrounds, larvae at the L1 and L4 stages (see Aln 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
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://writingcenter.unc.edu/handouts/plagiarism/
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?