The Department of Biology
Comprehensive Feasibility of Dissertation and Doctoral Oral Exam

Timing:
  The comprehensive exam must be held before the end of the spring semester of the third year.

Outline of Expectations for Students:
- Schedule a thesis committee meeting for the exam. Scheduling includes finding a date that works for each of your committee members and finding a room to hold the exam in.
- Prepare an NIH/NSF style written grant proposal on your thesis project (just the science and bibliography parts – not any of the other forms). While the proposal should include some preliminary data, the primary emphasis should be on the logic and feasibility of the project. It is also important for both the student and the committee to understand that the proposal is not a contract – the goals of the proposal may evolve over the course of the student's graduate experience. An exemplary proposal is attached to this document.
- Distribute the proposal to the committee 1-2 weeks prior to the meeting.
- Obtain a copy of the Doctoral Exam Report Form (DERF) from the Biology Department's Student Services Administrative Assistant (see attached).
- Conduct the oral exam during the meeting (see below for details).
- Have your committee members sign the DERF.
- Return the DERF to the Biology Department's Student Services Administrative Assistant.

The Oral Exam:
  Comprehensive exams are managed by the student's thesis committee. During the exam your PI cannot serve as the chair, instead another committee member must be appointed as the exam chair. At the beginning of the meeting, the student will be asked to leave the room for a few minutes and their PI will give the committee a very brief summary of the student's progress. The student will then be invited back into the room and will begin an oral presentation of their proposal (typically accompanied by a PowerPoint presentation). The committee members are expected to frequently question the student during the presentation. Questions can be specific to the proposal or they can broadly test the student's knowledge of key topics the committee expects the student to have mastered (i.e. genetics, cell biology, biochemistry etc.). It is important for the PI to remain as silent as possible during the exam (i.e. they shouldn't come to the student's rescue if they are struggling with a question), though they are encouraged to challenge the student with their own questions. After the presentation and questioning are finished the student will be asked to leave the room again. The chair will lead a discussion of the student's performance on the exam and will issue a pass/fail grade. Students must receive a pass from 2/3 of the committee (typically 4 out of 5 members). The student will then be invited back into the room and the committee will render its decision.

  The committee has the option of passing a student but requiring some additional work

including but not necessarily limited to: i) re-writing the proposal, ii) taking an additional course or iii) reading some set of material.

Other Considerations:
- Some students or committees are at located remote locations, making an in-person meeting very difficult. Under such circumstances a video-conference is acceptable.
- Faculty scheduling conflicts (sabbaticals etc.) may interfere with reaching a quorum for the exam. Under such circumstances the student may hold the exam with a sub-quorum and arrange for a separate exam with the missing member(s). In these cases the exam chair must confidentially communicate with the missing member(s) after the separate exam so that a final pass/fail grade can be issued.

Failure and Re-examination:
Students who fail the comprehensive exam may take it a second time. A minimum of three months must separate the first and second exam attempts. Students who fail the comprehensive exam twice become ineligible for further graduate work. Upon request of the Director of Graduate Studies (DGS), the Graduate School may permit a student a third and final opportunity to take the examination. Such requests are NOT automatic and are at the discretion of the DGS after consultation with the student's PI.
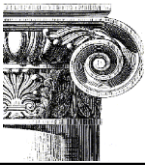
THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
The Graduate School
DOCTORAL EXAM REPORT FORM
NOTE: The Committee Composition form should be on file with the Graduate School **before** exam results are reported.

Student's Name _____ PID# _____

Department/Curriculum/School: _____

PART I: REPORT OF PRELIMINARY WRITTEN EXAMINATION

On behalf of a majority of the examining committee, I certify that the above named student:

_____ successfully passed the examination

_____ failed to pass the examination   _____   _____
                                          signature of committee chair   *date*

Check here if student previously failed exam. Date(s):_____

☐ **By initialing, the committee chair certifies that this student was registered as required during the term this work was completed.**

PART II: REPORT OF ORAL EXAMINATION

On behalf of a majority of the examining committee, I certify that the above named student:

_____ successfully passed the examination

_____ failed to pass the examination   _____   _____
                                          signature of committee chair   *date*

Check here if student previously failed exam. Date(s):_____

☐ **By initialing, the committee chair certifies that this student was registered as required during the term this work was completed.**

PART III: REPORT OF THE FINAL ORAL EXAMINATION (defense of dissertation)

A majority of the committee for the above named student has judged the dissertation defense to be:

_____ acceptable

_____ unacceptable   _____   _____
                                          signature of committee chair   *date*

Committee member signature/date	Pass/Fail	Committee member signature/date	Pass/Fail
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Check here if student previously failed exam. Date(s):_____

☐ **By initialing, the committee chair certifies that this student was registered as required during the term this work was completed.**

PART IV: REPORT OF THE FINAL DISSERTATION (can be completed at the same time as Part III as appropriate)

A majority of the committee for the above named student has judged the dissertation to be:

_____ acceptable

_____ unacceptable   _____   _____
                                          signature of committee chair   *date*

Committee member signature/date	Pass/Fail	Committee member signature/date	Pass/Fail
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

☐ **By initialing, the committee chair certifies that the required edits were made and the final document is approved for electronic submission.**

- Submit to the Graduate School after all activities have been successfully completed
- Keep copies for your files

Rev 7/09

The Role of Canoe in Morphogenesis

Jessica K. Sawyer

Specific Aims: Cell-cell adhesion is critical for development. The adherens junctions (AJs) are thought to form mechanical attachments between cells by linking actin bundles in neighboring cells together through the cadherin-catenin complex. However, recent work by the Nelson and Weis labs has challenged the idea that cadherins and catenins form this link. Additional proteins are recruited to AJs whose function is less well understood. These proteins may act partially redundantly to regulate junctional plasticity by regulating connections to the actin cytoskeleton and to signal transduction machinery. To test this hypothesis I am defining the function of Canoe (Cno)/ Afadin (AF-6) in *Drosophila* morphogenesis. The experiments proposed here will elucidate Cno's role in morphogenesis and its relationship to AJs.

Aim I. Determine the Role of Cno During Morphogenesis. *Hypothesis: Cno acts redundantly to regulate junctional plasticity by regulating connections to actin and/or to signal transduction machinery.*

Aim II. Assess the Function of Known Cno Binding Partners and Determine Novel Ones. *Hypothesis: Cno associates with different partners throughout development to mediate multiple morphogenetic processes.*

Background and Significance

Morphogenesis requires dynamic regulation of cell-cell adhesion and cell shape change. These two processes are coordinated by AJs linking neighboring cells to each other and to the apical actin cytoskeleton. Over the past twenty years, the core molecular machinery of AJs has been uncovered and characterized (Gates and Peifer, 2005). Cadherins are Ca^{2+} dependent transmembrane proteins that mediate homophilic adhesion. The cytoplasmic tails of cadherins bind to β-catenin, which in turn binds α-catenin. Finally, α-catenin can directly bind actin filaments. Using the "commutative property" it has been long assumed AJs were linked to the cytoskeleton via the cadherin-catenin complex. However, recently the Nelson and Weis groups discovered this may not be the case. They found while the binary relationships are easily detectable, the cadherin-catenin complex could not bind to actin (Drees et al., 2005; Yamada et al., 2005). While there are several possibilities to explain this result, one is that other molecules mediate the direct connection between AJs and the cytoskeleton.

Luckily, several proteins reside at AJs whose functional roles are not well understood. One interesting candidate is the nectin-afadin complex. Afadin was originally isolated from rat brains as an actin filament binding protein. Two isoforms were identified: a long form (l-afadin) that has both a PDZ domain and a F-actin binding domain and a short form (s-afadin) that has only the PDZ domain. Afadin has 90% sequence identity to human *AF-6*, which was isolated as a translocation fusion partner to the *ALL-1* gene. This translocation is involved in some cases of acute leukemia. In addition, Afadin was shown to localize at AJs (Mandai et al., 1997). This localization suggested Afadin might play a role in adhesion. Two groups independently knocked out *afadin* in mice. Homozygous *afadin* mice were indistinguishable from wild-type (WT) mice prior to 7.5 days *post coitum* (dpc). However subsequently the embryos were impaired in ectoderm organization, migration of mesoderm, and lost some structures derived from ectoderm and mesoderm. This led both groups to conclude that Afadin is an important regulator of AJs during development (Ikeda et al., 1999; Zhadanov et al., 1999). However, it is worth noting that this phenotype is significantly less severe than that of mice lacking E-cadherin (Larue et al., 1994) or β-catenin (Haegel et al.,
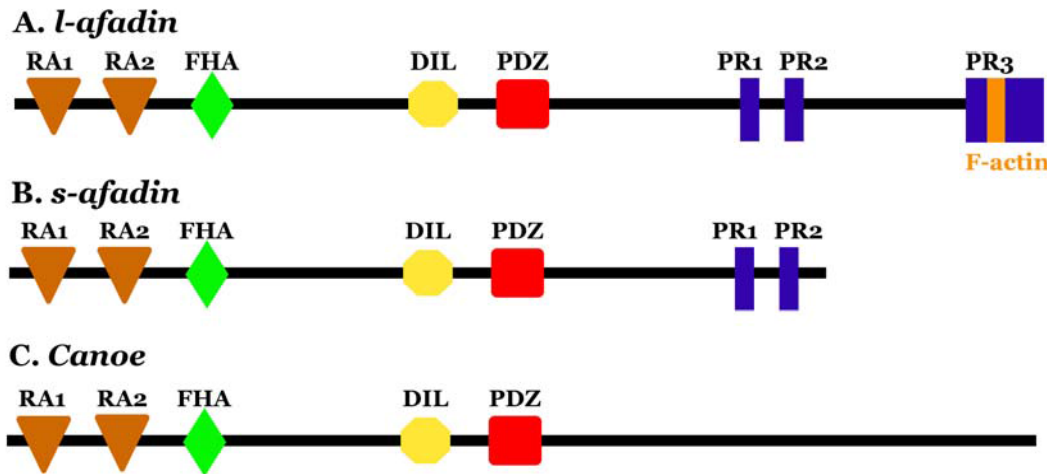
1995).

***Figure 1.* Domain structure of Afadin and Canoe.**
(A) l-afadin, 1829aa; rat (B) s-afadin, 1663aa; rat (C) Canoe, 2051aa; *Drosophila*

The structure of Afadin suggests a scaffolding role. It has two Ras association (RA) domains, a forkhead-associated (FHA) domain, a DIL domain, a PDZ domain, three proline rich domains, and an actin binding domain (*Fig. 1*). The RA domains bind H-Ras (Kuriyama et al., 1996) and Rap1A (Linnemann et al., 1999), although Rap1A is considered to be the preferred binding partner. The FHA domain is known to function as a nuclear localization signal in other proteins, however thus far Afadin has not been observed in the nucleus. The DIL domain is a protein interaction domain. Afadin DIL domain-interacting protein (ADIP) has been identified as a binding partner. ADIP can also bind to α-actinin, thus providing a potential link between the actin cytoskeleton and AJs (Asada et al., 2003). The PDZ domain binds a transmembrane Ca^{2+} independent immunoglobulin (Ig)-like cell-adhesion molecule (CAM) called Nectin. Nectin and Afadin colocalize at AJs (Takahashi et al., 1999). Nectins function in a similar fashion as cadherins, first forming cis-dimers and subsequently forming trans-dimers. Unlike cadherins, Nectins often form hetero-trans-dimers (Takai and Nakanishi, 2003). Thus far Ponsin (Mandai et al., 1999) and Profilin (Boettner et al., 2000) have been identified to bind to the proline-rich domains, providing another way for Afadin to link AJs and the actin cytoskeleton. Afadin has an actin-binding site, providing a direct link between AJs to cystoskeleton (Mandai et al., 1997). Finally, Afadin can bind to α-catenin; however, this interaction is only observed under certain conditions (Tachibana et al., 2000). While biochemical data suggests that Afadin

could function as a link between AJs and the cytoskeleton, there is little functional data to support this hypothesis. Afadin is highly conserved, with homologs in humans, mice, flies, and worms.

Drosophila provides a tractable genetic system to understand Afadin function *in vivo*. The Afadin homolog, *canoe* (*cno*), is encoded by a large gene with 19 exons covering ~70 kb of genomic sequence. Three mRNAs exist (7.0, 7.5, and 8.2kb), probably due to different polyadenylation sites. *cno* mRNA levels are highest in the embryo, decline in larval development, and increase in pupae and adults (Miyamoto et al., 1995). There are two Cno isoforms: a long form of 2051 amino acids and a short form of 1882 amino acids (aa), which lacks an internal 169aa thus deleting part of the DIL domain. It is unclear if there is an important biological difference between these two isoforms.

cno was first found in the Nüsslein-Volhard /Wieschaus screen for mutants affecting the pattern of the larval cuticle. The gene was so named because the cuticle had a dorsal open phenotype and had the appearance of a canoe (Jürgens et al., 1984). Dorsal closure (DC) occurs during mid-embryogenesis, when the dorsal portion of the embryo is covered by the amnioserosa, a squamous epithelium that will not be incorporated into the larva. Two migrating lateral epithelial sheets meet at the dorsal midline and fuse, enclosing the animal in epidermis. The establishment of the leading edge (LE) is important for DC. This subset of cells elongates on the dorsal-ventral axis and extends filopodia that help guide the sheets together. One pathway essential for this process is the Jun amino-terminal kinase (JNK) signaling pathway (Noselli and Agnes, 1999). The *cno* phenotype is enhanced when in the background of JNK pathway members. Expression of the JNK target gene *decapentaplegic* (*dpp)* is reduced in LE edge cells in *cno* mutants, leading to the hypothesis that Cno was either upstream or parallel to the JNK pathway. In addition, *cno* enhances eye and wing phenotypes of Notch pathway mutants (Miyamoto et al., 1995).

Several Cno binding partners have been identified. Cno directly binds Polychaetoid (Pyd)/Zona Occludens-1 (ZO-1). These proteins genetically interact; when *pyd* was placed in a sensitized *cno* background, it enhanced the *cno* dorsal open phenotype (Takahashi et al., 1998). This result was also observed with *Rap1*. However, a *cno* transgene lacking the two RA domains partially rescues the DC

phenotype, which suggests that Cno has both Rap1-dependent and Rap1-indenpendent functions in DC (Boettner et al., 2003). Cno also binds H-Ras/Ras1 and this interaction is important for regulation of cone cell formation in the *Drosophila* eye (Matsuo et al., 1997). Finally, Cno directly binds Echinoid (Ed), a nectin-like protein. Cno is mislocalized in *ed* mutant clones in wing discs, suggesting Ed helps to localize Cno to AJs. Interestingly, Ed also binds to Bazooka (Baz/PAR-3), an apical polarity protein, suggesting *ed* may also help to establish polarity (Wei et al., 2005).

These studies suggest that Cno may play a complex role regulating adhesion or the cytoskeleton, but do not clarify its mechanism of action. One difficulty is most studies of Cno have focused on zygotic loss of function, effects on late stages in embryogenesis, and/or the use of partially functional alleles in imaginal disc tissues. However, it is likely a significant maternal protein pool is present in the embryo. In order to determine the true *cno* null phenotype, we removed the maternal and zygotic protein.

Preliminary Results

Different hypotheses for Cno function seemed plausible. Cno could be a core adhesion molecule, essential for adhesion. At the other end of the spectrum, Cno may solely regulate signaling during late morphogenesis. We sought to remove both maternal and zygotic protein to address this issue. We wanted to use a null allele, to fully eliminate Cno function. We sequenced the cno^{R2} allele; it results from an early stop codon (K221Stop) after the first RA binding domain, suggesting it is a null allele. We utilized the FLP/FRT and dominant female sterile system to make germline clones lacking Cno function (Chou et al., 1993). First, we used cuticles as a way to assess the severity of the phenotype. Similar to what was previously found with other *cno* alleles, the zygotic cno^{R2} phenotype is somewhat variable; less severe embryos have head defects or head holes (88%) and a more severe class has head holes and ventral and/or dorsal holes (11%). The cuticle phenotype of maternal and zygotic mutants (m/z) was much more severe than the zygotic phenotype alone. In addition to head and/or dorsal holes, many had holes in the ventral epidermis (68%), while others had only dorsal cuticle (32%) (*Fig. 2*). However, the cuticle phenotype was not as severe as seen in mutants lacking the core adhesion molecules, *E-cadherin* or *armadillo/ß-catenin*. This suggests Cno is not a core

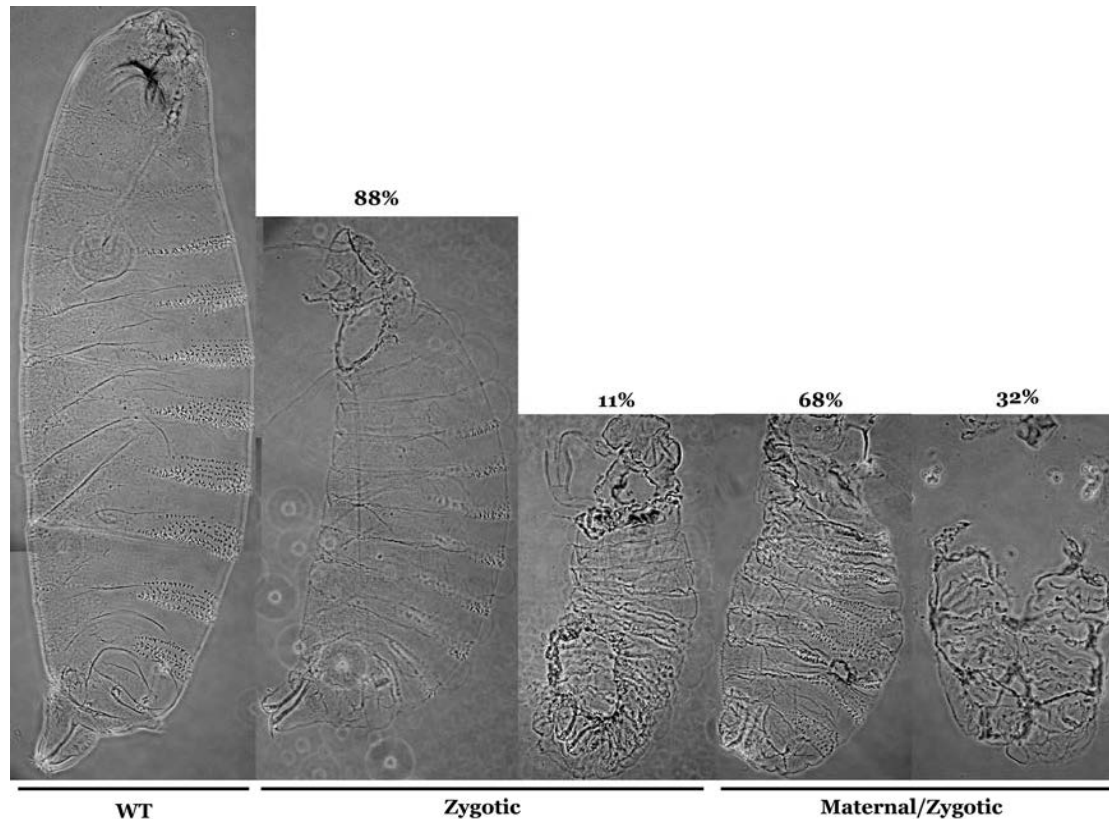adhesion molecule, but plays an important role in embryogenesis.

*Figure 2. **cno** zygotic and maternal/zygotic phenotypes*.
cno zygotic mutants have head holes and/or dorsal holes. *cno* m/z mutants include a class with head holes and ventral holes and a more severe class with only dorsal cuticle.

In order to understand the terminal *cno* phenotype, we analyzed earlier stages in embryogenesis and found that mesoderm invagination is compromised in *cno*$^{M/Z}$ mutants. Mesoderm invagination is the first step in *Drosophila* gastrulation. Mesoderm forms from the most ventral cells of the embryo. Cells destined to be mesoderm accumulate apical actin and myosin and apically constrict, creating a bend in the epithelium. The mesoderm is then internalized as a tube and subsequently undergoes an epithelial-to-mesenchymal transition and migrates throughout the embryo (Dawes-Hoang et al., 2005; Sweeton et al., 1991).

cno$^{M/Z}$ mutants do not completely internalize the mesoderm. After gastrulation should be complete, many mesodermal cells remain on the embryo surface and begin to divide in this aberrant location. One reason that mesodermal cells might not be getting internalized completely is a failure to initiate apical constriction. Therefore, I analyzed earlier stages to examine the initiation of the furrow, using still fixed images and live imaging of embryos expressing moesin::GFP, which binds to actin filaments (data not

shown). $cno^{M/Z}$ mutants do initiate furrow formation by apical constriction (*Fig.3G,H.*) and cells constrict synchronously, unlike defects seen in mutants like *folded gastrulation (fog)* and *concertina (cta)*, in which apical constriction is uncoordinated and an abnormal furrow forms (Sweeton et al., 1991).
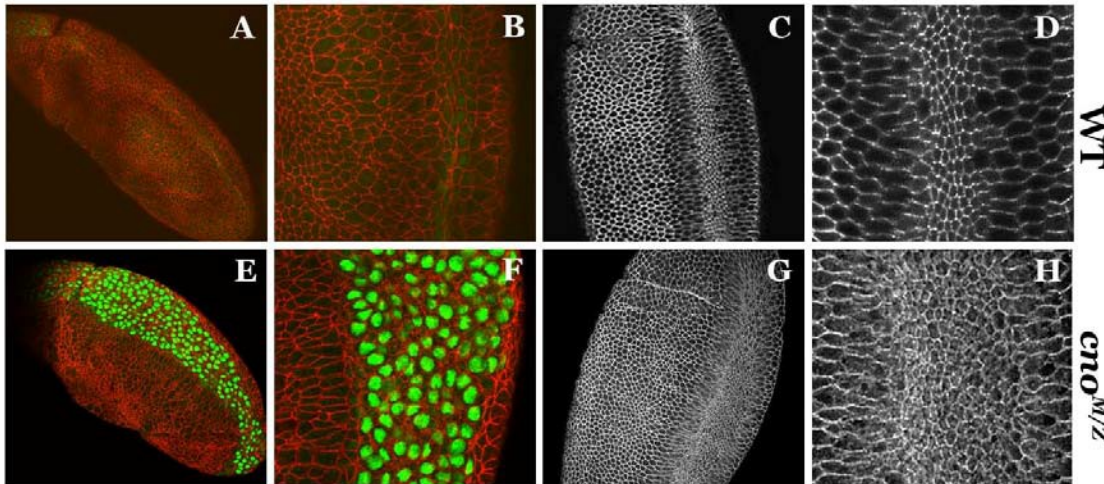

Figure 3. $cno^{M/Z}$ **mutants do not completely internalize the mesoderm, but initiate furrow formation.**
(A-D) WT. (E-H) $cno^{M/Z}$.
(A, B, E, F) PTyr (red) and Twist (green) staining. Some mesodermal cells (green) are trapped on the surface in $cno^{M/Z}$ mutants.
(C, D, G, H) Ptyr (white) staining. $cno^{M/Z}$ mutants do initiate constriction and ventral furrow formation.

While there is a defect in mesoderm invagination, AJs are not disrupted and overall epithelial integrity remains largely intact. Neither E-cadherin nor Pyd localization is disrupted in $cno^{M/Z}$ mutants (*Fig 4.*). E-cadherin and Pyd staining is reduced in mesodermal cells that remain on the surface of a $cno^{M/Z}$ mutant (right side of *Fig. 4C,D*). This reduction is normal, aiding the mesodermal epithelial-to-mesenchymal transition. This suggests cell fates are not altered in $cno^{M/Z}$ mutants. Consistent with this, $cno^{M/Z}$ mutant mesoderm also expresses Twist, the transcription factor confering mesodermal fate (*Fig.3*).
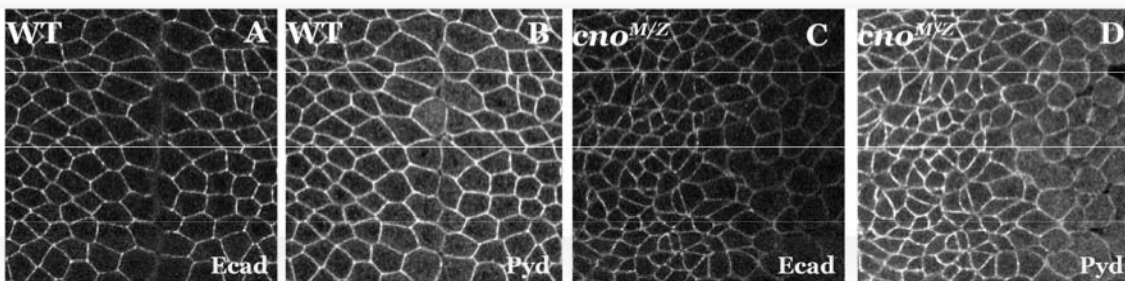

Figure 4. **AJ localization is not disrupted in *cno* mutants.**
(A,B) WT. (C,D) $cno^{M/Z}$.
(A, C) E-cadherin staining. E-cadherin localizes normally in $cno^{M/Z}$ mutant embryos.
(B, D) Pyd staining. Pyd localizes normally in $cno^{M/Z}$ mutant embryos.

We next looked in detail at mesoderm invagination. While $cno^{M/Z}$ mutants initiate apical construction correctly, defects in the localization of actin and myosin arise as constriction proceeds. Instead of an even sheet-like accumulation on the top of constricting cells, $cno^{M/Z}$ mutant cells have large "balls" of actin and myosin that form in the center of the apical portion of the cell (*Fig. 4E, 4F*, and data not shown). This is also apparent in sections of the ventral furrow (*Fig. 4C,D,G,H*). Thus, it appears $cno^{M/Z}$ mutants initiate constriction but do not complete the process; we hypothesize that the actin/myosin ring detaches from AJs and continues to constrict, which results in "balls" of actin or myosin at the apical portion of the cell. Experiments in the Weischaus lab are consistent with this; they demonstrated that the actomyosin contractile ring still constricts in the complete absence of AJs (Dawes-Hoang et al., 2005). I hypothesize that in $cno^{M/Z}$ mutants, cells begin to apically constrict, and this tension breaks the weakened link between AJs and the actomyosin ring; thus the ring detaches from AJs and constricts into a ball on the apical end without truly constricting the cell. Therefore, the mesodermal cells are not efficiently internalized. Thus, Cno may modulate cell-cell adhesion by facilitating proper attachment to the contractile ring.
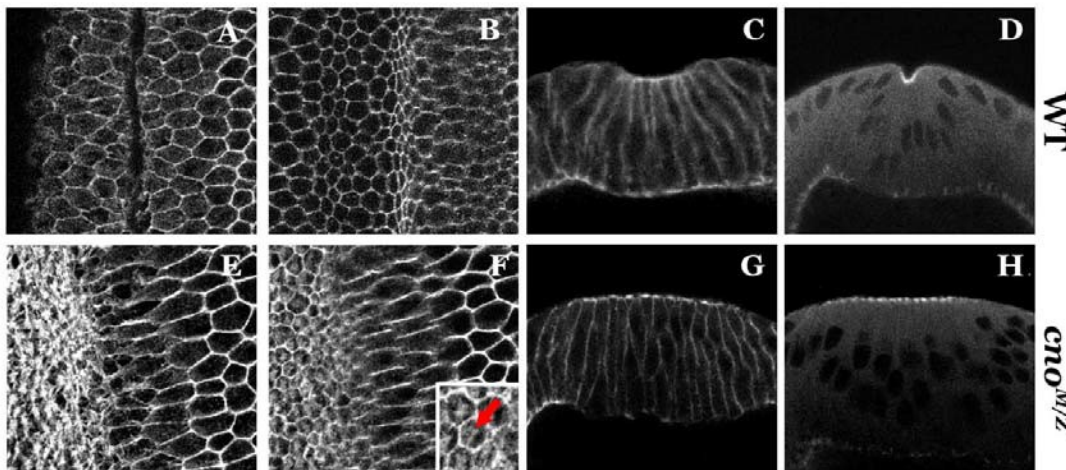

Figure 5. **AJs in $cno^{M/Z}$ mutants are not properly attached to the actin/myosin ring.**
(A-D) WT. (E-H) $cno^{M/Z}$.
(A-B, E-F) Actin.
(A, E) Apical section. Large actin balls are apparent in the $cno^{M/Z}$ mutants.
(B, F) Basal section. Inset: close up of cells in the ventral furrow. The actin ball is located in the middle of the cell, as if it slipped up and off the AJs (red arrow).
(C, G) Actin staining in sections. In $cno^{M/Z}$ mutants, the actin/myosin ring still constricts even though the cells themselves do not constrict.
(D, H) Myosin staining in sections. In $cno^{M/Z}$ mutants, the actin/myosin ring still constricts even though the cells themselves do not constrict.

Aim I. Determine the Role of Cno During Morphogenesis.

Hypothesis: Cno acts redundantly to regulate junctional plasticity by regulating connections to actin and/or to signal transduction machinery.

Cell-cell adhesion is critical for development. AJs are thought to form mechanical attachments between cells by linking actin bundles in neighboring cells together through the cadherin-catenin complex, but recent data from the Nelson and Weis labs suggests this link may not be direct. However, additional proteins are recruited to AJs whose function is less well understood. We hypothesize that these additional proteins act partially redundantly to regulate junctional plasticity by regulating connections to actin and to signal transduction machinery. To test this hypothesis we are defining the function of Canoe (Cno).

A. Precise Localization of Cno Throughout Development

Rationale: Initial characterization of Cno localization revealed an apical accumulation early in cellularization and throughout development, with a diffuse distribution in the cytoplasm. Cno co-localizes with Arm and α-catenin, suggesting that it is in AJs (Takahashi et al., 1998). Recently, the Hsu lab found Ed can bind Cno as well as Baz (Wei et al., 2005), while Tony Harris found that Baz localizes at AJs, below the more apical αPKC and PAR-6 complex (Harris and Peifer, 2005). I propose to do a more detailed localization of Cno in wild type (WT) embryos to determine if it localizes precisely with AJs or has a broader distribution. In addition, we do not have any information about Cno's dynamic behavior during morphogenesis. I will make a Cno::GFP fly to observe Cno live and compare its dynamics relative to a stable resident of AJs (E-cadherin) and a more dynamic resident (Adenomatous polyposis coli 2/APC2; Cliffe et al., 2004). Understanding Cno localization and dynamics will help elucidate its function in morphogenesis.

Methods: For the localization study we have an aliquot of Cno antibody used in previous studies (Takahashi et al., 1998). By combining confocal microscope and deconvolution software, I will determine the precise localization of Cno throughout development. I will do an in depth study of the localization of Cno during cellularization. This is a simple stage where epithelial cells first form and is ideal to determine the precise

localization of a protein. I will also survey embryogenesis to see if there are stages where the localization differs from what I observe in cellularization. I will use two sets of markers. To determine if Cno co-localizes precisely with AJs, I will use DE-cadherin to mark AJs and Discs Large (Dlg) to mark the lateral membrane. To determine if Cno overlaps with the apical polarity complex, I will use atypical Protein Kinase C (aPKC). I will also make my own anti-Cno antibody, using the same epitope as Takahashi, et al. 1998, to supplement the existing antibody.

To make Cno::GFP, I will utilize the Gateway® System. Using the *cno* cDNA available from the Drosophila Genomics Resource Center, I will construct an entry vector. Once in the entry vector, bacterial recombination can be used to place *cno* in multiple cassettes. Once I have made the Cno::GFP construct, I will inject it into flies and map the transgenes. Then, I will determine if the transgene can rescue the zygotic phenotype by placing it in a *cno* background. I will stain the Cno::GFP embryos with Cno antibody to determine if there is any difference between the localization of endogenous protein and the fusion protein; if the localizations are similar, I will then use the Cno::GFP transgene to further understand the dynamics of Cno. Using the spinning disk laser confocal microscope, I will observe Cno localization live. I will use fluorescence recovery after photobleaching (FRAP) to determine if Cno is stably associated with AJs or is more dynamic. I will use FRAP on live embryos expressing the Cno::GFP, Ecad::GFP (stably associated with AJs), and GFP-E-APC (dynamic localization with AJs) using techniques developed by Cliffe, et al.

Expected Results: I hypothesize that Cno will co-localize with AJs throughout development, consistent with previous studies. If however, Cno overlaps with the apical polarity complex or with Dlg, a basolateral marker, this may be important to Cno's function. Cno is proposed to be a scaffolding protein, suggesting a structural role at AJs. I hypothesize that Cno will be stably associated with AJs like E-cadherin. If Cno's dynamics are more similar to APC, this may suggest Cno plays an important role in the cytoplasm.

B. Does Cno Link the Actomyosin Ring to AJs?

Rationale: Thus far, our data suggest Cno may modulate cell-cell adhesion by facilitating proper attachment to the actomyosin ring. I will characterize this connection between the AJs and the actomyosin network

more completely by analyzing sections of the ventral furrow in WT and *cno^{M/Z}* mutant embryos. Using markers for actin, AJs, and the plasma membrane, I will test my hypothesis that the contractile ring becomes physically separated from the AJs during ventral furrow formation in *cno^{M/Z}* mutants.

In addition to confirming my preliminary data in the ventral furrow, I will analyze two other processes that require epithelial folding and invagination to determine if connecting AJs to the cytoskeleton is a global role for Cno; these are formation of the wing imaginal disc and formation of the salivary gland. The wing imaginal disc has a highly stereotypical folding pattern before it undergoes unfolding and shaping of the appendages (Fristrom and Fristrom, 1993; Nikolaidou and Barrett, 2004); since this folding pattern is precise, it is highly unlikely that it arises from passive processes. Instead, it is believed that the epithelial folds arise from apical cell constriction (Nikolaidou and Barrett, 2004). I hypothesize that Cno is required for this folding process. The salivary glands form from two placodes found on the ventral-lateral sides of the embryo. Cells in the posterior rim of the placode begin to invaginate and form a tube, eventually internalizing the entire salivary gland (Myat and Andrew, 2000; Skaer, 1993). I further hypothesize that Cno is required for salivary gland invagination.

Methods: To examine the ventral furrow in *cno^{M/Z}* mutants, I will section embryos as described in Dawes-Hoang, et. al., 2005. I will cross arm::GFP into the *cno^{M/Z}* background. Using antibodies to GFP, actin, and a membrane marker I will be able to determine the relationship between the actomyosin ring and AJs.

To determine if Cno is required for epithelial folding in the wing disc, I will make *cno* mutant clones in wing discs of third instar larvae. With this analysis I can compare WT and mutant tissue side by side. For example, I can see if a fold in WT tissue is continued through mutant tissue. In addition, I will stain the wing discs with Cno to confirm that the mutant clones have no residual protein present. I will also stain the discs with DE-cadherin and Dlg to see if polarity or AJs are disturbed. In addition, I will stain with Myosin, Arm, and Dlg to see if the actomyosin ring becomes separated from the AJs in the mutant tissue.

If Cno is required for salivary gland invagination, they should not be internalized in *cno^{M/Z}* mutants. I will stain with a salivary gland specific marker, CREB-A, and observe salivary gland morphology. There are

four stages of internalization: (I) the two salivary gland placodes form on the anterior ventral surface (II) the more dorsal cells at the posterior end of the gland begin to invaginate (III) the cells that invaginate first form a narrow tube and the more ventral cells form a more rounded structure (IV) the more ventral cells continue to invaginate, until the salivary glands are completely inside the embryo as a uniform cigar shaped tube (Myat and Andrew, 2000). I will examine all of these stages to determine where Cno function is required. In addition, I will stain with Myosin, Arm, and Dlg to see if the actomyosin ring becomes separated from the AJs in the mutant tissue. Both assays were used to determine the role of DRhoGEF2 in epithelial folding (Nikolaidou and Barrett, 2004) and should be an excellent way to answer this question for Cno.

Expected Results: I hypothesize that Cno is required to link AJs to the actomyosin ring and thus should be required for apical constriction in all three of these processes. If Cno is required in some processes but not others that may indicate these processes do not truly use the same mechanism. Perhaps in tissues where the invagination is smaller or shallower, the cadherin-catenin system is sufficient to keep AJs attached to the actomyosin ring. In the ventral furrow, I hypothesize that AJs will detach from the actomyosin ring, indicating that the force required for apical constriction is too great when Cno is not present. I hypothesize that Cno will be required for epithelial folding in the wing disc. However, I think AJs and other polarity markers will be undisturbed in mutant wing disc clones. I hypothesize that Cno is required for Stage II in salivary gland morphogenesis; therefore, glands will not be internalized and will remain as a rounded group of cells on the ventral surface. It may be that Cno function is required for a later stage of salivary gland morphogenesis; I can examine the morphology of the salivary gland to determine what stage requires Cno function.

C. Further Characterize the *cno* Maternal/Zygotic Phenotype

Rationale: While we know that ventral furrow formation is impaired in $cno^{M/Z}$ mutants, this does not entirely explain the dorsal cuticle only phenotype (*Fig. 2*). From my data, during gastrulation epithelial integrity remains intact, and despite severe problems with ventral furrow formation, embryos continue through initial events such as germband extension. We seek to understand if other processes later in

embryogenesis require Cno function, or if the cuticle phenotype is a culmination of secondary effects from defective furrow formation. I will thus examine two other dynamic morphogenetic remodeling processes.

First, I will examine the ventral epidermis while it undergoes neuroblast delamination. Mutant *E-cadherin/shotgun (shg)* embryos have a similar ventral cuticle phenotype to *cno* (Tepass et al., 1996). In the case of *shg* this is due to problems with neuroblast invagination. In WT, 30% of the neuroectoderm cells delaminate in a fairly regular pattern to form neuroblasts; neuroectoderm cells on the surface close the gaps and form the ventral epidermis. In *shg* mutants, this is disturbed. Neuroblasts delaminate in irregular clusters leaving large gaps that cannot be resolved; however, development of the dorsal ectoderm is normal. Disruption of the ventral ectoderm led to widespread cell death; however, even when cell death was suppressed, the ventral epidermis still broke down (Tepass et al., 1996). I will examine neuroblast invagination in $cno^{M/Z}$ mutants. Second, I will examine the malphigian tubules (MTs) in $cno^{M/Z}$ mutants. This epithelium requires convergent-extension to form long tubules. In *shg* mutants, convergent-extension is disrupted because loss of adhesion triggers fragmentation of the MT epithelium. In contrast, convergent-extension in *cut (ct)* (a transcription factor required for MT morphogenesis) mutants is blocked, but cells remain epithelial. Therefore, MTs remain as clumps of columnar cells (Tepass et al., 1996). Examining both of these processes will lead to a better understanding of the function of Cno in later embryogenesis.

Methods: To determine if neuroblast invagination is affected in $cno^{M/Z}$ mutants, I will examine embryos stained with the apical membrane marker Crb and the basolateral marker Dlg. I can assess two aspects of the process with these markers (1) Can neuroblasts delaminate and polarize properly? (2) Are ventral epidermal cells able to close the gaps after neuroblasts invaginate? To determine if Cno is required in the ventral epithelium because it is dynamic, I will reduce the morphogenetic activity of this tissue. *Notch* is required to specify epidermal precursors in the ventral epithelium. Loss of *Notch* results in all cells taking on a neuronal fate, while overexpression of *Notch* results in all cells becoming epidermal. Using an activated form of *Notch*, N_{intra}, I can convert the ventral epithelium into a less dynamic tissue (Tepass et al., 1996). I will express N_{intra} in the $cno^{M/Z}$ background, and determine if it can rescue the ventral cuticle phenotype.

To determine if Cno is required for the formation of MTs, I will examine embryos stained with Cut (Ct, a transcription factor that marks the MTs), Crb, and Dlg. I will assess stage 11, when the MTs begin to invaginate from the hindgut, and stage16 when the tubules should be fully elongated (Skaer, 1993). I will compare the phenotypes of *shg* zygotic mutants, *ct* zygotic mutants, and *cno*$^{M/Z}$ mutants.

Expected Results: I hypothesize that Cno will not be required for neuroblast invagination or polarization, but will be required for the ventral epidermis to close the gaps in the epithelium after neuroblasts invaginate. Similar to *shg*, Crb staining will appear fragmented in the ventral epidermis of *cno*$^{M/Z}$ mutants. One complicating factor in this experiment is that some mesoderm is still present on the ventral surface in *cno*$^{M/Z}$ mutants. While the severity of the mesoderm gap varies from embryo to embryo, it may make this analysis difficult to interpret. If this is the case, I can stain the embryos with Twist, a mesoderm marker, and Crb. This should clarify whether the fragmented Crb staining is in the mesoderm and/or the ventral epidermis.

I hypothesize that Cno will not be required for convergent-extension of MTs, but will be required for the initial apical constriction from the hindgut. *cno*$^{M/Z}$ embryos still complete germband extension, which is a process that requires convergent-extension. Therefore, I suspect the *cno*$^{M/Z}$ phenotype will be more similar to the *ct* phenotype because loss of *cno* will likely interfere with apical constriction. If *cno*$^{M/Z}$ mutants have a similar phenotype to *shg* mutants, it will suggest Cno function is important for epithelial integrity in this tissue. If however, MTs look normal in *cno*$^{M/Z}$ mutants that would indicate that other AJs components are sufficient to facilitate the connection between AJs and the actomyosin ring in MTs.

C. Localization of Cno in other AJs Mutants

Rationale: It has been proposed that the cadherin-catenin and nectin-afadin adhesion systems work together. To what extent the two systems cooperate is not clear. One way to address this question is to determine if their localization is dependent on one other. From my data, E-cadherin localizes properly in a *cno* mutant background (*Fig 4.*). I will examine the localization of Cno in *ß-catenin (arm)* maternal/zygotic and *E-cadherin (shg)* zygotic mutants. These experiments will help determine if the localization of Cno is dependent on cadherin-catenin adhesion system.

Methods: To examine Cno localization, I will utilize laser scanning confocal microscopy. I will use the strong *arm^{XP33}* allele to make the m/z *arm* mutants. They lose epithelial integrity early in gastrulation, so I will examine embryos prior to this stage during cellularization, where epithelial cells first form (Harris and Peifer, 2004). I will examine the apical surface and cross-sections of these cells with Cno, DE-cadherin, and Dlg as markers. I will also examine zygotic mutants of a null allele of *DE-cadherin (shg^{R69})*, balanced on a chromosome with a GFP marker so I can clearly compare Cno localization in WT and mutant flies. Again, I will use Cno, DE-cadherin, and Dlg as markers and analyze embryos during dorsal closure, when maternal contribution of DE-cadherin should be absent. I will examine localization on the surface and in section.

Expected Results: I hypothesize that Cno localization depends on the cadherin-catenin complex. In *arm^{XP33}* m/z mutants, I hypothesize that Cno localization will be disrupted, as was seen with DE-cadherin; Tony Harris found only weak DE-cadherin staining and it did not co-localize with Baz, whose localization at AJs is not affected in these mutants (Harris and Peifer, 2004). In addition, I hypothesize that Cno will be mislocalized in *shg* zygotic mutants in later stages of embryogenesis. If however Cno localizes normally, that suggests Cno can localize to AJs independently of the cadherin-catenin system. Recently, it has been shown that Ed, a transmembrane protein, localizes at AJs and binds both Baz and Cno (Wei et al., 2005). Since Baz localizes to the AJs independently of Arm function, it is possible that Cno could as well, if Ed's localization is also unaffected by loss of *arm*. If Cno does localize to AJs independently of the cadherin-catenin system, I will assess the localization of Ed in *arm* m/z mutants and *shg* zygotic mutants.

Specific Aim 2: Assess the Function of Known Cno Binding Partners and Determine Novel Ones

Hypothesis: Cno associates with different partners throughout development to mediate multiple morphogenetic processes.

The domain structure of Cno suggests that it is scaffolding protein. A few binding partners in *Drosophila* are known: Ras, Rap1, Echinoid (Ed), and Polychaetoid (Pyd). I will examine Ed function with Cno during morphogenesis to further understand how Cno may link AJs to the actomyosin network. Nathan Harris, a graduate student in our lab, will examine the role of Rap1 in morphogenesis and how it cooperates

with Cno; our collaborator Alan Fanning will analyze Pyd function in morphogenesis. Along with my studies of Ed, this should give us greater insight to how these proteins work together during morphogenesis. Additionally, it seems likely other unknown Cno binding partners exist. Learning the identity of other binding partners will give us a better understanding of Cno's role in morphogenesis. I propose to identify novel Cno binding partners and test to see if their interaction is biologically relevant. Specifically, I would like to understand how Cno links the AJs to the actomyosin network.

A. Analysis of Echinoid

Rationale: As mentioned previously, the Hsu lab found that Ed is a Cno binding partner and may be a distant relative of Nectin. They also show evidence that Ed is required for the proper localization of Cno in wing discs (Wei et al., 2005). I propose to characterize if the Cno-Ed interaction is biologically relevant in the embryo. I will determine if there is a zygotic genetic interaction between *ed* and *cno*. In addition, I will make *ed* m/z mutants and examine the localization of Cno in these embryos. If Ed-Cno functions similarly to the nectin-afadin system in mammals, using *Drosophila* as an *in vivo* system will be useful to answer questions about how this adhesion system works.

Methods: First, I will examine a genetic interaction between *cno* and *ed*. I will use the cno^{R2} allele, with an early stop codon (aa221) after the first RA domain and the ed^{IF20} allele that has an early stop codon (aa63) in the first Ig domain. Since the ed^{IF20} allele should only produce a small portion of the extracellular domain, it is proposed to be a null allele (Escudero et al., 2003). I will examine the phenotypes of ed^{IF20}/ ed^{IF20}; $cno^{R2}/+$, $ed^{IF20}/+$; cno^{R2}/cno^{R2}, and ed^{IF20}/ ed^{IF20}; cno^{R2}/ cno^{R2} embryos.

I will also generate germline clones lacking both maternal and zygotic Ed function (Chou et al., 1993). I will examine *ed* m/z mutants and determine if Cno localization is disrupted. I also will assess whether or not *ed* m/z mutants have any defects in ventral furrow formation.

Expected Results: Data from the Hsu lab suggest that Ed is upstream of Cno. I hypothesize that *ed* and *cno* will genetically interact. ed^{IF20} is larval or early pupal lethal; I hypothesize when cno^{R2} is heterozygous in the background it will be embryonic lethal. In addition, with ed^{IF20} in the background, I hypothesize that the

cno[R2] zygotic phenotype will be enhanced. If there is no significant enhancement of the zygotic phenotypes, it may suggest that Cno cooperates with other partners at AJs.

In *ed* m/z mutants, I hypothesize there will be subtle defects in ventral furrow formation. In addition, I hypothesize that Cno localization will be altered but not totally mislocalized. Instead, less Cno protein may localize to the AJs, but it may localize correctly. This would imply that other proteins present at AJs are capable of binding Cno. Data from the Nilson lab is consistent with this hypothesis; during dorsal closure Ed protein is absent from the amnioserosa (Laplante and Nilson, 2006), however, Cno is present in this tissue. In addition, Baz is required for localization of AJs proteins early in embryogenesis (Harris and Peifer, 2004). Based on data from the Hsu lab, one might conclude that Ed may be required to localize Baz apically early in embryogenesis. If this were the case, one would expect the phenotype of the *ed* m/z mutants to be quite severe. However, the Nilson lab found that *ed* m/z mutants have defects in dorsal closure (Laplante and Nilson, 2006). This is a much later stage in embryogenesis and argues against Ed having an essential role in polarity establishment. It may be that Baz can bind to Ed, but has additional binding partners at AJs.

B. Screen For Additional Binding Proteins

Rationale: While there are a few Cno binding partners known in *Drosophila*, it seems likely more exist. Studies in mammalian systems revealed that Afadin can bind to several members of the actin cytoskeleton: α-catenin, profilin, ponsin, and F-actin (Boettner et al., 2000; Mandai et al., 1997; Mandai et al., 1999; Tachibana et al., 2000). However, functional information is lacking. It is unclear from these experiments if these interactions are important in the cell or in the organism as a whole. I propose two approaches to address this question. First, I will use a candidate approach to determine if Cno can bind to Profilin/Chickadee (Chic), α-catenin, and/or IrreC-rst. Determining if Profilin and α-catenin are binding partners will indicate whether Cno also has a connection to the cytoskeleton. As mentioned previously, data from the Nilson lab suggests Ed may not be the only integral membrane protein that can bind Cno. Therefore, I will determine if Cno can bind to IrreC-rst. IrreC-rst is a transmembrane protein of the immunoglobin superfamily and was found to be important for cell adhesion during cell sorting in the

Drosophila pupal eye. Similar to Nectin and Ed, it has a conserved consensus binding site for PDZ domains; making it a good candidate for a Cno binding partner (Grzeschik and Knust, 2005). Second, I will perform a screen to uncover novel binding partners of Cno.

Methods: For the candidate approach, I will specifically determine if Cno binds Profilin/Chickadee (Chic), α-catenin, and/or IrreC-rst. Chic and α-catenin antibodies are available from the Developmental Studies Hybridoma Bank. I will acquire IrreC-rst antibody made by the Fischbach lab and has been used in several studies of the protein's function (Schneider et al., 1995). Using co-immunoprecipitations (co-IPs) and immunoblotting, I will determine if Cno can associate with these proteins.

To determine novel Cno binding partners, I will prepare extracts from embryos expressing Cno::GFP and immunoprecipitate proteins using beads coupled to anti-GFP antibodies. For the negative control, I will use embryos that do not express the fusion protein and use the same procedure. I will use SDS-Page and stain with SYBRO Ruby to identify all proteins that are recovered. I will choose promising bands that co-IP specifically with Cno::GFP for mass spectrometry analysis at the UNC-Duke Michael Hooker Proteomics Center. Jeremiah Roeth, a post-doc in our lab, has successfully used this technique using cadherin::GFP to pull down components of the cadherin-catenin complex. For promising candidates, I will confirm the results using co-IPs and immunoblotting if antibodies available. For candidates with no antibody available, I will cut out the band in the Cno::GFP lane and the control lane and send them for analysis. Proteins unique to the Cno::GFP lane will be tested further. If unknown proteins are identified and are unique to the Cno::GFP IP, I will blast the sequence to look for conserved domains of interest and homology to other organisms. I will prioritize the list of candidates that pass these tests and then assess whether the interaction is biologically significant using zygotic genetic interactions, if a mutant is available.

Expected Results: For the candidate approach, I hypothesize that Cno will bind to Chic and/or α-catenin. My preliminary data suggests that Cno can act as a link between AJs and the cytoskeleton and both of these proteins are good candidates for this link. However, determining if Cno can bind α-catenin may be difficult. In mammalian cells, Cno does not co-IP with full-length α-catenin, but only with its carboxyl-terminus; it

was reasoned that α-catenin and Afadin have a complex interaction that may involve another accessory proteins or additional posttranslational modifications (Tachibana et al., 2000). I further hypothesize that Cno will bind to IrreC-rst. While Ed is likely an important binding partner, data from the Nilson lab suggests that Cno has Ed independent functions and requires other proteins to localize to AJs.

If the mass spectrometry screen is successful, I should see a band of ~226kDa that corresponds to Cno::GFP. In addition, I might expect to see the following bands based on known binding partners: ~21-25kDa Ras1 (predicted), ~20-25kDa Rap1 (predicted), ~147kDa Ed (predicted), ~170-180kDa Pyd (observed). I suspect that transmembrane proteins, in addition to Ed and IrreC-rst, will be uncovered that Cno can associate with at AJs. In addition, I suspect other proteins that associate with the actin cytoskeleton will be found in this screen; it would be satisfying if some of the proteins found are known Afadin binding partners, such as Profilin and Ponsin. This would provide more evidence that Cno is an important link between the actin cytoskeleton and AJs. Additionally, proteins identified could be assessed *in vivo* to determine what biological processes are affected when these partners are not able to interact. While mapping the binding sites of identified proteins is beyond the scope of this proposal, identifying the binding partners will make a future structure-function study of Cno more instructive.

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