AD NUMBER

ADB222576

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Oct 96. Other requests shall be referred to Commander, U.S. Medical Research and Materiel Command, Attn: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

AUTHORITY

USAMRMC ltr dtd 17 Feb 2000

AD_____

GRANT NUMBER DAMD17-94-J-4266

TITLE: Control of Cell Morphology: Signalling by the Receptor Notch

PRINCIPAL INVESTIGATOR: Edward Giniger, Ph.D.

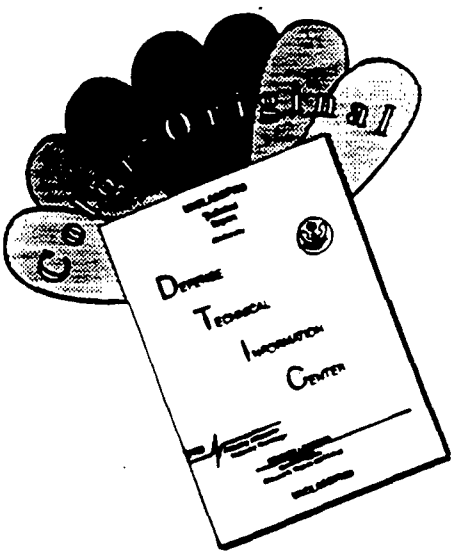
CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, Washington 98104-2092

REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF COLOR PAGES WHICH DO NOT REPRODUCE LEGIBLY ON BLACK AND WHITE MICROFICHE.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE Control of Cell Morphology: Signalling by the Receptor Notch

5. FUNDING NUMBERS
DAMD17-94-J-4266

6. AUTHOR(S)
Edward Giniger, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center
Seattle, Washington 98104-2092

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Oct 96). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The goal of our study is to understand how the *Drosophila* gene *Notch* promotes axon extension. The specific second year tasks were to finish establishing assays for *Notch* function by characterizing the effects of activated NOTCH proteins in various developmental contexts, and to begin localizing signaling domains of NOTCH that are required for its control of axon extension.

In the past year, we have completed a detailed analysis of the roles of *Notch* in oogenesis and myogenesis. In each case, we have identified multiple steps at which *Notch* controls these processes. We have also taken advantage of evidence that the morphogenetic function of *Notch* may be executed *via* the ABL tyrosine kinase, to identify two small regions of NOTCH that are likely to be its morphogenetic control domains, by virtue of being interaction sites for ABL and associated proteins.

The observation that we can disrupt *Notch*-dependent axon extension without perturbing cell identity strongly supports our original hypothesis that these are independent functions of *Notch*. This is further supported by the biochemical identification of ABL-interaction domains in NOTCH. Finally, our results provide us with unforeseen and powerful biochemical and genetic tools to advance our experimental program.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer; Cell Morphology; Differentiation; Drosophila; Notch; Abl; Signal Transduction	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__X__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__X__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__X__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 10/29/96 Date

TABLE OF CONTENTS

This page contains unpublished data

5. INTRODUCTION

NOTCH is a transmembrane receptor that is required for the proper differentiation of a wide variety of cell types during *Drosophila* embryogenesis [1, 2]. Mutations in the *Notch* gene lead to severe defects in cell identity in the nervous system, musculature, gonads and many other tissues. Recent work has suggested the following model for the molecular mechanism by which *Notch* regulates one process, the segregation of neural precursors [3, 4]. In the absence of extracellular signals, a specific transcription factor, Su(H), is tethered to the intracellular domain of NOTCH and therefore is unable to enter the nucleus. When NOTCH is stimulated by its ligand, DELTA, Su(H) protein translocates to the nucleus and binds to the promoters of the *E(spl)* genes, activating their transcription and in turn repressing neurogenesis. Equivalent mechanisms are thought to act in other tissues where *Notch* controls cell fate, and also in the function of the *Notch* homologs in other metazoans, including humans. However, two lines of evidence indicate that there are likely to be other signal transduction pathways acting downstream of *Notch*. First, in an embryo that lacks all maternal and zygotic Su(H), *Notch* is nonetheless capable of activating a different process, midline development, by turning on expression of the gene *sim* [5]. Second, the protein NUMB regulates sense organ differentiation by intercepting the signal produced by NOTCH . However, expression of NUMB does not block the NOTCH signal that regulates neuroblast segregation [6], perhaps suggesting the existence of another (*numb*-insensitive) *Notch* signaling pathway.

In addition to their effects on cell identity, *Notch* mutations also cause defects in cell morphology. For example, there is a specific set of axon guidance decisions in the *Drosophila* embryo that require the function of *Notch*, and of one of its ligands, *Delta* [7]. There are two classes of models that could explain the requirement for *Notch* in axon guidance. It could be that *Notch* controls axon extension indirectly, by virtue of its role in determining the identities of neurons and/or substratum cells. Alternatively, NOTCH may work directly in the growth cone, as an axonal guidance molecule, separately from its control of cell identity. The second of these models suggests that we should be able to uncouple these two roles of *Notch*. Thus, we can ask whether we can identify different signaling domains of NOTCH for the control of cell fate *vs* cell morphology; alternatively, we can ask whether we can identify different signaling proteins downstream of NOTCH for the control of cell fate *vs* morphology. Over the past two years, we have been investigating the functions of constitutively active derivatives of NOTCH, and their interactions with other signaling proteins, in order to ask these two questions.

During the course of our experiments, an unrelated project in our lab has identified an excellent candidate for a protein that may be a specific effector of the morphogenetic function of *Notch*. Studies we performed investigating the effects of protein kinase mutations on the phenotypes of loss-of-function mutants of *Notch* have implicated the ABL protein tyrosine kinase in *Notch* signaling. In order to present these results and discuss their significance, it is first necessary to digress briefly into a description of the *Drosophila abl* gene.

The *abl* gene of *Drosophila* encodes a protein that is closely related to the Abl oncogene of mammals [8]. The genetics of *Drosophila abl* have been studied extensively, and are a classic example of genetic redundancy [9]. Homozygous *abl* mutant animals are semi-viable and show no discernible embryonic defects. It is believed that the inessential nature of *abl* reflects the existence of one or more additional tyrosine kinases in the fly that can substitute for the function of *abl*. The role of *abl* can be uncovered, however, by reducing the amount or activity of other proteins which normally collaborate with ABL. For example, if the dosage of the *disabled (dab)* gene is reduced by half in a homozygous *abl* mutant background, as in a fly with the genotype $dab^{-}/+$; abl^{-}/abl^{-}, then such an individual is embryonic lethal and dies with gross abnormalities of axon extension [10, 11]. Consistent with this phenotype, ABL protein is concentrated in axons, as is DAB [11, 12]. There is no evidence that the embryonic defects seen in *abl* mutants reflect aberrations of cell fate or overall differentiation. Rather, it is believed that *abl* has a direct role in control of the axonal cytoskeleton. Genes that interact synergistically with *abl* are collectively termed HDA loci (haploinsufficient, dependent on *abl*). While it has not been shown conclusively that HDA loci

encode proteins that physically interact with ABL *in vivo*, similar synergistic genetic interactions are often found in genes whose products interact directly, such as the different constituents of a multiprotein complex [13]. Consistent with this, there is good reason to think that DAB is a direct substrate of the ABL kinase [11].

In experiments supported by the March of Dimes Birth Defects Foundation (EG, unpublished), we have found that *Notch* has the genetic and developmental properties of an HDA locus. First, partial reduction of the level of *Notch* activity causes embryonic lethality of *abl* mutant animals, just as is seen for the classic HDA loci. Second, examination of the nervous system of embryos that die from the *Notch/abl* genetic interaction reveals severe axonal defects. Moreover, the axonal defects observed are restricted just to those axon tracts which are known to require *Notch* for their proper development, arguing that the phenotype is apt to be specific. Finally, we have not observed any neurogenic defects arising as a consequence of the *Notch/abl* interaction. The observation that the axonal defects in these animals occur in the absence of detectable changes of cell fate argues that the axonal defects cannot be secondary to disruption of cell identity, and thus that the NOTCH and ABL proteins probably collaborate directly in the axon to promote axon extension.

We realized that our discovery of a genetic interaction between mutations of *Notch* and *abl* offered us the opportunity to dramatically advance the goals of our DOD-supported Breast Cancer Research Project. Therefore, in experiments supported by this DOD grant, we have extended our *Notch/abl* experiments in two ways. First, we have biochemically identified two portions of NOTCH that are likely to constitute ABL interaction domains. Second, we have performed genetic experiments which verify that one of the putative ABL-binding domains is apt to contribute to the *Notch/abl* genetic interaction. Together, these observations suggest a modification to our overall strategy that will serve both to increase the efficacy of our experiments and to focus them more sharply on the elements of the *Notch* pathway that are important for the control of cell morphology.

6. BODY

METHODS

Immunocytochemistry:

Antibody staining of embryos was performed by standard methods, as described previously [14, 15].

Protein binding *in vitro* :

The GST-mouse DISABLED PTB domain fusion protein, coupled to Affigel (BioRad), was generously provided by B. Howell and J. Cooper. The *Notch* intracellular domain was linked to a histidine-tag by cloning into the vector pET3a (Invitrogen), and two copies of a consensus protein kinase A recognition site was inserted at the amino terminus of the construct with a synthetic oligonucleotide. The construct was transformed into *p-lysS E. coli*. Cells were grown to early log phase (OD^{600}=0.4), induced 4 hours with 1mM IPTG and harvested by centrifugation. Cells were lysed by freeze/thaw and then resuspended in 10 ml of 8M urea, 50 Na Phosphate pH 7.5, 1mM PMSF. Extract from 100 ml *E. coli* was incubated with 1 ml nickel beads (ProBond Resin; Invitrogen) for 1 hr at 4°. Beads were washed with lysis buffer containing 1 M NaCl, then with 8M Urea, 50mM Na Phosphate, pH 7.5, and finally renatured by overnight dialysis into 25mM Hepes pH 7.6, 100mM NaCl, 5mM β-mercaptoethanol, 1% NP40, 0.1mM PMSF.

Notch protein was labelled by incubating 100µl renatured NOTCH beads in 250µl 20mM Tris, pH 7.5, 100mM NaCl, 12mM $MgCl_2$, 1mM DTT with 20µCi γ-^{32}P-ATP and 25 units heart muscle kinase (Sigma). Labelled NOTCH protein was washed several times, and then released from beads by incubating in a minimum volume of binding buffer including 25mM EDTA.

Protein binding was performed as follows. DAB-beads or GST-beads were blocked by incubation in binding buffer (see below) containing 2% BSA for 30 min at 4°, then washed with binding buffer. Simultaneously, labelled NOTCH was diluted into 750 µl binding buffer,

preincubated in Sepharose beads coupled to a nonspecific protein (Protein A) that had been similarly blocked, and clarified at 17,000g for 10 minutes at 4°. Labelled NOTCH was then incubated with DAB- or GST-beads (10 µl packed bed) for 90 minutes at 4° with gentle rocking. Beads were collected by brief centrifugation and washed 5 times in binding buffer . Beads were finally resuspended in 20 µl Laemmli sample buffer and boiled 3 minutes. Half of the eluted material was electrophoresed through a 7.5% SDS (Laemmli) polyacrylamide gel. The gel was dried-down and analyzed by autoradiography.

Various binding buffers were investigated; all gave similar results. Typical binding conditions were: 50 mM Hepes, pH 7.5; 100mM NaCl; 1% NP40; 0.1% SDS; 14mM β-mercaptoethanol, 0.1mM PMSF.

RESULTS.

The work we have performed under this grant in the past year falls into two general areas. First, we have completed experiments designed to characterize the actions of *Notch* in two developmental systems that will be useful as assays of *Notch* function, oogenesis and myogenesis (Task 1). Second, we have used biochemical and genetic approaches to localize signaling domains of NOTCH that are implicated in its interaction with ABL and thus the control of axon extension (Task 2).

1. *Notch* in oogenesis.

Previous experiments have implicated *Notch* in several steps during oogenesis in *Drosophila* [15]. Temperature shifts of a temperature-sensitive *Notch* allele lead to mis-specification of the fates of specialized somatic follicle cells that normally surround the developing oocyte, and as a consequence, the anterior-posterior axis of the oocyte fails to be specified. In concert with these cell fate defects, alterations of the morphologies and migrations of follicle cells have been observed. Given the requirement for *Notch* in the coordinated changes in cell fate and morphology during oogenesis, and the convenience of the experimental system, we reasoned that oogenesis might be an attractive assay system for testing the effects of consitutive *Notch* derivatives. We therefore characterized the consequences of expressing activated *Notch* in ovarian follicle cells at various stages in oogenesis.

The phenotypes induced by activated *Notch* verified that this gene acts at multiple steps during oogenesis (Figure 1). In wild type development [15], follicle cells are first segregated into stalk cells and polar cell precursors. Polar cell precursors are then further segregated into polar cells (thought to have a role in specifying the primary body axes in the oocyte) and flanking cells. Expression of activated *Notch* late in oogenesis prevents segregation of polar cells from flanking cells, while expression earlier in oogenesis transforms polar cell precursors into stalk cells (Fig 1, A-D). At each of these decision points, the phenotype observed is the opposite of the phenotype reported previously for loss-of-function alleles of *Notch* [15].

Unexpectedly, we also observed an additional phenotype of activated *Notch*. When we examined the mature, hyperplastic stalks produced by very early expression of activated *Notch*, we observed that the morphology of the cells resembled the morphology of stalk precursor cells, not the morphology of fully differentiated stalk cells. This interpretation was verified by examination of a specific molecular marker. In stalk precursor cells, the protein BIG BRAIN is localized to the apical surface of the cell, whereas it is found at the lateral membranes of differentiated cells. In the hyperplastic stalk cells induced by activated Notch, BIG BRAIN is apically-localized, consistent with these cells being arrested at a precursor stage of their development (Fig 1, E-G).

These experiments were performed with two different activated derivatives of *Notch*. *Notch*^{intra} lacks a transmembrane domain and becomes localized to the nucleus, while *ΔE-Notch* retains the transmembrane domain and thus is tethered to the plasma membrane. Moreover, the *ΔE-Notch* used in these studies is truncated at amino acid 2236. Thus, it retains the RAM23 domain that interacts with Su(H) [16] and the ankyrin repeats that bind DELTEX [17], but lacks the C-terminus, possibly including the DISHEVELLED binding site [18]. In all assays performed,

Notch^{intra} and *ΔE-Notch* behaved similarly, though *Notch*^{intra} in all cases gave a more penetrant phenotype.

We reported the results of these experiments in Larkin, *et al* (Development, in press).

2. *Notch* in myogenesis

Myogenesis in *Drosophila*, like neurogenesis, initiates with the segregation of a limited number of precursor cells (termed "founder cells") out of a larger pool of competent cells [19]. Also as in neurogenesis, it has been proposed that the selection of founder cells from the precursor-competent pool in myogenesis is regulated by the *Notch* pathway: loss-of-function *Notch* mutations lead to hyperplasia of founder cells, as assayed by founder cell markers such as NAUTILUS [20]. Based on this observation, it has been widely assumed that activation of *Notch* would block the segregation of founder cells altogether. To test this hypothesis, we expressed activated *Notch* at a variety of stages of myogenesis, and in a variety of cells. Unexpectedly, this experiment revealed that *Notch* can regulate multiple stages of myogenesis.

Early high-level expression of activated *Notch* prevents expression of the founder marker NAUTILUS, consistent with expectation (Figure 2 A,B). In these experiments, we used two different GAL4-expressing lines (GAL4 24b or twi-GAL4) to drive expression of *ΔE-Notch*, and assayed *nau* expression both by *in situ* hybridization to RNA and with anti-NAU antibodies. In all cases, the results were identical.

Analysis of a second founder marker, the homeobox protein S-59, revealed a second and unexpected activity of *Notch*. In the same assay systems used to examine NAUTILUS, expression of *ΔE-Notch* did not block the initial expression of S-59, but that expression was transient and terminated prior to the initiation of myoblast fusion (Fig 2, C-E). Thus, it appears that *Notch* activity must be kept off throughout myogenesis in order for founder marker expression to be maintained. While it is possible that the difference between the phenotypes observed with *nau* and *S-59* reflect real differences in the regulation of these two genes, we suggest that a simpler model is that these genes are regulated similarly, but that *S-59* expression is more robust. Thus, it escapes from the initial effects of *Notch* and is capable of revealing a second role of *Notch* in maintenance of muscle identity. Preliminary experiments further increasing *Notch* activity are consistent with this interpretation: initiation of *S-59* is not completely resistant to *Notch* but rather can be blocked by higher levels of *Notch* signal (data not shown).

In the course of examining the roles of *Notch* in the ectoderm, we uncovered yet a third effect of *Notch* on myogenesis. Remarkably, driving expression of *ΔE-Notch* in the developing epidermis at relatively late embryonic stages, using either of two GAL4-expressing lines (GAL4-69b or GAL4-112A) led to a turn-off of muscle founder markers in the underlying mesoderm, with consequent failure of myocyte fusion (Fig 2, F,G). The mechanism responsible for this effect remains obscure. Examination of the ectoderm reveals no gross perturbation of epidermal differentiation. The cuticle pattern of such embryos is normal (Fig 2 H), as are a variety of markers for specialized epidermal cells (FAS III, GROOVIN, ENGRAILED; data not shown). Apparently, proper epidermal development produces a signal that is required for maintenance of muscle identity and initiation of muscle fusion, and expression of activated *Notch* in the epidermis disrupts this hypothetical inductive signal (or alternatively, *Notch* induces an epidermally-derived signal that represses founder identity in the mesoderm).

A manuscript describing the results of these experiments is currently in preparation, and will be submitted before the end of the year (S. Fuerstenberg and E. Giniger, in preparation).

3. Activated *Notch* and ectopic axon outgrowth

As we reported in our Annual Report last year, expression of activated *Notch* in postmitotic neurons is capable of inducing ectopic neurite outgrowth. Unfortunately, two aspects of this phenotype rendered it of limited utility as an assay for the morphogenetic effects of *Notch* truncations, or in a screen for interacting, second-site mutations. First, the axonal phenotypes were morphologically inconsistent and of low penetrance. Second, expression of activated *Notch* also caused defects in neurogenesis, confounding the interpretation of axonal defects seen in these

embryos. We tried a variety of approaches to circumvent these difficulties, including both use of different GAL4-expressing lines and a variety of temperature-shift protocols [21] to try to accentuate axonal defects and minimize neurogenic defects. None of the protocols we tried bypassed these difficulties sufficiently to make expression of activated *Notch* a particularly attractive approach to our eventual goal, the identification of morphogenetic domains and effectors of *Notch*. However, an alternative and far more promising approach became available at this time, as we will now discuss.

4. *Notch* and *abl*

As described in the Introduction, it came to our attention that *Notch* and *abl* loss-of-function mutations interact synergistically to cause embryonic lethality and to block the extension of *Notch*-dependent axons, specifically, without discernable defects in cell identity, demonstrating that *Notch* has the properties of an HDA locus.

Three classes of models could explain the genetic interaction of *abl* and *Notch*. *abl* could act upstream of *Notch*, it could act in parallel or it could act downstream. If *abl* kinase were upstream of *Notch*, the simplest notion is that *abl* either controls the level of NOTCH protein or phsophorylates it on tyrosine. Western blotting of homozygous *abl* mutant animals showed no change in NOTCH level (data not shown). Others have examined the phosphorylation state of NOTCH , and have never detected evidence for tyrosine phosphorylation of the protein. Moreover, NOTCH has no sequence that resembles a consensus ABL phosphorylation site. Thus, we do not think it very likely that *abl* acts directly upstream of *Notch*.

If *abl* acts downstream of *Notch*, we might be able to detect association of NOTCH protein, biochemically, either with ABL itself or with one of the other HDA proteins. It is unlikely that ABL itself binds to NOTCH , since NOTCH does not possess consensus recognition motifs for either the SH2 or SH3 domains of ABL. In contrast, the DISABLED (DAB) protein contains a PTB protein interaction domain closely related to the PTB domain of the NUMB protein. The NUMB PTB has recently been shown to bind directly to two sites in the NOTCH intracellular domain [22, 23]. Significantly, though PTB domains were originally identified as phosphotyrosine binding moieties, both the numb PTB and the PTB domain of mouse DISABLED bind to protein substrates that lack phosphotyrosine [22; 23; B. Howell and J. Cooper, pers. comm]. (The target sequences for the NUMB and mouse DAB PTB domains have not yet been characterized.) We therefore asked whether the DAB PTB binds to NOTCH.

We have now shown that the NOTCH intracellular domain binds to the PTB domain of mouse DAB in vitro. A GST fusion of the mDAB PTB, bound covalently to beads, was generously provided to us by Howell and Cooper. The *Drosophila* NOTCH intracellular domain was tagged with two protein kinase A recognition sites and a histidine-tag, and isolated from bacteria. The NOTCH protein was purified on a nickel column, renatured by dialysis and labeled with ^{32}P. NOTCH protein was then incubated with GST-DAB beads, or GST beads alone. The beads were washed extensively in binding buffer, boiled in Laemmli sample buffer, the samples were separated by PAGE and analyzed by autoradiography. NOTCH protein bound to the GST-DAB PTB beads, but not to beads bearing GST alone, under a variety of conditions (Figure 3). We have now expressed and purified the homologous domain of *Drosophila* DAB as a fusion to GST, and are currently repeating this experiment.

Given the sequence similarity between the NUMB and DAB PTB domains (which is underestimated in the literature due to sequence errors in the published *dab* sequence; Gertler and Howell, pers. comm), it seemed likely that DAB binds to the same sequences recognized by NUMB. These have been mapped near the amino terminus of the NOTCH intracellular domain (the so-called RAM23 region) and at the extreme C-terminus of NOTCH . Consistent with the former of these sites, two labelled proteolytic products of NOTCH containing the amino-terminal 80 and 45 Kd, respectively, of the NOTCH intracellular domain also associate with GST-DAB in the *in vitro* binding experiment. We decided to further test the putative C-terminal binding site genetically, taking advantage of *Notch* alleles that disrupt this portion of the protein.

This page contains unpublished data

notchoid (*nd*) alleles of *Notch* are a special class of *Notch* alleles that produce wing margin defects but no known embryonic neurogenic defects [24]. Several of the molecularly characterized *nd* alleles are missense mutations or small deletions at the extreme C-terminus of NOTCH, and lie within the minimal region that includes the C-terminal binding site for the NUMB PTB. Interestingly, we observed that wing margin defects similar to those produced by *nd* alleles can be generated as a synthetic phenotype by appropriate viable combinations of hypomorphic *Notch* and *abl* alleles (data not shown). We therefore asked whether *nd* alleles could interact synergistically with *abl* mutations to produce embryonic axonal defects.

Embryos doubly homozygous for *nd1* and strong *abl* alleles displayed axonal defects similar to those seen with strong *Notch* alleles, albeit at low penetrance (Figure 4). The low penetrance may arise either because the *nd1* mutation does not completely inactivate the C-terminal DAB binding site, or because the N-terminal binding site is intact in this mutant. Nonetheless, the *nd/abl* interaction phenotype is consistent with the notion that the C-terminal DAB-binding domain of NOTCH contributes to *Notch*- and *abl*-dependent signaling in axonogenesis.

We expect a manuscript describing the results of these experiments to be prepared before the end of the year (E. Giniger).

DISCUSSION

The goal of the second-year experiments under this proposal was to complete the characterization of *Notch* function in a variety of potential assay systems (Task 1), and to initiate the localization of signaling domains in the protein, focusing on signaling domains specific to the control of cell morphology (Task 2). In the past year, we have completed an analysis of *Notch* function in oogenesis and myogenesis. Attempts to localize signaling domains by the methods described in the original proposal met with unexpected difficulties, but an alternate approach has proven to be far more successful and direct. We will discuss the significance of these results in turn.

1. Oogenesis

There are three main conclusions to be drawn from our studies of oogenesis. First, in this as in a variety of other systems, expression of the cytoplasmic domain of *Notch* induces phenotypes that are opposite to the *Notch* loss-of-function phenotype. Second, these experiments verify that *Notch* is involved in several, sequential cell fate decisions during follicle cell development. Third, they provide direct evidence that *Notch* is capable of arresting a cell at a precursor stage of development.

The notion that *Notch* may act in a variety of developmental contexts to inhibit differentiation and arrest cells at a precursor stage has been proposed previously [33]. It has been further proposed that the oncogenic potential of *Notch* is based on this property: precursor-type cells are often only loosely associated with their substrata and thus are more likely to be invasive than fully differentiated cells, and they are also typically still dividing. However, few cell types afford markers that are specific for the precursor stage of development, and thus the model that *Notch* explicitly blocks differentiation has been difficult to test. In general, the notion that *Notch* arrests differentiation rests only on the negative result of a failure to observe expression of particular differentiated markers. In our experiments, we have both morphological and molecular markers that are specific for precursor cells *per se*, and we see that the expression of these persist in cells that contain activated *Notch*. Therefore, our experiments provide the first positive evidence that *Notch* does indeed arrest differentiation of these cells at a precursor stage.

We observed that $Notch^{intra}$ and *ΔE-Notch* gave identical phenotypes for multiple developmental decisions in oogenesis. Since $Notch^{intra}$ becomes localized to the nucleus, we can infer from this that all the functions of *Notch* in oogenesis can likely be performed directly in the nucleus. Put differently, this suggests that there is no specifically cytoplasmic function of *Notch* in oogenesis, as might be expected if the control of follicle cell morphology involved direct control of the cytoskeleton by *Notch*. We do not yet know whether the same is true of *Notch* in other developmental contexts.

This page contains unpublished data

2. Myogenesis

The process of myogenesis in *Drosophila* has a number of parallels to the process of neurogenesis. For that reason, it seemed that it would be a valuable system for characterizing the roles of *Notch*. As in neurogenesis, but contrary to previous notions of the function of *Notch* in myogenesis, we found that *Notch* was involved in multiple steps in muscle development. The action of *Notch* to block initial segregation of muscle founders was not unexpected. In contrast, its role in the maintenance of founder identity had not previously been suspected. This role may be another parallel to the functions of *Notch* in neurogenesis: it has long been suspected (though never directly demonstrated) that *Notch* activity is necessary for maintenance of epidermal identity, and that suppression of *Notch* is the mechanism by which extra neuroblasts are recruited from the epidermis when a neuroblast is ablated experimentally [25, 26].

Our finding of a tissue-nonautonomous ability of epidermal activated *Notch* to block muscle fusion was completely unexpected. We cannot distinguish whether this reflects a wild-type function of the NOTCH protein, or whether it is a neomorphic effect of activated *Notch*. In either event, what it does show unambiguously is the existence of an activity in the epidermis that controls late events in muscle development, either a muscle-inducing activity that is blocked by *Notch* or a muscle-repressing activity that is stimulated by *Notch*.

Why might the epidermis regulate the course of muscle development? Clearly, since the somatic musculature must attach to the epidermis, it is critical for muscle and epidermal development to be somehow coordinated. In retrospect, evidence for such coordination has been observed previously [27, 28]. Treatments that disrupt epidermal development are correlated with a variety of ill-characterized defects in muscle development, though the mechanisms underlying these defects remain obscure. Perhaps, for example, there is a mechanism that monitors epidermal integrity, and delays muscle fusion until the successful completion of epidermal differentiation. Regardless of whether or not this process employs *Notch* in wild type development, our experiments demonstrate that expression of activated *Notch* can be used as a reagent to probe the mechanism that coordinates muscle development with epidermal development.

Thus, our experiments of the past year, taken together with the characterization of *Notch* function in neurogenesis and in adult sense organ determination by us and by others, complete the job of providing us with an excellent and diverse set of assays to dissect the structure of *Notch* and of *Notch* signaling pathways, as proposed for Task 1.

3. *Notch*, *abl* and axon extension.

The observation of a genetic interaction between loss-of-functions mutations of *Notch* and *abl*, made during the course of an unrelated project in the lab, presented us with an unexpected opportunity to bypass some of the most time-consuming and difficult steps in our original DOD proposal and jump directly to some of the central issues that we set out to address in this project. In particular, it provided direct evidence that the cell identity and morphology phenotypes of *Notch* could be genetically uncoupled, and suggested direct biochemical and genetic tests to localize the morphogenetic signaling domain(s) of *Notch*.

Given that *Notch* indeed has a direct morphogenetic activity, the challenge of our experimental program is essentially to uncover the link between NOTCH and the cytoskeleton (Tasks 3 and 4). We know from work in vertebrate systems that the Abl tyrosine kinase and its associated proteins are quite directly involved in control of Rho-family GTPases [29], and thus presumably of the polymerization of actin [30] and the activity of cytoplasmic myosins [31]. Therefore, clarifying the link between NOTCH and ABL would imply a simple and testable model for the molecular connection between *Notch* activity and actin-based motility. We have now shown that DISABLED can bind directly to NOTCH. Since we know that DAB is likely to be a direct substrate of ABL kinase, and that ABL can bind to phosphorylated DAB, our data raise the possibility that ABL is part of a signaling complex that forms on the intracellular domain of NOTCH. We would then expect this molecular machine to lie at the heart of the morphogenetic activity of *Notch*.

* This page contains unpublished data

Localization of the morphogenetic control domain of NOTCH (Task 2) permits a genetic test of our model that this function of the protein is based on its association with DAB. Based on experiments with the NUMB PTB domain, we suspect there to be two DAB-binding regions, one near the transmembrane domain and one at the extreme C-terminus of the protein. The observation that a *nd* allele of *Notch* which alters only the extreme C-terminus of the protein interacts genetically with *abl* to perturb axon extension is consistent with this region being involved in the morphogenetic activity of *Notch*. Clearly, additional experiments will be necessary to test this idea more thoroughly.

Another of the original goals of our experimental program was to ask whether the various *Notch* signaling pathways act independently, or alternatively, whether their activities are either competitive or synergistic (Task 2). If the *Notch/abl* genetic interaction indeed arises from the direct binding of DAB to NOTCH, this suggests possible biochemical models for the interaction of the *abl* pathway with other *Notch* signaling pathways. For example, given the sequence similarity between the DAB and NUMB PTB domains, it is likely that they bind the same sequence on NOTCH. Thus, if these two pathways were active in the same cell, we would expect them to be anatagonistic due to competition for binding to NOTCH. Moreover, based on work done with NUMB, we imagine that the N-terminal PTB binding site on NOTCH is in the RAM23 domain that is also the binding site for the Su(H) protein. It will therefore be interesting and enlightening to ask whether binding of Su(H) and DAB to this domain is independent or whether either protein alters the affinity or activity of the other. We are currently planning experiments to investigate these issues.

Our discovery of a *Notch/abl* interaction suggests a powerful and direct approach to identifying additional members of this signaling pathway genetically (Task 3). Rather than the relatively laborious and indirect approach of screening for suppressors of activated *Notch* phenotypes, and then sifting these to separate those which are involved in cell identity from those involved in the control of morphology, we can now look for modifiers of the *Notch/abl* interaction itself. Since this genetic interaction only affects the morphology pathway, modifiers of the interaction should similarly be specific just to the pathway we wish to study, the morphology pathway. Thus, in the future we will screen for suppressors of the lethality produced by appropriate combinations of *Notch* and *abl* alleles. In addition, we have observed that some, but not all, allelic combinations produce an adult-viable synthetic phenotype in the wing margin ("notched wings"). Thus, we can also screen for enhancers of wing notching. Both of these are extremely convenient F1 screens, and should be substantially more efficient than the genetic screens we originally designed. We expect pilot tests of these screens to be performed during the coming year.

7. CONCLUSIONS

In the second year of this grant, we have completed the analysis of a diverse set of the developmental functions of Notch. The possession of well-characterized assays for *Notch* function is clearly a necessary prerequisite to any detailed dissection of *Notch* signaling. These studies have also shed light on fundamental aspects of *Notch* function. Most significant is the evidence that *Notch* is capable of inhibiting differentiation *per se*, that is, arresting cells in a precursor stage of development. As discussed above, such an activity has been proposed previously, and has been considered central to the oncogenic potential of *Notch*, but it has never been demonstrated directly. Our use of the well-studied *Drosophila* oogenesis system to study *Notch* action afforded us both morphological and molecular markers that made this finding possible.

In the past year, we have also obtained biochemical and genetic evidence that the morphogenetic activity of *Notch* arises from its interaction with Abl kinase and associated proteins. In addition to the importance of this result for understanding the mechanism of axon extension, it is also potentially of great significance for understanding a variety of malignancies. Abl has been the subject of intense investigation for nearly 20 years, since it was one of the first cellular oncogenes to be characterized. To date, however, the receptor(s) that activate Abl have remained elusive. Our data raise the possibility that these two known oncogenes, both of which are associated with T-cell

* This page contains unpublished data

leukemias when activated inappropriately, are actually two parts of a single signaling pathway. It remains to be seen how many other cancers, perhaps including breast cancer, may reflect the ectopic activation of this ubiquitous signal transduction pathway.

8. REFERENCES

1. Artavanis-Tsakonis, S., *et al.*, *The Notch locus and the cell biology of neuroblast segregation.* Annu. Rev. Cell Biol. (1991). **7**: p. 427-452.

2. Campos-Ortega, J.A. and Jan, Y.N., *Genetic and molecular basis of neurogenesis in Drosophila melanogaster.* Ann. Rev. Neurosci. (1991). **14**: p. 399-420.

3. Fortini, M.E. and Artavanis-Tsakonis, S., *The suppressor of Hairless protein participates in Notch receptor signalling.* Cell (1994). **79**: p. 273-82.

4. Artavanis-Tsakonis, S., *et al.*, *Notch Signaling.* Science (1995). **268**: p. 225-32.

5. Lecourtois, M. and Schweisguth, F., *The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling.* Genes Dev (1995). **9**: p. 2598-608.

6. Spana, E.P. and Doe, C.Q., *Numb antagonizes Notch signaling to specify sibling neuron fates.* Neuron (1996). **17**: p. 21-26.

7. Giniger, E., *et al.*, *Specifying the path of the intersegmental nerve of the Drosophila embryo: a role for Delta and Notch.* Development (1993). **117**: p. 431-440.

8. Henkemeyer, M.J., *et al.*, *DNA sequence, structure and tyrosine kinase activity of the Drosophila Abelson proto-oncogene homolog.* Mol. Cell. Biol. (1988). **8**: p. 843-53.

9. Hoffmann, F.M., *Drosophila abl and genetic redundancy in signal transduction.* TIGS (1991). **7**: p. 351-355.

10. Gertler, F.B., *et al.*, *Drosophila Abl tyrosine kinase in embryonic CNS axons: A role in axonogenesis is revealed through dosage-sensitive interactions with disabled.* Cell (1989). **58**: p. 103-13.

11. Gertler, F.B., *et al.*, *Dosage-sensitive modifiers of Drosophila abl tyrosine kinase function: prospero, a regulator of axonal outgrowth, and disabled, a novel tyrosine kinase substrate.* Genes Dev. (1993). **7**: p. 441-453.

12. Henkemeyer, M., *et al.*, *A novel tyrosine kinase-independent function of Drosophila abl correlates with proper subcellular localization.* Cell (1990). **63**: p. 949-960.

13. Bruno, *et al.*, *dosage sensitive interactions of dynein and dynactin mutations in Neurospora.* Proc Natl Acad Sci, USA (1996). **93**: p. 4775.

14. Bodmer, R. and Jan, Y.N., *Morphological differentiation of the embryonic peripheral neurons in Drosophila.* Roux's Arch Dev Biol (1987). **196**: p. 69-77.

15. Ruohola, H., *et al.*, *Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila.* Cell (1991). **66**: p. 433-450.

16. Tamura, K., *et al.*, *Physical interaction of a novel domain of the Notch receptor with the RBP-Jκ transcription factor.* Curr Biol (1995). **5**: p. 1416-23.

17. Diederich, R.J., *et al.*, *Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway.* Development (1994). **120**: p. 473-481.

18. Axelrod, J.D., *et al., Interaction between wingless and Notch signaling pathways mediated by dishevelled.* Science (1996). **271**: p. 1826-32.

19. Bate, M., *The embryonic development of larval muscles in Drosophila.* Development (1990). **110**: p. 791-804.

20. Corbin, V., *et al., A role for the Drosophila neurogenic genes in mesoderm differentiation.* Cell (1991). **67**: p. 311-323.

21. Speicher, S.A., *et al., The Serrate locus of Drosophila and its role in morphogenesis of the wing imaginal discs: Control of cell proliferation.* Development (1994). **120**: p. 535-44.

22. Guo, M., *et al., Control of daughter cell fates during asymmetric division: Interaction of Numb and Notch.* Neuron (1996). **17**: p. 27-41.

23. Zhong, W., *et al., Asymmetric localization of a mammalian Numb homolog during mouse cortical neurogenesis.* Neuron (1996). **17**: p. 43-53.

24. Lindsley, D.L. and G., Z.G., *The Genome of Drosophila melanogaster.* 1992, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press.

25. Doe, C.Q. and Goodman, C.S., *Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells.* Dev Biol (1985). **111**: p. 206-19.

26. Hoppe, P.E. and Greenspan, R.J., *The Notch locus of Drosophila is required in epidermal cells for epidermal development.* Development (1990). **109**: p. 875-85.

27. Bate, M., *et al., A dual requirement for neurogenic genes in Drosophila myogenesis.* Development (Supplement) (1993). : p. 149-61.

28. Baker, R. and Schubiger, G., *Ectoderm induces muscle-specific gene expression in Drosophila embryos.* Development (1995). **121**: p. 1387-98.

29. Maru, Y. and Witte, O., *How does the chimeric BCR-ABL oncogene function in human myelogenous leukemia?,* in *Application of Basic Science to Hematopoiesis and Treatment of Disease,* E.D. Thomas, Editor. 1993, Raven Press: New York.

30. Hall, A., *Ras-related GTPases and the cytoskeleton.* Mol. Biol. Cell (1992). **3**: p. 475-9.

31. Kimura, K., *et al., Regulation of myosin phosphatase by Rho and Rho-associated kinase.* Science (1996). **273**: p. 245-8.

32. Brand, A.H. and Perrimon, N., *Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.* Development (1993). **118**: p. 401-415.

33. Coffman, C.R., *et al., Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos.* Cell (1993). **73**: p. 659-671.

This page contains unpublished data

9. APPENDICES

FIGURE LEGENDS

Figure 1. Expression of activated *Notch* affects three distinct aspects of oogenesis.

A,B: Ovaries expressing the stalk cell marker *93F* were stained with XGAL. Wildtype females produce ovarioles with a small number of stalk cells separating successive egg chambers (A), while expression of activated *Notch* causes hyperplasia of stalk cells (B). Stained stalks are indicated with brackets.

C,D: Ovaries were double-stained with an antibody that recognize the oocyte nucleus (arrow), and with anti-FASIII to label mature polar cells (triangles). Wildtype egg chambers have 1-2 FASIII-positive polar cells at each end (C), while expression of activated *Notch* late in oogenesis inhibits development of polar cells (D). The positions of the missing polar cells are indicated by the triangles in this panel.

E-G: Ovaries were stained with anti-BIG BRAIN to identify stalk cell precursors. In wild type ovarioles (E), immature stalk cells initially form a double row of cells and accumulate BIG BRAIN protein primarily at the apical end of the cells (stalk indicated by triangle; depicted by #1 in the inset). As the stalks mature, the cells intercalate to form a single row with BIG BRAIN protein at the lateral membranes of the cells (older stalk indicated by arrow; depicted by #2 in the inset). Persistent expression of activated *Notch* from very early stages of oogenesis arrests stalk cells at the precursor stage (F,G), as assayed by their retention of a double-row organization with apically-localized BIG BRAIN protein.

Figure 2. Expression of activated *Notch* reveals three functions in myogenesis.

A,B: Embryos were stained for expression of the founder marker *nautilus*, either by *in situ* hybridization (A) or with anti-NAU antibodies (B); RNA and protein analysis gave identical results. Wild type embryo (A) initially expresses *nau* in a single myoblast per hemisegment (arrow). Expression of activated *Notch* under control of *ptwist*-GAL4 (B) prevents initiation of *nau* expression. A single *nau*-positive myoblast that has escaped from *Notch* inhibition (probably due to inhomogeneity of the expression system [32]) is indicated by arrow.

C-E: Embryos were stained with antibodies against the founder marker *S-59*. C and D are lateral views of stage 11 embryos; E is a ventral view of a stage 13 embryo. At stage 11, wildtype embryos (C) express *S-59* in 3 myoblasts per hemisegment. Expression of activated *Notch* (*ΔE-Notch*; also called *Notch* TM in the figure) in the mesoderm under the control of *ptwist*-GAL4 does not affect the initial expression of *S-59* (D). In such embryos, S-59 expression is lost in the mesoderm by stage 13 (E), still prior to myoblast fusion. At this stage, *S-59* should be present in a substantial number of cells in the ventrolateral mesoderm (indicated by meso); but only one cell (arrow) escapes from *Notch* inhibition. In contrast, in this embryo *S-59* expression is readily detectable in a number of cells of the central nervous system (CNS, indicated by bar).

F,G: Embryos were stained with antibodies against the founder marker *vestigial*. At stage 13, *vg* is expressed in several CNS neurons (alongside bar) as well as a substantial number of myoblasts (meso) in a wildtype embryo (F). Expression of activated *Notch* in the ectoderm (G), under control of the enhancer trap GAL4-112 inhibits neurogenesis, as expected (CNS expression of *vg* is blocked), but surprisingly also shuts down mesodermal expression of *vg*.

H: Cuticle of an embryo expressing activated *Notch* in the ectoderm. Expression of activated *Notch* apparently does not grossly disrupt epidermal differentiation, since cuticle forms and is patterned normally. Note the regular bands of denticles (arrow) that are diagnostic for proper epidermal differentiation.

Figure 3. NOTCH binds DISABLED *in vitro* .

NOTCH protein was labelled with ^{32}P and incubated with beads covalently coupled to either a fusion of mouse *disabled* to glutathione-S-transferase (m-DAB) or else glutathione-S-transferase alone (Gst), in binding buffer containing the indicated detergent(s). After extensive washing,

bound material was eluted in Laemmli sample buffer and analyzed by PAGE. In this experiment, two proteolytic products of NOTCH were present in the labelled material. The higher band contains the amino-terminal 80 Kd of NOTCH; the lower band contains the amino-terminal 45 Kd of NOTCH.

Figure 4. Genetic interaction of null and hypomorphic alleles of *Notch* with *abl* mutations.

Embryos were stained with anti-HRP to visualize the surfaces of all neurons. In each panel, ventral view of embryo showes the axon scaffold of the central nervous system (CNS), with arrow indicating the position of one of the longitudinal axon tracts that connect the successive segmental ganglia.

A: Wild type embryo. CNS axons form an unbroken scaffold throughout the thorax and abdomen. *abl* null mutant animals have a CNS axonal pattern indistinguishable from the wildtype.

B: $Notch^{ts}$ mutant embryo, shifted to restrictive temperature at mid-embryogenesis. Loss of *Notch* function leads to a distinctive pattern of CNS defects, with a failure to form longitudinal connections between successive segmental ganglia. In consequence, CNS axonal pattern resembles a series of separate 'squares' rather than an unbroken ladder.

C,D: CNS axonal pattern arising from the *Notch/abl* genetic interaction. *abl* null animals that are heterozygous for a *Notch* deletion (C) or homozygous for the hypomorphic *Notch* allele *nd1* (D), give a pattern of CNS axonal defects that closely resembles the pattern induced by complete removal of *Notch* function at mid-embryogenesis.

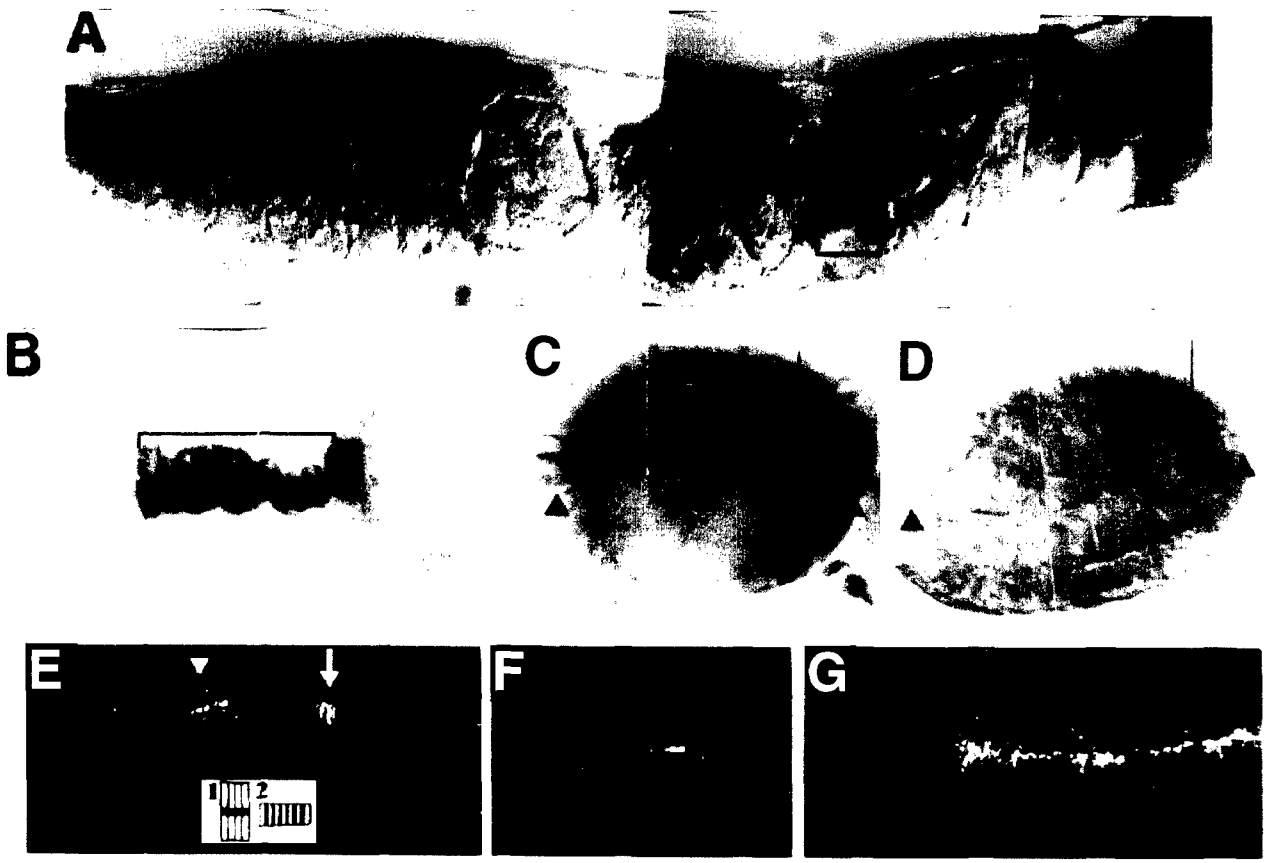


Figure 1
Expression of activated Notch affects three distinct aspects of oogenesis.

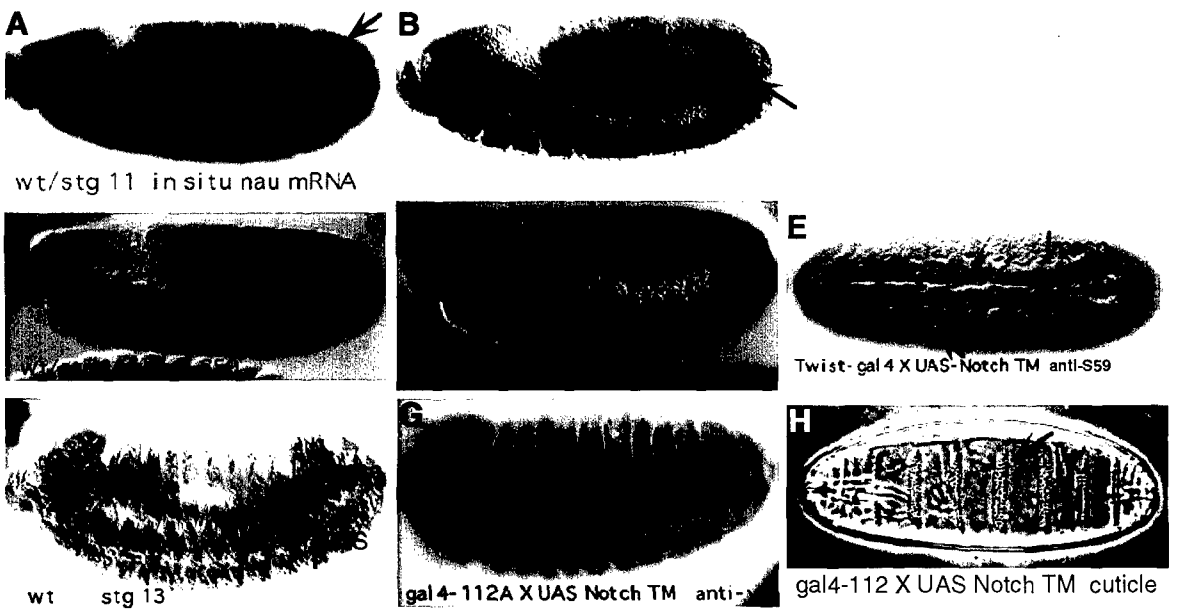


Figure 2
Expression of activated Notch reveals three functions in myogenesis.

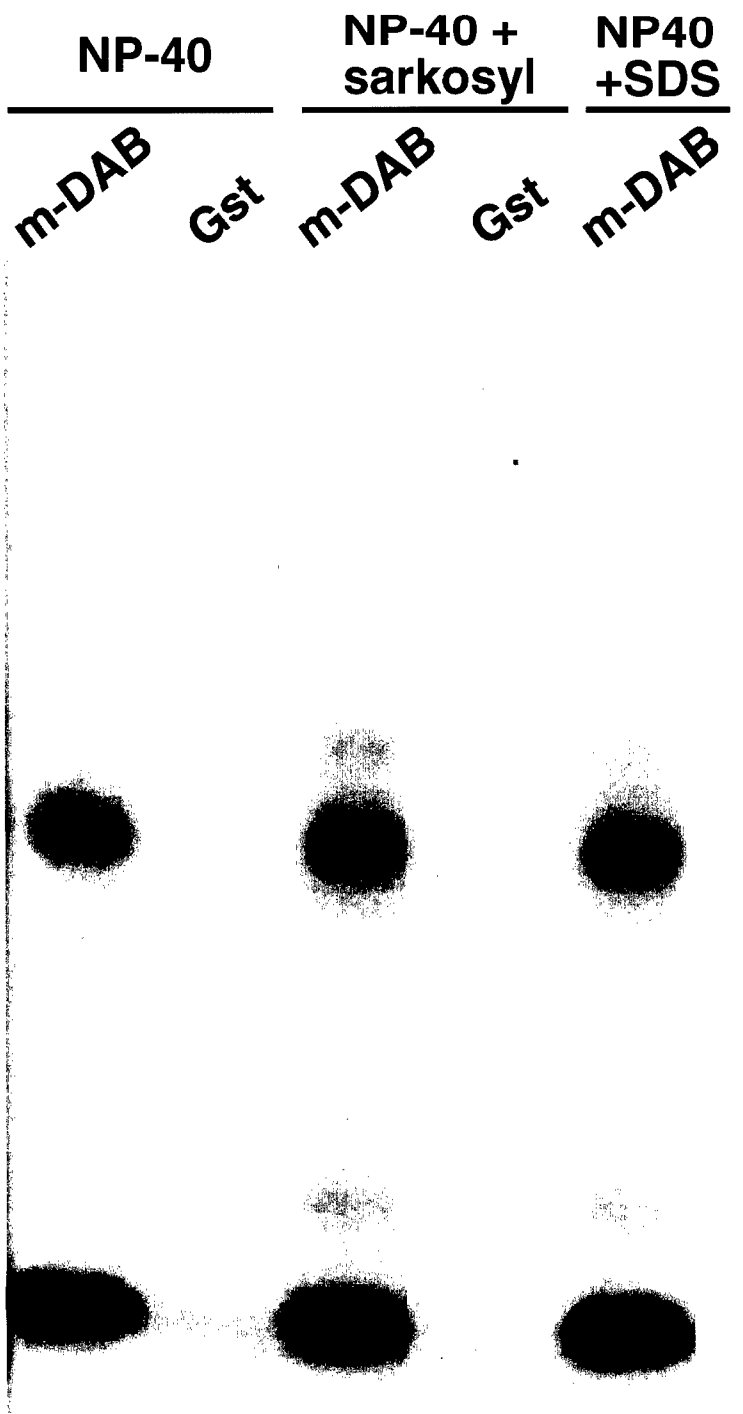


Figure 3
NOTCH binds DISABLED in vitro.

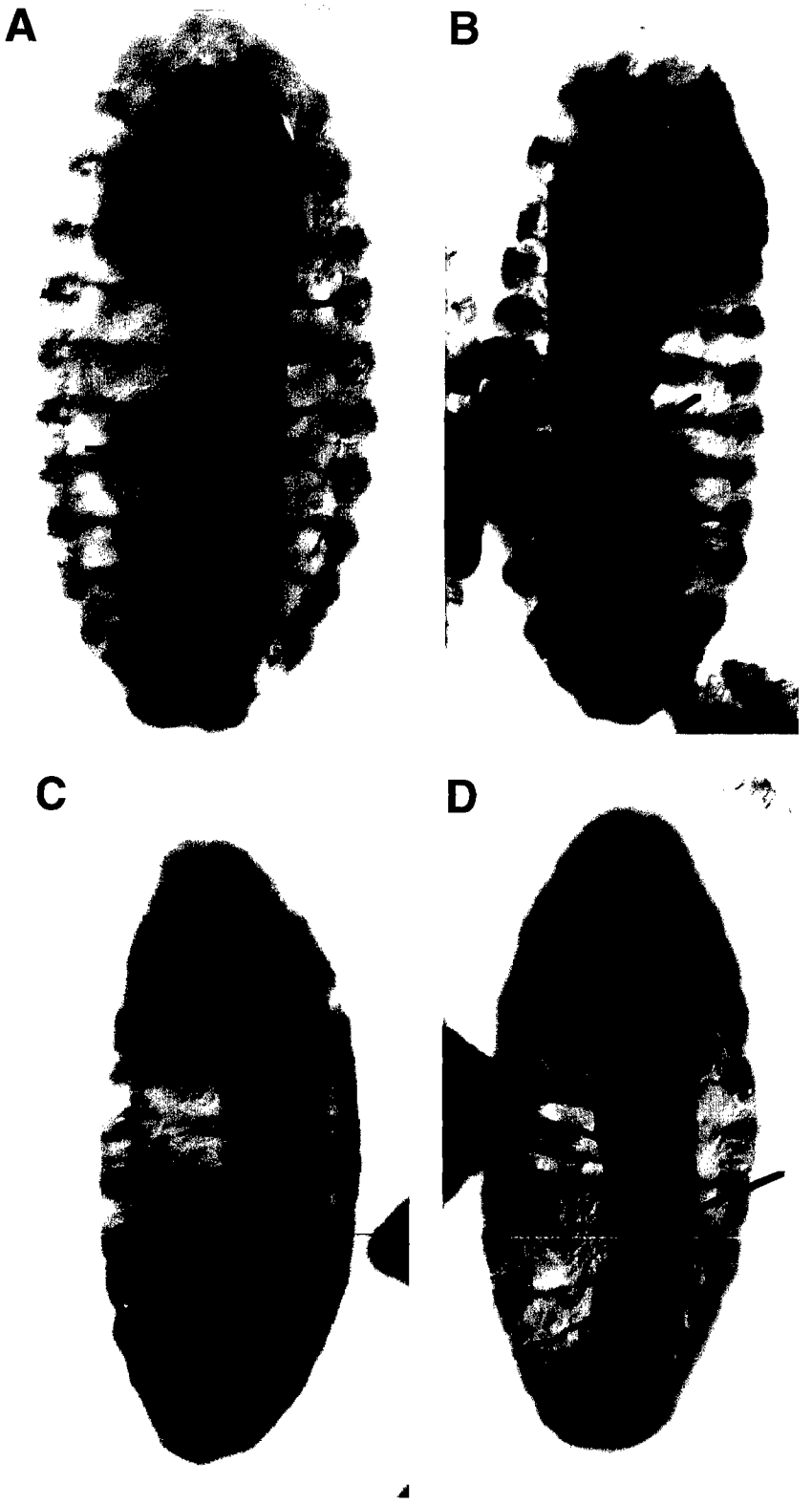


Figure 4
Genetic interaction of null and hypomorphic alleles of Notch with abl mutations.

Recd 2/25/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Feb 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical report written for Grant DAMD17-94-J-4266 and DAMD17-94-J-4301. Request the limited distribution statement for Accession Document Numbers ADB222576, ADB221779, and ADB235519, be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management