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THE NEUROGENIC GENES IN *DROSOPHILA* OOGENESIS

by

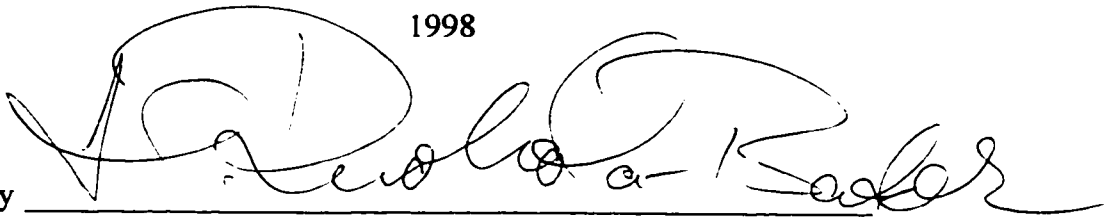
Michele Keller Larkin

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by 

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Doctoral Dissertation

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Abstract

THE NEUROGENIC GENES IN *DROSOPHILA* OOGENESIS

by Michele Keller Larkin

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Department of Biochemistry

The Notch receptor of *Drosophila* and its homologues in other organisms mediate cell-cell interactions required for the correct partitioning of cell fates within equivalence groups. Genes related to Notch and other components of the Notch signaling pathway represent a well conserved system for signal transduction, having been isolated from organisms as diverse as flies, worms, sea urchins, frogs, fish, chickens, mice, rats, and humans (reviewed by Lardelli *et al.*, 1995) The expression and requirements for Notch signaling are pleiotropic through development, in contrast to other tissue or cell type specific receptors. How the Notch signaling cascade mediates pattern formation in so many tissues and cell types is not well understood. The research contained herein increases the understanding of Notch signaling by studying its role during *Drosophila* oogenesis. Additionally, this research lends insight into several important processes that take place during *Drosophila* oogenesis, including determination of follicle cell fates surrounding the egg chamber and in stalk formation, establishment of the oocyte anterior-posterior axis, and the control of germ-line mitosis.

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List of Abbreviations

AC. anchor cell

A-P. anterior-posterior

bam. bag-of-marbles

bcd. bicoid

bib. big Brain

caN. constitutively-active Notch

cni. cornichon

Dl. Delta

Egfr. Egf receptor

enc. encore

FasIII. Fasciclin III

fc. follicle cell

Flp. Flipase

FRT. Flipase recognition target

grk. gurken

HS. Heat shock

hts. hu-li tai shao

MTOC. Microtubule organizing center

N. Notch

neu. neuralized

osk. oskar

pc. Polar cells

PKA. protein kinase A

pum. pumilio

Ser. Serrate

stau. staufen

Su(H). Suppressor of Hairless

top. torpedo

UAS. Upstream activating sequence

VU. ventral uterine precursor

w. white

wg. wingless

y. yellow

Dedication

I wish to dedicate this dissertation to my family, without whom I never would have been able to do this.

Introduction

The *Drosophila Notch* gene encodes an ~300kD transmembrane receptor, with an extracellular ligand binding domain and an intracellular domain required for signaling (reviewed by Weinmaster, 1997). Notch was initially identified by the mutant phenotype from which it takes its name - flies heterozygous for this mutation exhibit notches in their wing margins (Mohr, 1919). Later work revealed that most of the epidermis of *Notch* mutant embryos is transformed to the neural fate (Poulson, 1940); it is this phenotype which now defines some members of the Notch-signaling cascade as "neurogenic". Although the Notch signaling pathway has long been associated with neurogenesis, the requirements for Notch-signaling are pleiotropic, affecting tissues derived from all three germ layers. Notch is widely expressed in the *Drosophila* embryo, and expression persists in uncommitted and proliferative cell groups in later stages of development (reviewed by Artavanis-Tsakonas *et al.*, 1995). Notch signaling is generally involved in mediating cell-cell interactions required for the correct partitioning of cell fates.

STRUCTURE OF THE NOTCH RECEPTOR

The Notch receptor is a very large transmembrane receptor, containing several specific domains identified in other proteins (reviewed by Egan *et al.*, 1998). The extracellular domain of Notch contains an N-terminal signal peptide, 36 tandem EGF-like repeats, and 3 tandem cysteine rich repeats termed the Notch/LIN-12 repeats. The signal peptide is required for passage through the secretory pathway on its way to the plasma membrane. EGF-like repeats are protein-protein interaction motifs; each is approximately 40 amino acids long and contains 6 cysteine residues with conserved spacing to form 3 disulfide bridges. The Notch/LIN-12 repeats are also cysteine rich domains; it has been

suggested that they are required to prevent dimerization of the receptor prior to ligand binding and activation. The extracellular domain is followed by a transmembrane domain, then the intracellular domain. This cytoplasmic domain contains a Ram domain, six cdc10/ankyrin repeats, which are protein-protein interaction motifs, and a PEST sequence, important for protein stability.

COMPONENTS OF THE NOTCH PATHWAY

Many components of the Notch-signaling cascade have been identified, and like Notch, some of the other constituents are encoded by so-called neurogenic genes (reviewed by Blaumueller and Artavanis-Tsakonas, 1997 and Egan *et al.*, 1998). There are two known ligands for Notch in *Drosophila*: Delta and Serrate. They are both membrane bound and are homologous to one another as well as to Notch. The Notch-signaling cascade also includes the nuclear protein Mastermind, the membrane protein Big brain, the bHLH genes of the *Enhancer of Split* complex, *Deltex*, *Hairless*, *numb*, *groucho*, and *Suppressor of Hairless*. In addition, different interaction and phenotypical studies have also linked *neuralized*, *almondex*, *fringed*, *daughterless*, *wingless*, *strawberry notch*, *vestigial*, *shaggy*, *Star*, *bearded*, *scabrous*, and *canoe* to the Notch pathway (reviewed by: Blaumueller and Artavanis-Tsakonas, 1997; Kimble and Simpson, 1997; Young and Wesley, 1997; Egan *et al.*, 1998). An interaction pathway for neurogenic gene action was proposed by de la Concha *et al.* (1988) by modifying the dosage of neurogenic genes individually and analyzing the effects. More specifically, they examined whether over-expression of the different neurogenic genes could rescue the neurogenic phenotypes resulting from mutations in other neurogenic genes. Further studies have supported this conception of the pathway (Lieber *et al.*, 1993). It appears that Delta, Big brain, Neuralized, and Mastermind act in elaboration of a signal upstream of the Notch receptor, while genes of the *Enhancer of split* complex act after Notch. A speculative model for Notch signaling is illustrated in Figure 1 (Artavanis-Tsakonas *et al.*, 1995). Suppressor of

Hairless is sequestered in the cytoplasm, presumably by its interaction with the cytoplasmic domain of Notch (Fortini and Artavanis-Tsakonas, 1994). Either Delta or Serrate may bind to the extracellular domain of the Notch receptor, resulting in recruitment of the cytoplasmic protein Deltex (Fehon *et al.*, 1990; Rebay *et al.*, 1991; Lieber *et al.*, 1992, de Celis *et al.*, 1993; Diederich *et al.*, 1994; reviewed by Artavanis-Tsakonas *et al.*, 1995). Both Deltex and Suppressor of Hairless are able to bind to the intracellular Ankyrin repeats of Notch, leading to the suggestion that recruitment of Deltex leads to displacement Suppressor of Hairless, and its subsequent translocation to the nucleus (Diederich *et al.*, 1994; Fortini and Artavanis-Tsakonas, 1994; reviewed by Artavanis-Tsakonas *et al.*, 1995). Furthermore, it is thought that ligand binding results in dimerization of the receptor (reviewed by Artavanis-Tsakonas *et al.*, 1995 and Greenwald 1994), and that this dimerization is stabilized by binding of Deltex (Diederich *et al.*, 1994). Putative Suppressor of Hairless binding sites in the regulatory sequences of the Enhancer of Split genes suggest that transcription of these genes may be Suppressor of Hairless dependent (Tun *et al.*, 1994; Henkel *et al*, 1994; Grossman *et al.*, 1994). Hairless is believed to bind to Supressor of Hairless, preventing the transcription factor from binding to DNA (Schweisguth and Posakony, 1994, Brou *et al.*, 1994). In addition, recent studies have suggested that nuclear localization of the intracellular domain of Notch may be involved in Notch-pathway signaling (Fortini *et al.*, 1993, Aster *et al.*, 1994; Kopan *et al.*, 1994; Ahmad *et al.*, 1995; Zagouras *et al.*, 1995; reviewed by Blaumueller and Artavanis-Tsakonas, 1997; Egan *et al.*, 1998).

CONSERVATION OF THE NOTCH PATHWAY

Genes related to *Notch* and other components of the Notch signaling pathway represent a well conserved system for signal transduction, having been isolated from organisms as diverse as flies, worms, sea urchins, frogs, fish, chickens, mice, rats, and humans (Sherwood, 1997 and reviewed by Lardelli *et al.*, 1995). The overall structure

of these genes is highly conserved. Multiple paralogs of *Notch*, as well as the ligands for Notch, have been detected in worms, mice, rats, chicks, and humans, and have been called *Notch1*, *Notch2*, *Notch3*, and *Notch4*, *Jagged1* and *Jagged2* (Serrate homologues), and *Delta1* and *Delta2* (reviewed by: Lardelli *et al.*, 1995, Egan *et al.*, 1998; and Weinmaster, 1997). Homologues of *Suppressor of Hairless*, *big brain*, *groucho*, *scabrous*, and *Deltex* have been identified in mice and humans as well (reviewed by Blaumueller and Artavanis-Tsakonas, 1997), and *neuralized* related sequences have been identified in *C. elegans* and mammals (Zhou and Boulianne, 1994). Despite overlap in the patterns of paralog expression, differences have been observed (Weinmaster *et al.*, 1992; Lardelli and Lendahl, 1993; Lardelli *et al.*, 1994; Lindsell *et al.*, 1995; Mitsiadis *et al.*, 1995). It appears that in some cases the functions of different paralogs are distinct, but it is unclear whether this arises from differences in expression patterns or the mechanism by which different paralogs act. At least in *C. elegans*, ectopic expression of the two Notch homologues that have been isolated indicates that they are functionally interchangeable, despite that loss of function studies indicate that they regulate distinct functions during development (Mango *et al.*, 1991; Roehl and Kimble, 1993; Fitzgerald *et al.*, 1993). In addition, *Drosophila* Delta and Serrate both activate the same receptor and can functionally replace each other in some processes (Gu *et al.*, 1995). However, they result in different mutant phenotypes, have distinct patterns of expression, seem to function in different developmental decisions, and are not equal in some cell types (reviewed by Weinmaster, 1997, and Egan *et al.*, 1998).

Data abound demonstrating the requirements of the Notch pathway in development; the majority comes from studies with *Drosophila* and *C. elegans* - molecularly, genetically, and phenotypically (reviewed by Greenwald, 1994). Here I will focus on the effects of disturbing the Notch pathway in several well characterized processes during development of these two organisms in order to illustrate the function of the Notch pathway, and the diversity of cell types in which it acts.

During *Drosophila* neurogenesis, an ectodermal proneural equivalence group segregates into neuroblasts, which delaminate from the monolayer and migrate dorsally to produce neuronal cell types, and dermoblasts, which remain at the ventral surface and give rise to epidermal structures. Loss of Notch signaling results in failure of neuroblast and dermoblast fates to partition properly, leading to hyperplasia of neural cells. This hyperplasia occurs at the expense of epidermal cells - resulting in embryos that exhibit no ventral cuticle and hypertrophy of the central and peripheral nervous systems (reviewed by Muskavitch, 1994). Activation of the pathway results in the seemingly opposite phenotype, in that fewer neurons are detected (reviewed by Muskavitch, 1994; Artavanis-Tsakonas *et al.*, 1993).

Development of the small sensory bristles of *Drosophila* also relies on Notch signaling (reviewed by Simpson, 1997, and Kimble and Simpson, 1997). On the notum, microchaetes are arranged in rows separated by epidermal cells. The microchaete precursors arise from stripes of *achaete/scute* expression; the cells that express *achaete scute* must choose between epidermal and neural fates. *achaete scute* expression is sustained in neural precursors and ceases in epidermal cells. Loss of Notch function results in all cells within a stripe adopting the fate of bristle precursors, while activation of the Notch pathway causes all cells to adopt the epidermal fate.

Another well-characterized function of the Notch pathway is in the development of the *C. elegans* gonad (reviewed by Kimble and Simpson, 1997). Here, two somatic gonad cells interact to adopt one of two alternate fates: anchor cell (AC) or ventral uterine precursor (VU). In the absence of Notch signaling, both cells become ACs, whereas activation of the Notch pathway results in both cells becoming VUs.

Notch signaling is also required for the control of germ-line mitosis in *C. elegans*. In the *C. elegans* gonad, a somatic cell termed the distal tip cell signals the neighboring germ line to divide mitotically; localization of this signal establishes polarity in

the germ-line tissue (Kimble and White, 1981). This control of germ-line mitosis requires the Notch homologue in the germ-line cell, and the Delta homologue in the signaling distal tip cell (reviewed by Kimble and Simpson, 1997). In the absence of this signal the germ line enters meiosis.

As these examples illustrate, Notch signaling functions in numerous cell-cell interactions and regulates a multitude of cell fate decisions. A common theme seems to be that loss of Notch pathway function generally results in all cells of an equivalence group taking one of two possible fates or differentiation programs, while activation of the pathway results in all of the cells taking the alternate fate.

NOTCH SIGNALING IN VERTEBRATE DEVELOPMENT AND DISEASE

Coffman *et al.* (1990) isolated the first vertebrate *Notch*-family gene using a *Drosophila Notch* cDNA probe. However, the first indication that Notch signaling may be crucial to vertebrate development came from detection of oncogenic forms of one of the human homologues of *Notch*. Ellisen *et al.* (1991) showed that the breakpoint of a recurrent chromosomal translocation present in T cell leukemias is located in the *Notch1* gene (also called *tan-1*, Ellisen *et al.*, 1991; reviewed by Lardelli *et al.*, 1995). Further evidence came from the mouse *Notch* related gene, *INT3*, which is activated in some mammary tumors (Robbins *et al.*, 1992), and from the findings that human *Notch2* and *Notch3* genes are located in regions of chromosomal translocations associated with neoplasias (Larsson et al., 1994). One *Notch* homologue was found to be expressed in human bone marrow cells, suggesting a role for Notch signaling in cell-fate decisions during hematopoiesis (Milner *et al.*, 1994). A role such as this has been found for Notch signaling in mice; transgenic mice with activated *Notch1* or *Notch3* affect T cell lineage decisions and neuronal differentiation (reviewed by Weinmaster, 1997). Additionally, the human disease, CADASIL, characterized by strokes and dementia, maps to mutations in *Notch3* (Joutel *et al.*, 1996). Notch signaling has also been implicated in cervical and

colonic neoplasias, and the human homologue of *Suppressor of Hairless* is involved in the immortalization of B cells induced by Epstein-Barr virus (reviewed by Blaumueller and Artavanis-Tsakonas, 1997).

The expression patterns of Notch homologues in vertebrates extends our understanding of its role in these organisms. Both *Xenopus* and Zebrafish exhibit Notch expression patterns strikingly similar to those characterized in *Drosophila*: Notch is expressed quite ubiquitously in early development, and becomes localized to regions undergoing proliferation or cell-fate changes (reviewed by: Blaumueller and Artavanis-Tsakonas, 1997; Egan *et al.*, 1998). Notch homologues are also expressed in proliferating tissues or areas of cell-fate determination in mammals (reviewed by Blaumueller and Artavanis-Tsakonas, 1997). The mutant phenotypes and expression patterns of Notch pathway elements seem to indicate that in addition to structural homology among Notch pathway genes, the functions of Notch signaling have been conserved throughout evolution.

Further understanding of Notch function in vertebrates has come from studies in *Xenopus* neurogenesis (Chitnis *et al.*, 1995, Dorsky *et al.*, 1997, reviewed by Weinmaster, 1997, and Gridley, 1997) and retinal development in the chick. In *Xenopus*, ectopic expression of dominant negative forms of Delta results in an increase in the density of neural tissue, whereas activation of the pathway reduces the density of neurons. Austin *et al.* (1995) explored the effects of disturbing Notch signaling on differentiation of ganglion cells of the chick. They determined that the number of retinal ganglion cells that differentiated was inversely related to the level of Notch signaling.

The above observations suggest that in vertebrates, Notch activity is important for terminal differentiation and cell proliferation. To investigate the requirements for Notch activity during mammalian development, mutations of the endogenous genes have been introduced into the germ line of mice by gene targeting.

Mouse embryos lacking *Notch1* die before 11.5 days of development, presumably because of defects in somitogenesis (Swiatek et al., 1994, Conlon et al., 1995). Embryos lacking *Notch2* die at birth and exhibit reduction in lung and kidney size (reviewed by Lardelli et al., 1995).

MECHANISMS OF NOTCH SIGNALING

Since the Notch-signaling cascade acts in so many separate developmental processes, there are several models to explain how Notch signaling mediates pattern formation (reviewed by Kopan and Turner, 1996; and by Blaumueller and Artavanis-Tsakonas, 1997). Two capacities in which the Notch pathway may act are lateral specification and inductive signaling. When Notch signaling functions in lateral specification (or lateral inhibition), subsets of a group of initially equivalent cells choose a different differentiation program. Initially, both cell types express both ligand and receptor; it is believed that random fluctuations in the levels of ligand and receptor are amplified through feedback regulation such that some cells become signaling, or Delta-expressing, cells while others become receiving, or Notch-expressing, cells. Examples of cell-fate decisions that are believed to involve lateral specification include formation of the anchor cell and the ventral uterine precursor cell of the *C. elegans* hermaphrodite gonad and partitioning of neural and epidermal precursor cells in the ventral ectoderm of the *Drosophila* embryo (reviewed by Greenwald, 1994 and Artavanis-Tsakonas *et al.*, 1995). In other instances, the Notch-signaling cascade is required for inductive signaling. This type of signaling occurs between two different cell types, one of which produces a signal (the ligand) and induces the other, which contains the receptor for the signal, to adopt a particular fate (reviewed by Artavanis-Tsakonas *et al.*, 1995 and Simpson, 1997). In these cases, Notch signaling is responsible for refining a pattern rather than stochastically resolving cell fates. Examples of cell-fate decisions that require inductive cell-fate specification include induction of the vulval precursor cells by the anchor cell in the *C.*

elegans gonad and induction of the R7 cell by the R8 cell during *Drosophila* eye development (reviewed by Artavanis-Tsakonas *et al.*, 1995).

One unresolved issue concerning the Notch pathway is whether signaling is required for activation of a specific cell-fate program (acting "instructively") or inhibition of the alternate program (acting "prohibitively") (reviewed by: Greenwald, 1994; Muskavitch, 1994; Artavanis-Tsakonas, 1995; Blaumueller and Artavanis-Tsakonas, 1997). Studies in *C. elegans* have suggested that Notch signaling is responsible for activation of certain cell-fate programs (reviewed by Blaumueller and Artavanis-Tsakonas, 1997). However, work in *Drosophila* and *Xenopus* has suggested that Notch signaling represses cellular responses to other signals, delaying cellular differentiation (Coffman *et al.*, 1993; Fortini *et al.*, 1993; Struhl *et al.*, 1993). It has been suggested that this function of Notch signaling may serve to influence the type and number of cells responding to certain differentiation pathways, retaining subsets of cells for later differentiation events (Blaumueller and Artavanis-Tsakonas, 1997). This is an attractive model, since a general mechanism of delaying differentiation could possibly explain how one signaling cascade could accomplish such a range of tasks in so many tissues and cell types. Since the differentiation state of the cell is ultimately defined by the expression of various genes, such a differentiation delay would most likely accomplished by repression of certain genes and the simultaneous induction of others. Indeed, the cytoplasmic domain of Notch has been hypothesized to act as a molecular scaffold that promotes the assembly of a transcriptional regulating complex on specific gene promoters (Egan *et al.*, 1998).

It has been suggested that there is yet another function for the Notch pathway. It is possible that not all processes that require the function of the Notch pathway require its signaling activity. A role for Notch in cell-cell adhesion has been suggested by the finding that mutations in collagen genes can suppress the Notch mutant phenotypes in *C. elegans* (Kramer *et al.*, 1988; Johnstone *et al.*, 1992; reviewed by Lardelli *et al.*, 1995). A similar role has been suggested by studies of the intersegmental

nerve of the *Drosophila* embryo, where growing axons use Notch on their surfaces to recognize a path of Delta expression (Giniger *et al.*, 1993).

REGULATION OF THE NOTCH PATHWAY

The Notch pathway is regulated at a number of levels throughout development (reviewed by: Kimble and Simpson, 1997; Egan *et al.*, 1998). One widely used control is cell-specific expression of ligand and receptor, commonly utilized for inductive interactions - such as *C. elegans* germ-line induction, where the Delta homologue is required in the distal tip cell and the Notch homologue is required in the germ-line cells. With this type of regulation, the control of gene expression defines signaling and receiving cells. A second control that has shown to be important in the control of Notch signaling is feedback loops, affecting expression of both ligand and receptor. This method of regulation is often utilized in lateral specification events, insuring the adoption of two alternative fates by cells within a field of equivalent precursors. It is also employed during induction to maintain cell fate decisions. The AC/VU decision of *C. elegans* is a prime example of feedback regulation; here, it has been found that receptor activation down regulates expression of ligand in the receiving cell while maintaining receptor expression in the same cell (Wilkenson *et al.*, 1994). A third way the Notch pathway can be regulated is by asymmetric segregation of a pathway regulator. An example of this type of control is the protein Numb, a presumptive negative regulator of the Notch pathway. It is believed that after a cell divides, asymmetric segregation of Numb in one daughter cell down-regulates Notch specifically in that cell (Jan and Jan, 1995). A fourth type of control, proteolysis of the Notch receptor, has been found to be important through studies showing that Kuzbanian proteolytically activates Notch (Pan and Rubin, 1997). Lastly, interactions with other pathways can often regulate the function of Notch signaling; two examples which are widely cited are the *wingless* (*wg*) and Egfr (Egfr) pathways (reviewed by Kimble and Simpson, 1997). Interactions

with the *wg* pathway are context dependent: Notch and *wg* activities can be synergistic (Neumann and Cohen, 1996) or antagonistic (González-Gaitán and Jäckle, 1995; Axelrod *et al.*, 1996). The combined action of the Notch and Egfr pathways is required for the fate specification in the *C. elegans* vulva, as well as in *Drosophila* for eye development and axis formation (reviewed by: Duffy and Perrimon, 1996; Kimble and Simpson, 1997). How these two pathways relate to one another is not known.

Obviously, many of the fine points of Notch signaling have yet to be elucidated. We still do not know the exact effects on the cells in which Notch signaling acts, or the mechanism by which a single pathway can biochemically function in such a diverse range of tissues and have such a wide range of results. The research contained herein intends to increase the understanding of Notch signaling by studying its role during *Drosophila* oogenesis. Oogenesis lends itself to this type of study for several reasons. Aside from the ease of genetic manipulation, there are relatively few cell types, and the arrangement of the *Drosophila* ovariole represents a time-lapse view of development. Additionally, this research lends insight into several important processes which take place during *Drosophila* oogenesis, including determination of follicle cell fates surrounding the egg chamber and in stalk formation, establishment of the oocyte anterior-posterior axis, and the control of germ-line mitosis.

OVERVIEW OF *DROSOPHILA* OOGENESIS

The *Drosophila* ovary consists of 15-20 ovarioles: strings of egg chambers aligned in developmental order (Figure 2). At the anterior end of each ovariole lies the germarium, where the germ cell divisions take place. Here, each germ-line stem cell divides to produce two daughters; one daughter remains a stem cell while the other becomes a cystoblast. Through four successive rounds of mitosis, each followed by incomplete cytokinesis, this cystoblast forms a cyst of 16 cells interconnected by

cytoplasmic bridges. These bridges, called ring canals, are an indication of the number of cell divisions: two cells have four ring canals, two cells have three ring canals, four cells have two ring canals, and eight cells have a single ring canal. One of the two cells with four ring canals will become the oocyte, while the other 15 cells will differentiate as nurse cells that produce and transport components to the oocyte throughout oogenesis. The four mitotic divisions are accompanied by the growth of a branched cytoplasmic structure called the fusome, which extends through the ring canals and associates with one pole of the mitotic spindle. On completion of the fourth round of mitosis, germ-line cysts are surrounded by a somatic follicle cell monolayer, creating a defined egg chamber. As the egg chamber is released from the germarium, a subset of follicle cells intercalates between egg chambers to form an interfollicular stalk. Egg chambers then continue to develop, connected to one another by these stalks. Several other groups of follicle cells are morphologically discernible, including polar cells, two cells at the anterior and posterior of each egg chamber (For review of oogenesis see King, 1970; Spradling, 1992).

Figure 1: Diagram of a hypothetical model for Notch signaling

The signaling cell is diagrammed on the left and the receiving cell on the right. The Notch receptor is indicated in red, Delta in purple, Deltex in gray, Hairless in orange, Suppressor of Hairless in yellow, and Serrate in green. The genes of the Enhancer of Split complex are represented as a DNA strand. Suppressor of Hairless is sequestered in the cytoplasm. Upon binding of Delta or Serrate to the extracellular domain of Notch, Deltex is recruited, displacing Suppressor of Hairless, which is then translocated to the nucleus. It is thought that ligand binding results in dimerization of the receptor, and that this dimerization is stabilized by binding of Deltex. Putative Suppressor of Hairless binding sites in the regulatory sequences of the *Enhancer of Split* genes suggest that transcription of these genes may be Suppressor of Hairless dependent. Hairless is believed to bind to Supressor of Hairless, preventing the transcription factor from binding to DNA.

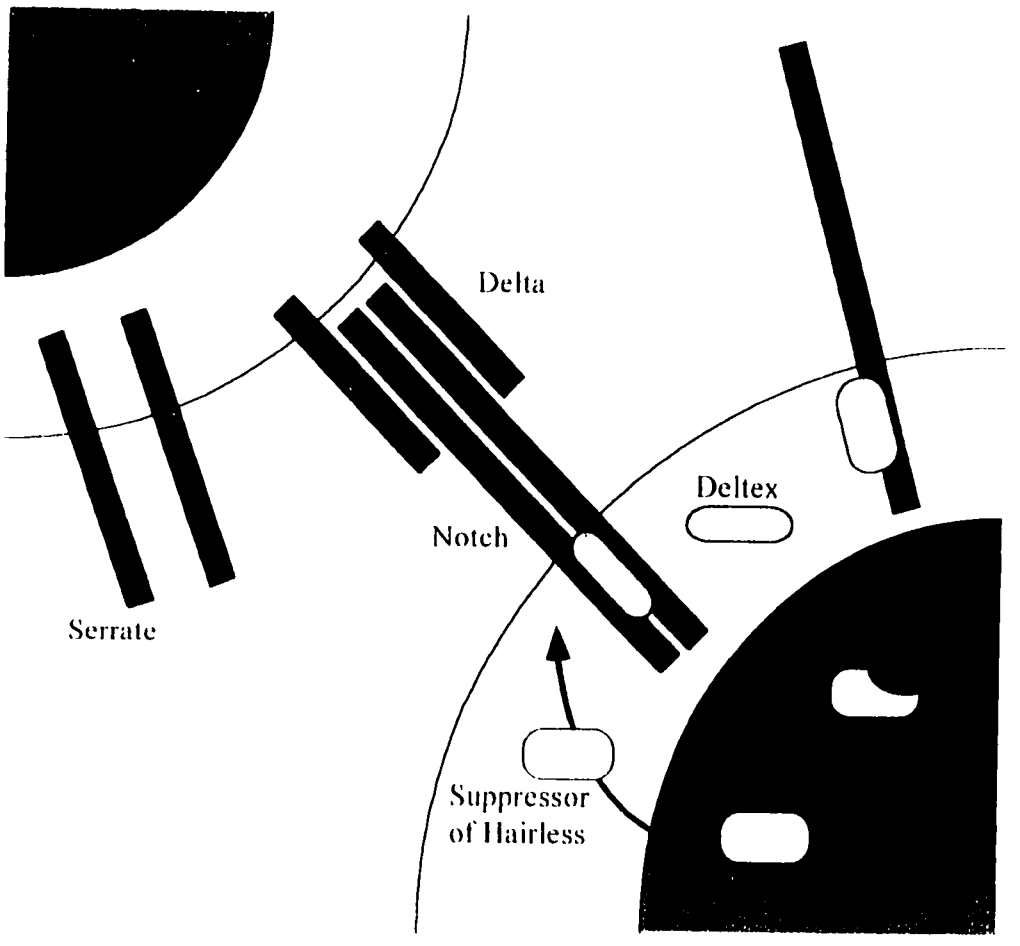
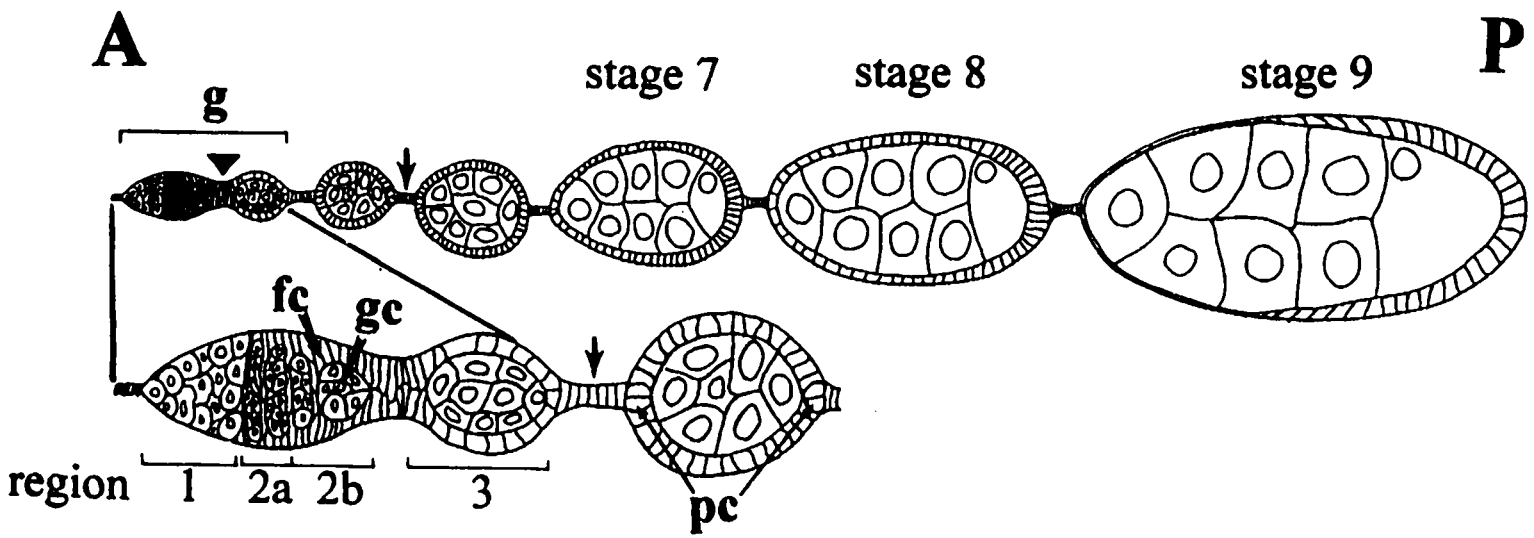

Delta
Deltex
Notch
Serrate
Suppressor
of Hairless

Figure 2: Developmental sequence of oogenesis: diagram of an adult wild-type ovariole.

Drosophila oogenesis is divided into 14 stages (stages 7, 8, and 9 indicated). The anterior-most structure, the germarium (g), contains the stem cells for germ line and the somatic follicle cells. The germarium is divided into four regions: In region 1 germ-line cells divide four times to produce a cyst of 16 germ cells (gc), 15 nurse cells and one oocyte. When the cyst has reached region 2, follicle cells (fc) migrate between two successive egg chambers and begin to intercalate, causing the region 3 egg chamber to "pinch off" from the germarium (arrowhead). These cells then take the fate of either stalk cells (arrows) or follicle cells which surround the egg chamber, including the polar cells (pc).


A
P
stage 7
stage 8
stage 9
g
fc
gc
region
1
2a
2b
3
pc

Chapter 1: The Neurogenic genes *Notch* and *neuralized* are required for the cessation of germ-line mitosis during *Drosophila* oogenesis.

INTRODUCTION

The cell-division cycle is controlled by the combined action of two families of proteins. The cyclin-dependent protein kinases (Cdk) function by phosphorylating selected proteins on serines and threonines, while the cyclins bind to Cdk molecules and control their ability to phosphorylate target proteins. The cell-division cycle is strictly controlled by the periodic assembly, activation, and disassembly of cyclin/cdk complexes (reviewed by Hartwell and Weinert, 1989; Murray and Kirschner, 1991; O'Farrell, 1992). This cycle is controlled at specific checkpoints that allow the cell cycle control system to be regulated by signals from the environment; the most prominent checkpoints are the G_2 checkpoint, the metaphase checkpoint, and the G_1 checkpoint. This is how regulatory information modifies the cycle.

The germ cells of the *Drosophila* ovary exhibit modification of the normal division cycle. Asymmetric division of a stem cell results in two daughter cells, one of which becomes a cystoblast. The cystoblast then undergoes four rounds of mitosis with incomplete cytokinesis to form a germ-line cyst of 16 cystocytes interconnected by ring canals. Ring canals are an indication of the number of cell divisions: two cells have four ring canals, two cells have three ring canals, four cells have two ring canals, and eight cells have a single ring canal (Figure 3). The genome of oocytes then undergoes meiotic reduction, while the interconnected nurse cells undergo substantial endoreplication. (reviewed by Lehner and Lane, 1997; Spradling, 1993).

Exactly how this precise program of germ-line division is regulated is poorly understood. The "ovarian tumor" class of mutants results in hundreds of undifferentiated germ cells, but the genes affected in these mutants most likely disrupt only germ-line sex determination (Pauli and Mahowald, 1990). Mutations in *pumilio* (*pum*) or *piwi* generate ovaries with less than the normal complement of germ-line cells by affecting the asymmetric division of germ-line stem cells (Lin and Spradling, 1997). *bag-of-marbles* is important for the switch to cystoblast division mode; it is required for differentiation of the cystoblast (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). Two genes that are likely to have a role in the control of the four rounds of germ-line mitosis are *Hu-li tai shao* (*Hts*), an adducin-like molecule, and *encore* (*enc*). Both are required for the correct number of divisions; mutations in *enc* result in exactly one extra round of germ-line mitosis (Hawkins *et al.*, 1996), while *Hts* mutants fail to complete four rounds of mitosis (Yue and Spradling, 1992). Both *Hts* and *enc* are components of the germ line, suggesting that control of germ-line cell division is intrinsic to the germ line. Nevertheless, germ-line mitosis could be controlled by an external signal from some other cell group. Intercellular signaling between germ-line cells and somatic cells has been shown to be important in *C. elegans*, where a signal from the somatic cells is necessary for the proliferation of neighboring germ-line cells (Kimble and White, 1981). Similarly, somatic cells play a role in the control of germ-line cells in mammalian oogenesis; factors within the somatic cells maintain the oocyte in meiotic arrest (reviewed by Buccione *et al.*, 1990).

The precise pattern of division suggests that *Drosophila* germ-line mitosis is well regulated. While investigating the requirements for the neurogenic genes in oogenesis, it was noticed that some mutant germ-line cysts contained more than the normal 16 cells (Feger, G., and Ruohola-Baker, H. personal communication). To gain insight into the Notch-signaling pathway and the control of germ-line mitosis, I analyzed the role of the neurogenic genes *Notch* and *neuralized* in control of germ-line mitosis.

RESULTS

MUTATIONS IN *NEURALIZED* RESULT IN DEFECTS IN GERM-LINE DIVISION

To investigate whether *Notch* (*N*) or *neuralized* (*neu*) is required for proper division of the germ line, we looked at the number of germ-line cells in ovaries mutant for *Notch* or *neu*. Because all published mutations in the neurogenic genes are lethal, we generated ovaries mosaic for loss-of-function mutations using the FLP-FRT system (Figure 3; Golic and Lindquist, 1989; Xu and Rubin, 1993) and analyzed them by staining the nuclei with propidium iodide and counting the number of germ-line cells present in each egg chamber. Ovaries harboring mutations in either *Notch* or *neuralized* exhibit a defect in the germ line. Egg chambers are present that contain more than the normal complement of 16 germ cells. Mosaic patches were not marked in these initial experiments so clonal frequency was not known; using $neuralized^{9B9}$, 17% (7/42 total chambers analyzed) of egg chambers displayed this defect, while using $Notch^{55e11}$ resulted in defects in 5% (2/38 total chambers analyzed) of egg chambers. To analyze whether the extra germ-line cells arose by fusion of egg chambers or from extra germ-line divisions, the ring canals were examined by staining with phalloidin. In wild-type egg chambers, germ-line cells contain between 1 and 4 ring canals, due to the precise program of division (Figure 3). In egg chambers mutant for *Notch* or *neu*, germ-line cells are detected that contain more than four ring canals, an indication of extra cell divisions (Figure 5).

To analyze the defect further, I used the neu^{R121} allele of *neuralized*. This allele of *neuralized* is homozygous viable and thus eliminates the complication of generating mosaic flies. To determine whether this allele behaves as expected for mutations in *neuralized*, I examined the bristles in flies transheterozygous for neu^{R121} and neu^{A101}, a strong allele of *neuralized*. Neurogenic signaling is required for bristle development; mutations in several of the neurogenic genes results in bristle defects (Hartenstein and Posakony, 1990; de Celis *et al*, 1991; Parks and Muskavitch, 1993; Bang

et al, 1995; Lyman and Yedvobnick, 1995; Parks *et al.*, 1997). Bristle defects were detected in 100% of neu^{R121} neu^{A101} flies and consist of missing macrochaetaes (Figure 6 B&D) and/or two bristles arising from a hole with no socket (Figure 6F). This phenotype is similar to that detected previously with in *neu* mutant clones, and indicates that neu^{R121} indeed behaves as expected for an allele of *neu*, at least in its role in bristle formation. I also analyzed the cuticles of neu^{R121} neu^{A101} embryos to determine whether this viable allele of *neu* behaves as expected during neurogenesis as well (Figure 6H). Embryos displayed no ventral cuticle, a "neurogenic" phenotype, which is the same phenotype detected previously in *neuralized* mutants, as well as mutants for *Notch*, *Delta*, *mastermind*, and *bigbrain* (Lehmann *et al.*, 1981; Lehmann *et al.*, 1983; Jurgens, 1984). This phenotype results from hypertrophy of neuroblasts at the expense of epidermiblasts, which give rise to the cuticle of the embryo. This result indicates that $neuralized^{R121}$ behaves as expected for an allele of *neuralized* in neurogenesis.

To analyze the germ-line-division defect, I crossed neu^{R121} to several other alleles of *neuralized*: neu^{A101}, neu^{KX9}, and neu^{9B9}. All three allelic combinations, neu^{R121} neu^{A101}, neu^{R121} neu^{KX9}, and neu^{R121} neu^{9B9}, exhibited germ-line division defects, in 39% (20/51), 63% (33/52), and 73% (38/52) of stage 6-10 egg chambers, respectively (Table 1). These results indicate that the strength of these alleles in oogenesis with respect to germ-line mitosis proceeds from neu^{9B9} as the strongest to neu^{A101} as the mildest, and that all alleles tested result in the same defect (with different expressivity). Further analysis of neu^{R121}/neu^{A101} chambers that had division defects reveals that they have an average of 32 cells per egg chamber (n = 37 chambers), twice the normal complement of 16 cells (Figure 6J). Occasionally there were chambers with more (1/37 chambers) or less (1/37 chambers) than 32 cells, but the majority of chambers have exactly 32 germ-line cells (35/37). The chambers usually had 1 oocyte, based on accumulation of phalloidin in the membrane and the size of the nucleus.

WHERE *NEURALIZED* IS REQUIRED

To investigate whether germ-line division is controlled by an intracellular or intercellular mechanism, I set out to determine which cells require the function of the *neuralized* for the control of germ-line division. This question could not be determined by the expression patterns of these genes in the germarium, as examination of their expression patterns reveals that they are expressed both in the germ line and in the surrounding somatic follicle cells (Markopoulou and Artavanis-Tsakonas, 1989; Ruohola *et al.*, 1991; Xu *et al.*, 1993; Boulianne *et al.*, 1991).

To determine where the *neuralized* gene is required for the control of germ-line mitosis, I generated marked clones. This method utilizes a *lacZ* marker on the *FRT* chromosome that does not harbor the mutated gene, such that cells homozygous for the mutation will not express β-galactosidase, while those heterozygous or homozygous for the wild-type chromosome will. Thus, patches of non-staining cells correspond to cells that are mutant in the respective gene (Figure 4). I carried out this analysis with two alleles of *neuralized*: neu^{KY9} and neu^{j9B9}. Using neu^{KY9}, 100% (47/47) of the chambers with a germ-line division defect did not stain the germ line (Figure 7C&D). In addition, 100% (24/24) of egg chambers mutant for neu^{j9B9} did not stain in the germ line (Figure 7E). A few egg chambers were also detected that had a germ-line clone but still had 16 cells: 11% (6/53) with neu^{KY9} and 8% (2/26) with neu^{j9B9}. With both alleles of *neu*, only 16 cells were detected in egg chambers in which no mosaic patches were observed (Figure 7A) or in which the germ line stained but the surrounding follicle cells did not stain (Figure 7B). These data indicate that with both neu^{KY9} and neu^{j9B9}, germ-line-division defects were only detected when the mutation was harbored by the germ line. These results reveal that Neuralized is required in the germ line for the control of germ-line division. In both experiments, the average number of cells in defective germ-line cysts was 32 (n=20), twice the normal complement of 16 cells. Most cysts had exactly 32 cells, but cysts were occasionally found with more or less than 32 cells. 94% (17/18) of the

defective chambers had only one oocyte (based on size of the nucleus and accumulation of yolk granules), and 6% (1/18) had two oocytes.

To confirm that *neuralized* is required in the germ line, I used the "dominant female sterile" technique in conjunction with the FLP-FRT system to generate ovaries that carry loss of function mutations in the germ line (Chou *et al.*, 1993). This second approach utilizes insertions of $P[ovo^{D1}]$. ovo^{D1} is a dominant female sterile mutation that blocks oogenesis at an early stage and is only required in the germ line. In a fly heterozygous for ovo^{D1}, a late stage egg chamber is formed only when a germ-line clone lacking ovo^{D1} is generated. Induction of mitotic recombination in a female transheterozygous for ovo^{D1} and the mutation of choice results in sister cells in which one is homozygous for ovo^{D1} and the other is homozygous for the mutation. Any eggs produced by such a female would therefore be homozygous for the mutation. Wild-type egg chambers and/or eggs would indicate that the gene is not required in the germ line.

More than 16 cells were detected in 87% (65/75) of stage 7- 10 egg chambers homozygous for neu^{9B9} in the germ line [5% (4/75) had too few cells, and 8% (6/75) had the normal 16] and 83% (45/54) using neu^{KY9}, indicating that Neuralized is indeed required in the germ line for the control of its division (Figure 7). To investigate the requirement for Neuralized in the germ line, females carrying potential germ-line clones were crossed to $neu^{A101}/TM3$ or wild-type males and eggs were allowed to develop at 25°C for 2 days. Only 3% (6/208) of fertilized eggs did not hatch when females carrying potential clones were backcrossed with wild type, but 49% (432/886) did not hatch when backcrossed to $neu^{A101}/TM3$ (Table 2). As a control, the ability of *FRT*, ovo^{D1} females to lay eggs was assessed. No eggs were laid, indicating that the ovo^{D1} construct had not reverted. Fertilized, non-hatching eggs were studied for embryonic defects. The embryos displayed no ventral cuticle, as observed previously with mutations in *neuralized* as well as in this study (Figure 8F; Lehmann *et al.*, 1981; Lehmann *et al.*, 1983; Jurgens, 1984). Removal of maternal and zygotic components of *neuralized* (Lehmann *et al.*,

1981; Lehmann *et al.*, 1983) results in the same phenotype as that which I have observed here, suggesting that the amount of maternally supplied *neuralized* does not affect neurogenesis.

WHERE NOTCH IS REQUIRED

To determine whether Notch is also required in the germ line, I generated germ-line clones of $Notch^{55e11}$ using the FLP/FRT-dominant female sterile technique described above. 94% (203/215) of egg chambers with Notch germ-line clones were wild type, suggesting that Notch is not required in the germ line for the control of germ-line mitosis (data not shown). I believe the few egg chambers which had more than 16 cells result from ovarioles that are mutant for Notch in the both the germ line and follicle cells since they are detected with such low frequency.

BAG-OF-MARBLES PROTEIN EXPRESSION IS PROLONGED IN *NEURALIZED* MUTANT OVARIES

Previous studies have shown that Bag-of-marbles (Bam) is involved in control of germ-line division (McKearin and Ohlstein, 1995; Hawkins, 1996; Ohlstein and McKearin, 1997). Studies with mutations in *encore* have lead to speculation that Bam could be a titratable factor whose levels determine when the germ-line cyst ceases to divide (Hawkins *et al.*, 1996). To determine whether the germ-line-division defect generated by loss of Neuralized function is due to a defect in Bam expression, I looked at Bam protein. Bam protein is normally expressed in the spectrosome and fusome of all germ-line cells located in the germarium and in nurse cells as well as in the cytoplasm of mitotically active cystocytes; antibodies specific to these two regions have been generated and have been called BamF and BamC, respectively (McKearin and Ohlstein, 1995). I chose to examine the expression of BamC in *neuralized* mutant ovaries, since this staining is more readily apparent and the level of BamC diminishes with each division of the germ line, making this the likely candidate for a titratable factor (McKearin and Ohlstein, 1995).

Incubation of BamC antiserum with fixed neu^{R121} neu^{A101} ovaries revealed prolonged expression of cytoplasmic Bam protein (Figure 9). Bam expression in this mutant background extended more posteriorly within the germarium - in cysts with more than 8 cells, and was even detected in some early stage egg chambers. I detected a similar phenotype in enc^{R7}/enc^{D1} ovaries (data not shown). A similar phenotype has been detected previously in ovaries mutant for *encore*, where *bam* mRNA expression was expanded to approximately double the width as that seen in wild-type ovaries (Hawkins *et al.*, 1996). This prolonged expression of Bam suggests that *neuralized* function is required for the depletion of Bam protein.

A null allele of *bam* behaves as a dominant suppressor of *encore* (Hawkins *et al.*, 1996). Since I found that mutations in *neuralized* generate similar defects at the protein level as those found with mutations in *encore*, I investigated whether *bam* could dominantly suppress *neuralized* as well. Because both *bam* and *neuralized* are located on the third chromosome, I recombined neu^{A101} with *bamΔ86*, a null allele of *bam*. I generated two recombinant lines, which I called neu^{A101},$bam^{\Delta 86}$*(7)* and neu^{A101},$bam^{\Delta 86}$*(11)*. The phenotype of *neuralized* mutant ovaries was indistinguishable from those harboring the null allele of *bam*; 33% (71/215) of neu^{R121} neu^{A101} chambers displayed a germ-line division defect, while 30% (83/280) and 35% (97/279) of neu^{R121} neu^{A101},$bam^{\Delta 86}$*(7)* and neu^{R121}/neu^{A101},$bam^{\Delta 86}$*(11)* displayed the same defect, respectively. Therefore, *bam* cannot suppress the germ-line division defect of viable *neuralized* ovaries.

DISCUSSION

I have shown that in *Drosophila* oogenesis, mutations in *neuralized* result in defects in germ-line mitosis. These mutations usually resulted in exactly one extra round of division, so that germ-line cysts now had 32 cells, and cells were found with more than four ring canals. Analysis of germ-line clones generated through the use of a

dominant female sterile mutation, as well as marked clones, revealed that Neuralized function is required in the germ line for control of its division. I found that expression of Bam protein, a marker for mitotically active cysts, was prolonged with mutations in *neuralized*.

Because it could be possible that the germ line could cease dividing and an extra round of division could occur sometime later in oogenesis, I looked to see how early in oogenesis I observed the defect. Because I frequently detect cysts with more than 16 cells in the germarium, I believe that these mutations result in prolonged mitosis within the germarium and that Neuralized function is therefore required for the cessation of normal germ-line division.

Egg chambers with more than 16 cells have been reported previously with mutations in *Notch*, *Delta*, *brainiac*, *pipsqueak*, *daughterless*, and *toucan* (Ruohola *et al.*, 1991; Goode *et al.*, 1992; Siegel *et al.*, 1993; Cummings and Cronmiller, 1994, Grammont *et al.*, 1997). The phenotype I observe here is different than the phenotype observed previously in these mutants, however; because these mutations affect the process by which follicle cells separate neighboring germ-line cysts. I have shown that the additional germ-line cells result from extra cell divisions, based on the finding of cells with more than the usual maximum of 4 ring canals. The phenotypes I report resemble those reported with mutations in *encore* (Hawkins *et al.*, 1996). *encore* mutations result in exactly one extra round of mitosis, so germ-line cysts contain 32 cells and an oocyte with 5 ring canals. The authors found that the germ-line mitosis phenotype of encore is mediated by *bag-of-marbles* (*bam*). In wild-type ovaries, *bam* mRNA is expressed in mitotically active cystocytes; mutations in *encore* result in expanded expression of *bam* mRNA. In addition, a null allele of *bam* acts as a dominant suppressor of the *encore* division defect. Because the mitotic defect of *neuralized* ovaries so closely resembles that of *encore* mutants, I analyzed whether *bam* could be mediating the germ-line-division defect of *neuralized* mutants as well. The domain of expression of Bam protein was

expanded in *neuralized* mutant ovaries. However, a null allele of *bam* cannot suppress the division defect of *neuralized* mutants as it can with mutations in *encore*. This could be for one of several reasons. It is feasible that the mutations in *neuralized* are too strong to be overcome by reduction in one copy of *bam*. Alternatively, the defect might not be mediated by Bam, but rather, Bam could be misexpressed due to defects in germ-line cell fate; the cells in mutant cysts could have the fate of early, mitotically active germ-line cells.

My analysis with marked clones and with ovo^{D1} revealed that *neuralized* is required in the germ line for the control of germ-line mitosis. It could still be possible, however, that *neuralized* could be required in the follicle cells as well as the germ line to mediate mitosis, if the clones analyzed were double clones (mutant in the follicle cells as well as the germ line). Analysis of *lacZ* marked clones showed that *neuralized* is not required in the follicle cells that surround the cyst: egg chambers were detected which had a germ-line division defect and which had a *neuralized* mutant germ line, but which did not have a *neuralized* clone in the surrounding follicle cells, based on *lacZ* expression. This does not exclude the possibility, however, that these ovarioles also have a clone in the stationary follicle cells at the anterior tip of the germarium since this region is difficult to analyze with the markers I used. This is not likely, however, with the frequency with which I found a defect associated with a germ-line clone.

It was previously not known whether control of germ-line division is intrinsic to the germ line or requires a signal from the somatic follicle cells. Mutations in *encore* result in phenotypes very similar to those I have reported here, but it was unknown whether *encore* was required in the germ line or soma (Hawkins *et al.*, 1996). The expression patterns of enhancer trap lines for *piwi* and *pumilio* suggest that a signal from the soma is required, at least for the asymmetric division of the germ-line stem cells. Both are required for control of this division; *pumilio* is expressed in the terminal filament while *piwi* is expressed in the germ-line cells (Lin and Spradling, 1997). My data indicate that

Notch is required in the somatic follicle cells for the cessation of germ-line mitosis, implicating a signal from the follicle cells in regulation of this process. Furthermore, my data show that *neuralized* is required in the germ line for the cessation of germ-line mitosis. Interaction studies have lead to the proposal that *neuralized* acts upstream of *Notch* (de la Concha *et al.*, 1988; Lieber *et al.*, 1993). There are several possible explanations for my results, which seemingly contradict this conception of the Notch-signaling pathway. The first possibility is that *neuralized* acts downstream of *Notch* in this process. *neuralized* encodes a zinc finger transcription factor. It has been suggested that Neuralized functions as a gene repressor because it shows homology known repressors (Price, 1993). It could be that Neuralized functions downstream of Notch, affecting the differentiation state of the cell by repressing signals involved in cellular differentiation. Other possibilities are that there is a signal from the germ line to the follicle cells preceding the follicle-cell-to-oocyte signal, or that *Notch* and *neuralized* act separately in this process.

Most of the egg chambers expressing mutant *neuralized* alleles that have a germ-line-division defect have only 1 oocyte. However, I have detected chambers with more than 1 oocyte, suggesting that Neuralized may be involved in oocyte specification. Because I detect this defect with such low frequency, I believe it is a secondary phenotype resulting from the defect in germ-line division. Further studies to test if the division defect can be separated from the multiple oocyte phenotype should shed light on this possibility.

Table 1: Alleles of *neuralized*

Allele	Allele class	Mutagen	germ-line division defect over neu^{R121}
neu^{4101}	-	P-element insertion	39% (20/51 egg chambers)
neu^{KX9}	amorph	-	63% (33/52 egg chambers)
neu^{9B9}	-	P-element insertion	73% (38/52 egg chambers)

Table 2: *FLP/FRT* ovo^{D1}-induced $neuralized^{KX9}$ germ-line clones

Genotype	n	unfertilized	fertilized, not hatched	hatched
FRT, neu^{KX9} *FRT,* ovo^{D1} *3R* x neu^{4101} *TM3*	925	39	49% (432/886)	51% (454/886)
FRT, neu^{KX9} *FRT,* ovo^{D1} *3R* x *Oregon R*	218	10	3% (6/208)	97% (202/208)
FRT, ovo^{D1} *3R TM3* x *Oregon R*	0	-	-	-

Figure 3: Schematic view of the germ cell lineage

Division of stem cells at the anterior end of the germarium produce two daughter cells; one of these daughter cells remains a stem cell, while the other becomes a cystoblast. Cystoblasts then go on to divide exactly four times and, through a precise program of division and incomplete cytokinesis, form a cyst of 16 interconnected germ-line cells. The cytoplasmic bridges connecting these germ-line cells are called ring canals. Ring canals mark the number of cell divisions: two cells have four ring canals, two cells have three ring canals, four cells have two ring canals, and eight cells have a single ring canal.

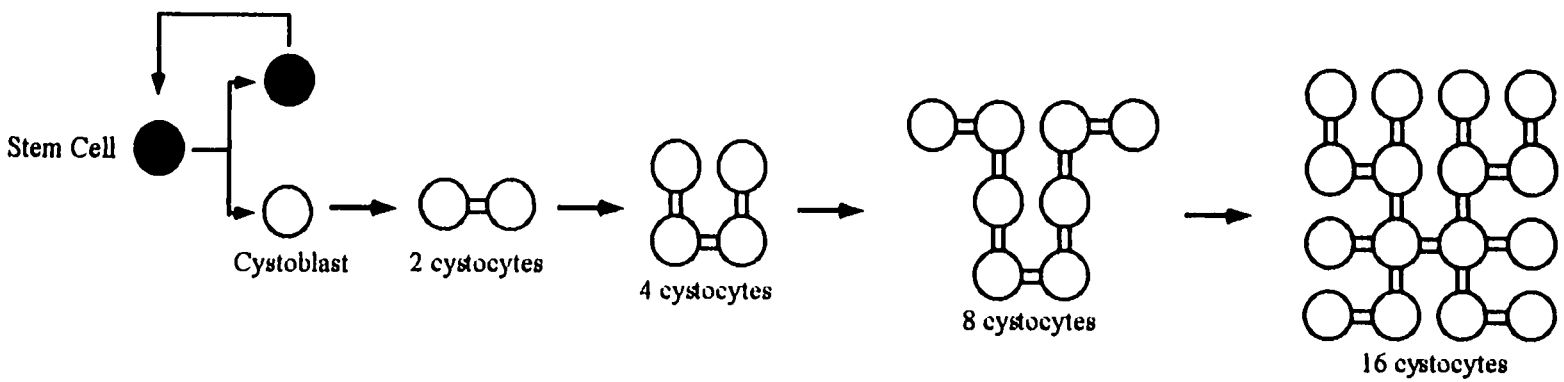

Stem Cell
Cystoblast
2 cystocytes
4 cystocytes
8 cystocytes
16 cystocytes

Figure 4: Producing homozygous clones of a given mutation

After generation of the recombinant chromosome that carries the *P[ry⁻, hs-neo, FRT]* element and the given mutation on the same arm, clones homozygous for the mutation can be produced by crossing this recombinant to a strain that carries the same *P[ry⁻, hs-neo, FRT]* element and a *hsFLP* element on a separate chromosome. Heat-shock induction of the FLP enzyme at the appropriate developmental time then induces mitotic recombination between *FRT* sequences. To mark the clones, the distal portion of the *FRT*-carrying arm (in the strain which does not carry the mutation) also carries an appropriate marker gene. For germ-line clones, the distal part of this arm carries the dominant female sterile mutation ovo^{D1} rather than the marker gene.

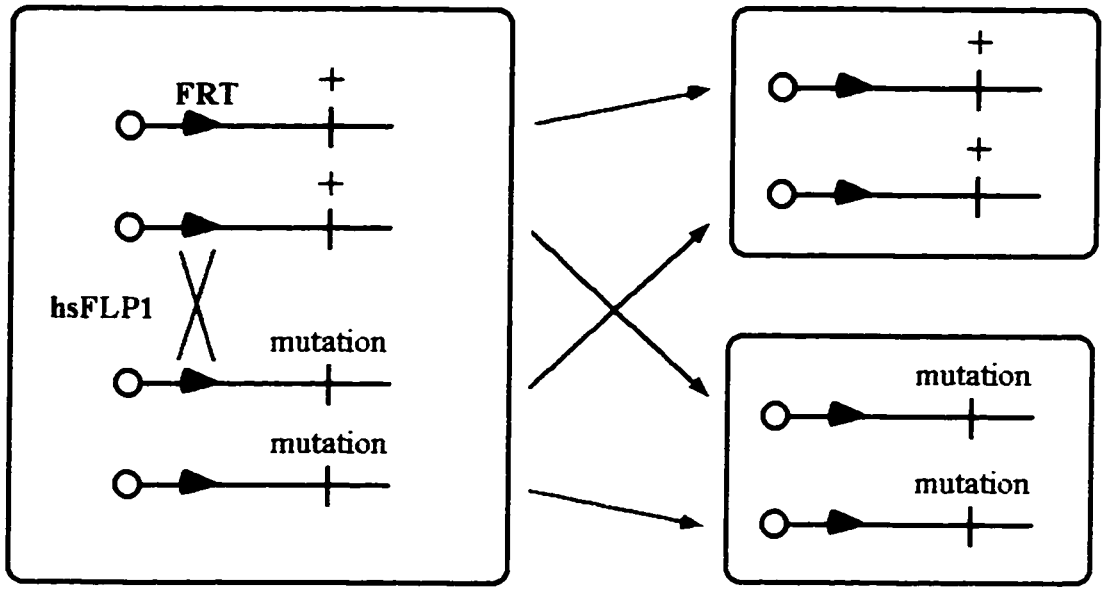

FRT
+
+
hsFLP1
mutation
mutation
+
+
mutation
mutation

Figure 5: Homozygous clones for mutations in *Notch* or *neuralized* result in germ-line division defects

Propidium Iodide staining on the left reveals the nuclei in each egg chamber (some nuclei underneath are obscured by those in front of them), while phalloidin staining on the right shows the ring canals, seen as rings, unless viewed from the edge. (A) Wild-type egg chambers have 16 germ-line cells, and none of these has more than four ring canals. (B) Arrowheads indicate the normal four ring canals of the oocyte. The inset shows another wild-type oocyte with the four ring canals indicated by arrows and arrowheads. (C&D) An egg chamber from a *yw, hsFLP*^{1369} *w; FRT, neu*^{KX9} *FRT,w* female. There are 32 germ-line cells (C), and the oocyte has 5 ring canals (D, inset shows higher magnification of oocyte showing the five ring canals), indicating that this cyst has gone through an extra round of division. (E&F) An egg chamber from a *yw, hsFLP*^{1369} *w; FRT, neu*^{9B9} *FRT,w* female. Here too there are 32 germ-line cells , and the oocyte has 5 ring canals (Inset shows higher magnification of the oocyte to show the 5 ring canals. The arrow indicates a ring canal berneath two in the foreground), indicating that another allele of neuralized generates the germ-line defect (F). (G) An egg chamber from *w*^{9} *Notch*^{55e11}, *FRT*^{101} *w*$^{-}$ *FRT*^{101} *w*$^{-}$; *FLP*^{38} – female. Once again, there are extra germ-line cells, as indicated by additional ring canals, shown enlarged in (H).

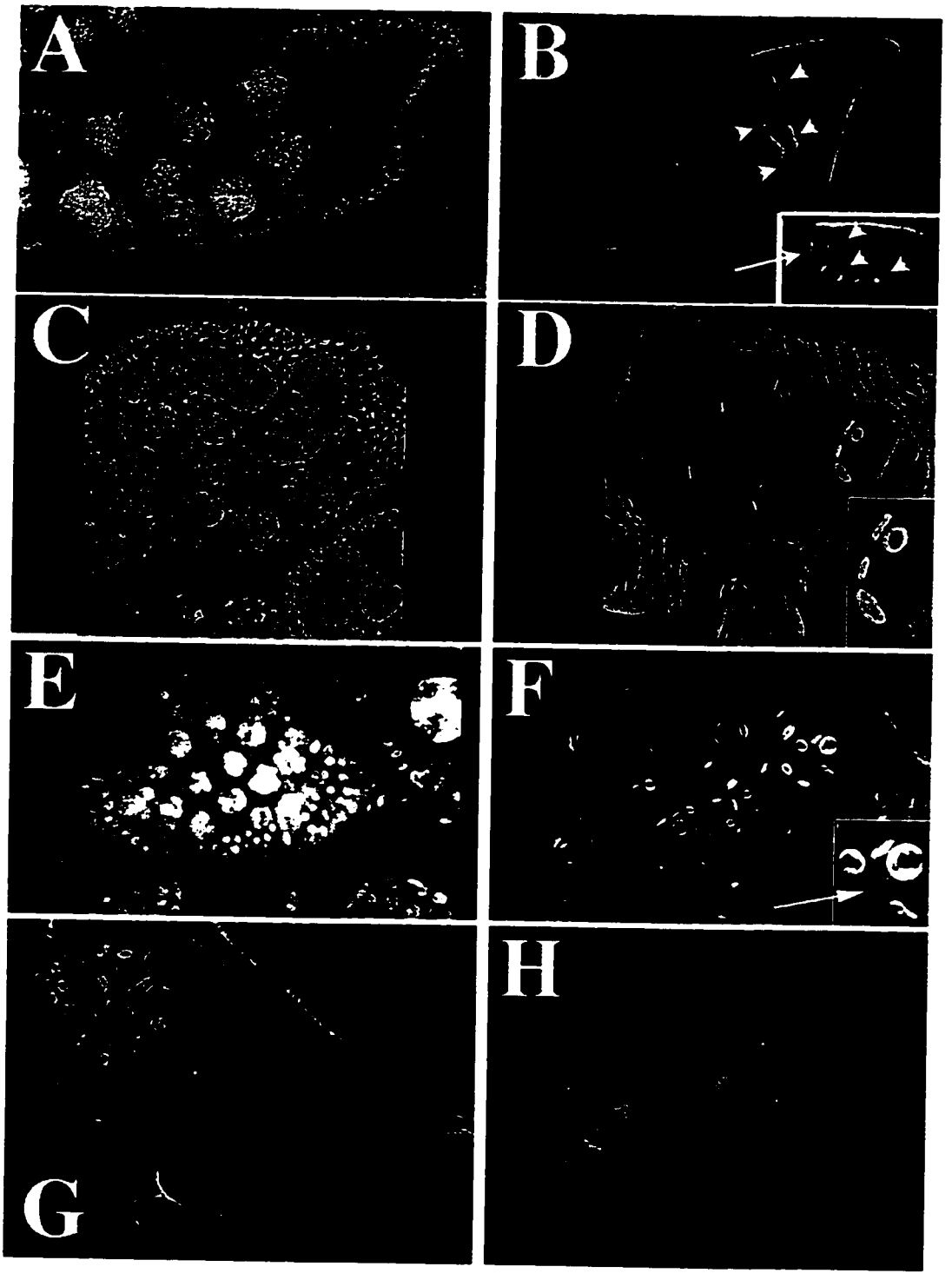

A
B
C
D
E
F
G
H

Figure 6: *neuralized*^{R121} behaves as expected for an allele of *neuralized*, and generates defects in germ-line division

Images on the left are wild type, while those on the right are *neu*^{R121} *neu*^{A101}. (A) Notum of wild-type fly showing the four scutellar bristles. (B) The notum of a *neuralized* fly, missing one of the anterior scutellar bristles and both of the posterior scutellar bristles. (C) Head of a wild-type fly. (D) Head of a *neuralized* fly missing the ocellar and orbital bristles. (E) The post-alar bristle of a wild-type fly. (F) A doublet of the post-alar bristle of a *neuralized* fly. (G) A wild-type embryo. (H) A *neuralized* embryo. Only a patch of dorsal cuticle is detected. (I&J) A *neuralized* egg chamber. There are 32 cells in the germ-line cyst, and the oocyte has more than the normal four ring canals. Inset shows enlargement of the ring canals; arrows indicate ring canals that are not seen as rings due to their angle.

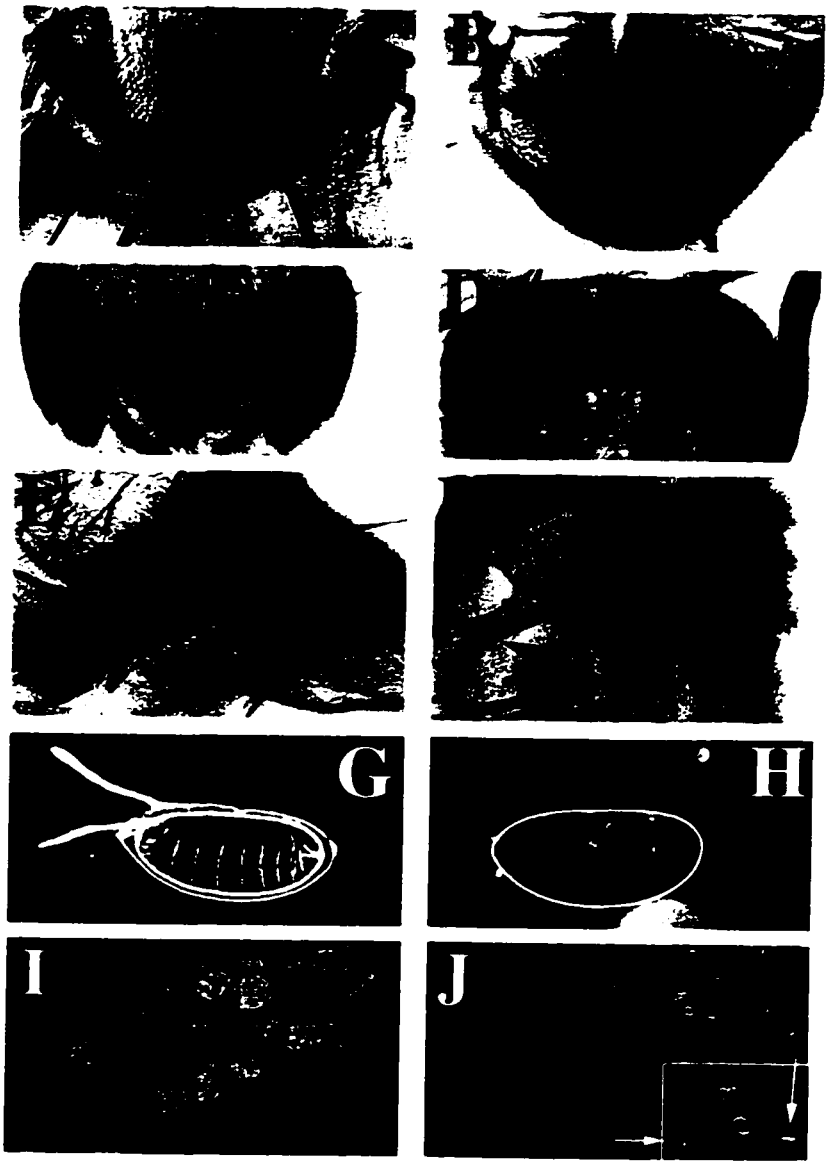

G
H
I
J

Figure 7: *neuralized* marked clones

Lack of nuclear β-galactosidase activity indicates homozygous *neuralized* mutant cells. β-galactosidase activity is nuclear. In come panels, cells may appear slightly blue because of staining in the overlying cells. (A-D) Egg chambers from ovaries of *yw, hsFLP*^{f369} *w; FRT, neu*^{KY9} *FRT,E3-26(tublacZ)* females. (E) Mosaic clones detected in ovaries of *yw, hsFLP*^{f369} *w; FRT, neu*^{9B9} *FRT,E3-26(tublacZ)* females. (A) The germ line appears normal (only 16 cells are detected) in egg chambers in which no mosaic patches are observed. (B) 16 germ-line cells are also detected when the follicle cells surrounding the oocyte are mutant for *neuralized* and the germ line has no mosaic patches. Egg chambers homozygous for *neu*^{KY9} (C&D) or *neu*^{9B9} (E) in the germ line (with no detectable clones in the follicle cells) display the division defect, suggesting that *neuralized* is required in the germ line for the control of germ-line division.

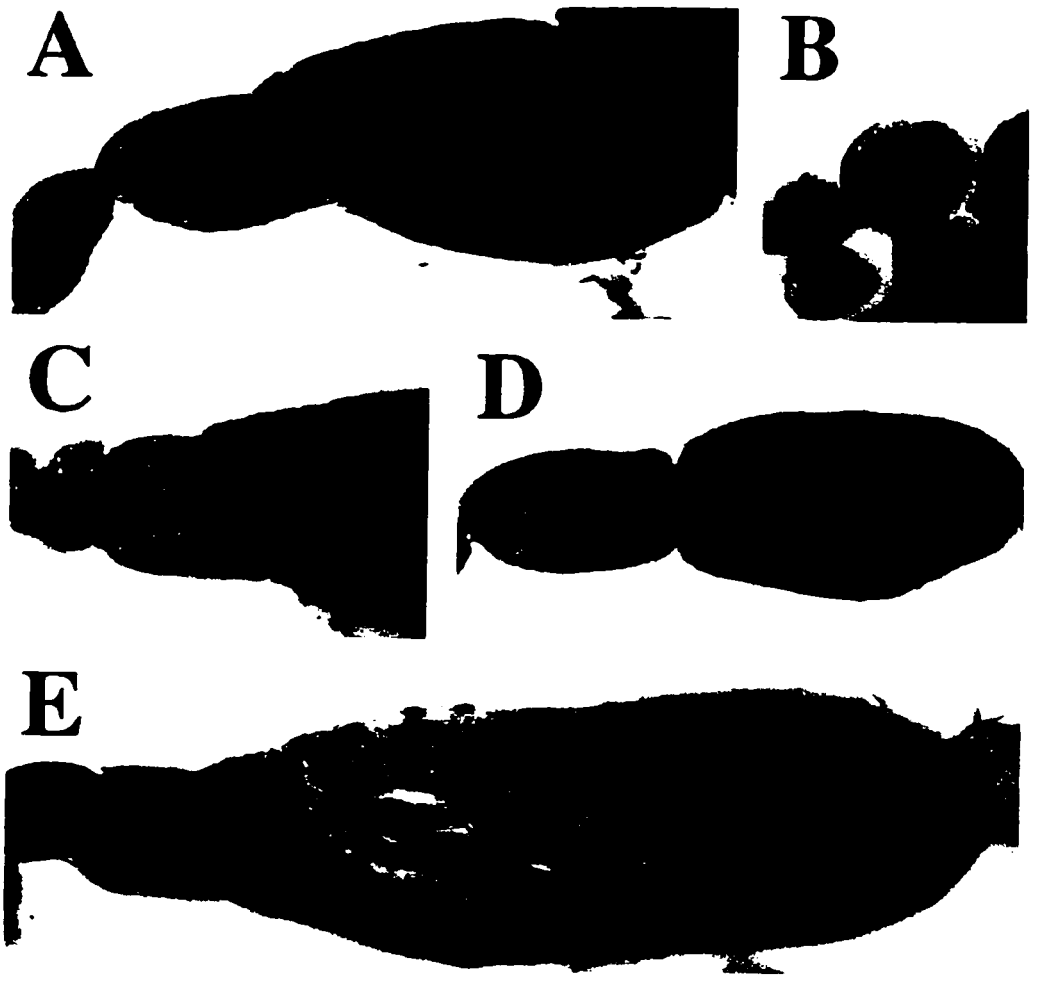

A
B
C
D
E

Figure 8: *neuralized* germ-line clones generated with *FLP*; *FRT*, ovo^{D1} and their embryonic phenotype

Germ-line clones of $neuralized^{KY9}$ (A&B) and $neuralized^{9B9}$ (C&D). Propidium Iodide staining of the nuclei shows that *neuralized* germ-line clones have 32 cells (A&C). Phalloidin staining of the same 2 chambers reveals that lack of *neuralized* from the germ line results in an oocyte with 5 ring canals (B&D). (B) Inset shows higher magnification of the oocyte to show the 5 ring canals. Arrows indicate ring canals that are turned on edge. (D) Arrows indicate all 5 of the ring canals. (E) A wild-type embryo. (F) An embryo from a $neuralized^{KY9}$ germ-line clone fertilized by a neu^{4101} *TM3* male. Only a patch of dorsal cuticle is detected.

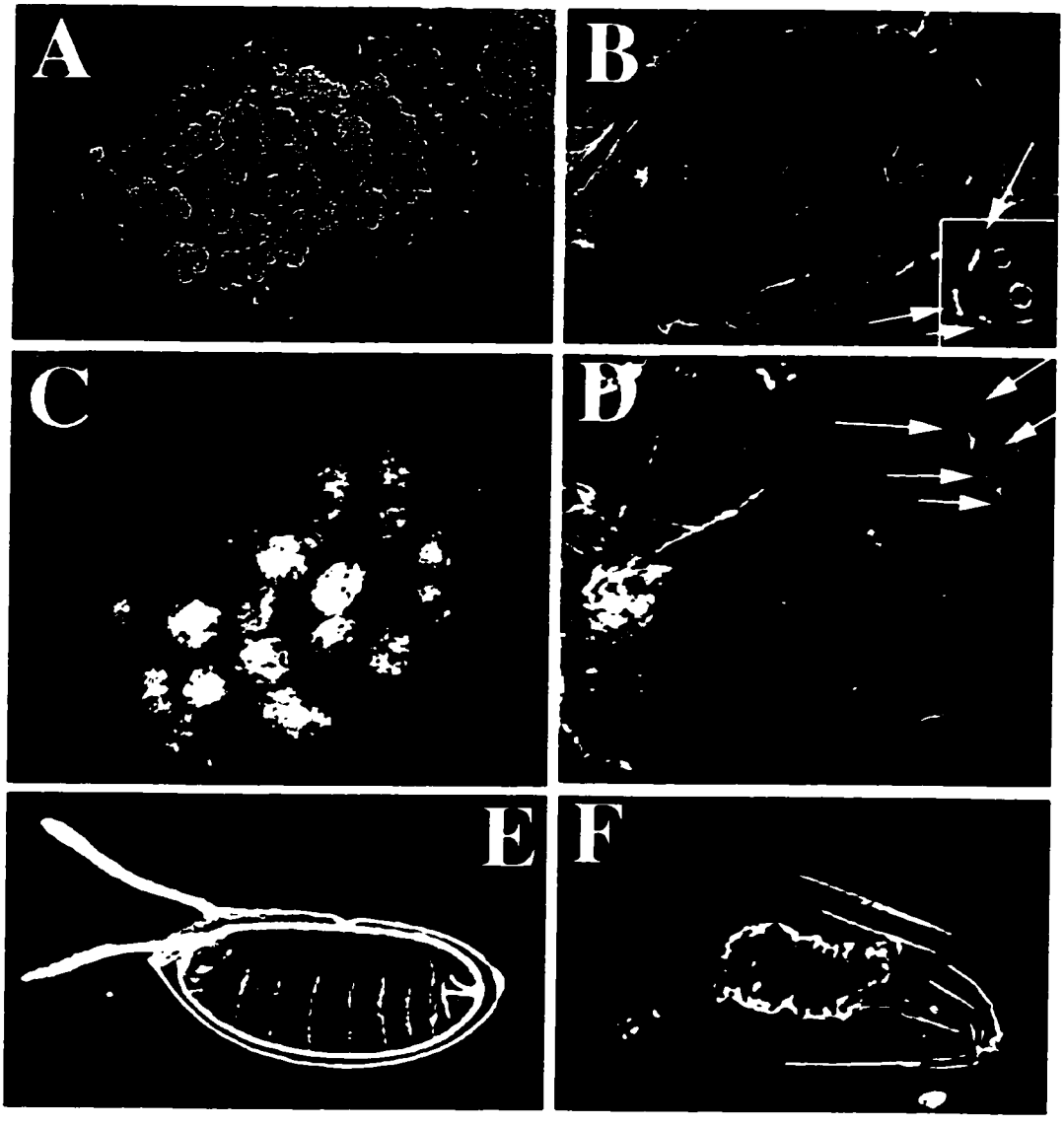

A
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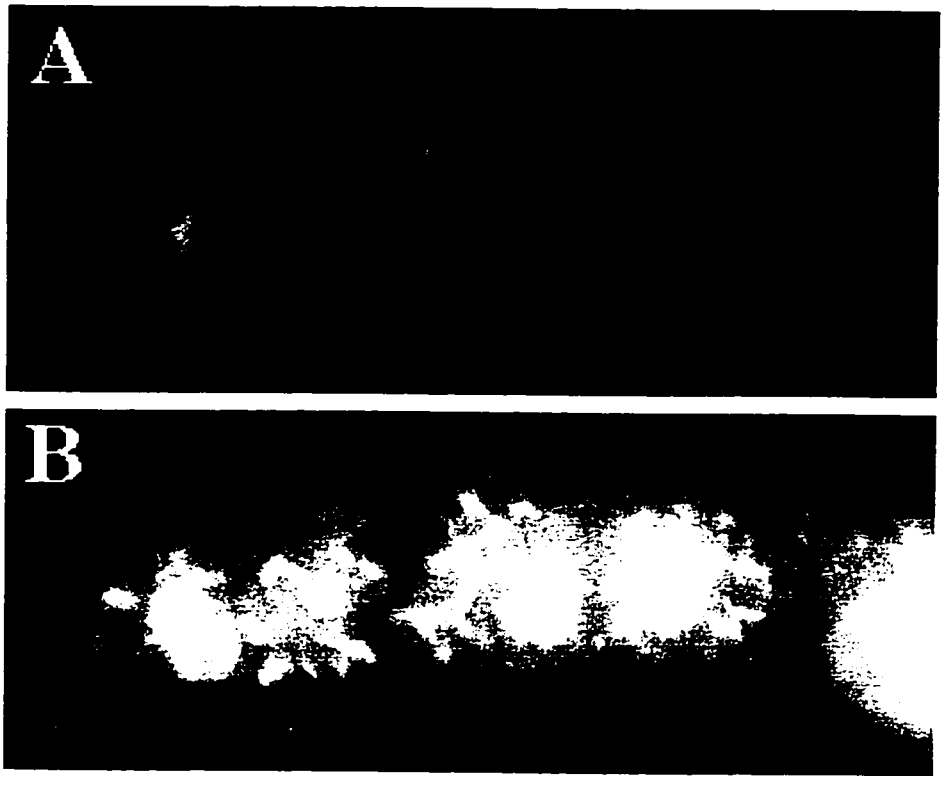
Figure 9: Expression of Bag-of-marbles (Bam) protein

Ovaries stained with anti-BamC antibodies. (A) In controls, BamC protein is expressed in the cytoplasm of mitotically active cystocytes, but is most readily observable in 8 cell cysts, appearing as a clump at the anterior of the germarium. (B) In *neuralized* mutant ovaries, neu^{R12I} neu^{A10I}, expression of BamC extends more posteriorly in cysts with more than 8 cells.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Chapter 2: Expression of Constitutively-active Notch Arrests Follicle Cells at a Precursor Stage During *Drosophila* Oogenesis and Disrupts the Anterior-Posterior Axis of the Oocyte.

INTRODUCTION

Despite the wealth of information about the loss-of-function phenotype of Notch in oogenesis, the precise role of Notch activity remains obscure. Here, I address this question by studying the effects produced by activation of Notch function in follicle cells during oogenesis. For this purpose, I have taken advantage of *Notch* mutations that uncouple intracellular signal transduction from ligand binding. Specifically, recent studies have shown that a fragment of Notch lacking the extracellular domain, or portions thereof, is constitutively-active. Persistent or transient ubiquitous expression of activated Notch in *Drosophila* causes an overproduction of epidermis at the expense of neural structures (Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993), a phenotype opposite to that seen with the loss-of-function allele of *Notch* (Poulson, 1940; Lehmann *et al.*, 1983; Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991). Activated Notch causes defects in developmental processes in *C. elegans* (Roehl and Kimble, 1993), *Xenopus* (Coffman *et al.*, 1993), chickens (Austin *et al.*, 1995), and humans (Ellisen *et al.*, 1991), as well as *Drosophila* eye development (Fortini *et al.*, 1993). Absence or delay of normal differentiation in some of these cases has led to the proposal that ongoing Notch activity prevents competent cells from undergoing differentiation *in vivo*. This has been hard to prove definitively, however, due to the lack of definitive molecular markers for the precursor stage.

Here I show directly by using molecular and morphological markers that in early oogenesis, constitutively-active Notch arrests follicle cells in a specific precursor

stage, blocking the development of both stalk cells and polar cells. Loss of Notch function eliminates this precursor stage. In a later stage of oogenesis, activation of Notch in follicle cells leads to aberrant formation of the oocyte anterior-posterior axis .

RESULTS

CONSTITUTIVELY-ACTIVE NOTCH CONSTRUCTS AND EXPERIMENTAL DESIGN

Two constructs of *Notch (N)* were used in these studies to express constitutively-active Notch either persistently or transiently: *N(intra)* and *ΔEN*. *N(intra)* consists of the intracellular domain of Notch, separated from an *actin* promoter by a cassette which is surrounded by two Flipase recognition target (*FRT*) sites and containing a stop codon. Flies carrying this construct were crossed to flies carrying *flipase* (*flp*) expressed via a *hsp70* promoter. Progeny were heat-shocked, resulting in a pulse of Flp expression that excises the cassette and allows *Notch(intra)* to be expressed under the control of the *actin* promoter (for details see Appendix A, Experimental Procedures and Struhl *et al.*, 1993).

ΔEN expression under the control of *GAL4* upstream activating sequence (UAS, Brand and Perrimon, 1994) leads to production of a protein fragment comprising the last four amino acids of the extracellular domain of Notch, the transmembrane domain, and the amino-terminal half of the intracellular domain, including the Su(H) interacting domain and the CDC10/ankyrin repeats. This construct is expected to behave as a constitutively-active form of Notch based on earlier studies (Lyman and Young, 1993; Coffman *et al.*, 1993; Fortini *et al.*, 1993; Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993; Roehl and Kimble, 1993; Kopan *et al.*, 1996), and I verified this hypothesis by analyzing its phenotype during embryonic neurogenesis (Appendix B, Fig. 1A-B).

Functionally, the significant difference between *ΔEN* and *N(intra)* is that excision of the *FRT* cassette separating *N(intra)* from the *actin* promoter results in persistent expression of constitutively-active Notch, while *ΔEN* is only expressed when GAL4 is present.

To induce *ΔEN* expression in oogenesis, the flies carrying the *UAS-ΔEN* construct were crossed either to flies expressing GAL4 under heat shock control (*HS-GAL4*), or to the enhancer trap GAL4 line, *109(3)9*. The GAL4 expression patterns in ovaries for both of these lines were examined by crossing them to a line carrying *UAS-lacZ* and staining the progeny with X-Gal, or by crossing them to *UAS-tau-GFP* and analyzing the GFP pattern by fluorescence microscopy. *HS-GAL4* induced *UAS-lacZ* expression in follicle cells beginning in region two of the germarium (Appendix B, Fig. 1D-E), whereas *109(3)9* induced follicle cell expression was not detected prior to stage 6 in oogenesis (Appendix B, Fig. 1G,H). No germ-line expression of GAL4 was detected (Appendix B, Fig. 1G-H; data not shown).

EXPRESSION OF CONSTITUTIVELY-ACTIVE NOTCH IN THE GERMARIUM FOLLICLE CELLS GENERATES LONG STALK STRUCTURES

Germ-line cysts are surrounded by a follicle cell layer and released from the germarium by "pinching-off" (Figure 1, arrowhead). This process results in the formation of two kinds of specialized follicle cells: stalk cells that intercalate between each other to form the stalk that separates two successive egg chambers, and polar cells that form a cap at the anterior end of the older egg chamber and the posterior end of the younger egg chamber. In loss-of-function $Notch^{ts}$ mutant ovaries, no stalk cells are detected at the restrictive temperature and the egg chambers never leave the germarium (Appendix B, Fig. 2E; Ruohola *et al.*, 1991; Xu *et al.*, 1993).

To address whether expression of constitutively-active Notch can induce long stalks between the egg chambers, a phenotype opposite to the loss of stalks observed with *Notch^{ts}*, I expressed constitutively-active Notch transiently in the germarial follicle cells using *HS-GAL4* induced expression of ΔEN. This expression lead to a dramatic phenotype in the early stages of oogenesis. Instead of a wild-type stalk of 6-8 cells (Appendix B, Fig. 2A, bracket), long stalk structures consisting of an average of 15 cells were detected (Appendix B, Fig. 2B, bracket).

The sequential nature of oogenesis allowed me to study whether ovarioles which include abnormally long stalks continue to develop, and at what stage of development the length of both wild type and long stalks is determined. If expression of ΔEN was induced by a 15-minute heat shock at 39°C and the flies were then allowed to develop for 24 hours at 18°C, long stalks were first detected between stages one and two (Appendix B, Fig. 2B, bracket). At this time point, long stalks were not observed between the later stages (Appendix B, Fig. 2B, arrowhead), indicating that the length of fully formed stalks was not altered. Previous studies have indicated that a new egg chamber pinches off every 24 hours at this temperature (10-12 hours at 25°C; King, 1970; Margolis and Spradling, 1995). Because long stalks were detected between stages 1 and 2 within 24 hours after induction of ΔEN, I concluded that constitutively-active Notch acts during or just before the beginning of the pinching-off event and has no effect on fully formed stalks. The expression pattern of *HS-GAL4* under these conditions indicated that it induced target gene expression in the follicle cells at regions 2 and 3 of the germarium (Appendix B, Fig. 1 D-F), the region where pinching-off occurs. Two days after the induction of constitutively-active Notch, the long stalks had progressed and were present between stage 2 and 3 egg chambers (Appendix B, Fig. 3B) and after three days, the long stalk had moved from the germarium region to the region between stages 3-6 in oogenesis (Appendix B, Fig. 4B). The long stalks detected in the later stages were of the same

average length as those seen earlier, indicating that the final number of cells in these long stalks was also determined in the germarium (Appendix B, Table 1).

To address whether constitutively-active Notch requires endogenous Notch to generate long stalks, I determined the phenotype produced when activated Notch is expressed in the background of *Notch^{ts}*. Only the long-stalk phenotype of constitutively-active Notch was detected in these ovaries (Appendix B, Fig. 2F, bracket). This result suggests that constitutively-active Notch can act without endogenous Notch. Similar results have been seen in other systems, such as the nervous system (Struhl *et al.*, 1993)

Long stalk structures are also observed when the FLP-FRT system was used to drive expression of constitutively-active Notch (Struhl *et al.*, 1993). One day after heat-shock induction of Flipase, long stalk structures were detected (Appendix B, Fig. 2C, bracket). The long stalks observed one day after heat-shock induction resembled the long stalks detected after GAL4-induced expression. The penetrance of the phenotype was higher and the stalks were longer (Appendix B, Table 2), however, indicating a stronger phenotype that could have been due to the different *Notch* construct or the experimental procedure. Multiple heat shock inductions of *ΔEN*, in order to avoid the transient nature of GAL4 expression, resulted in longer stalks that resembled those generated by *N(intra)* (Appendix B, Fig. 2G, bracket). This result indicates that longer stalks can be generated by longer expression of *ΔEN*, and that the stronger phenotype of *N(intra)* is most likely due to the fact that it is expressed persistently.

To examine whether the long stalks generated by *N(intra)* also progress through oogenesis, I looked at ovaries at later time points. Two days after heat shock, the long stalk progressed to between stages 3 and 6, and it was usually followed by a second long stalk. Six to seven days after heat shock, extremely long stalk structures were detected (Appendix B, Table 2 and Fig. 2D, bracket), which had presumably continued to grow for 3 days since the heat shock. Because they were detected at a later time point,

these extremely long stalks could be due to a stem cell carrying *N(intra)* under the control of the *actin* promoter, leading to progeny that all express constitutively-active Notch. The lengths of stalks generated in (1) *ΔEN* (37°C, 30 min or 39°C, 15 min), 2 days after induction, (2) *N(intra)*, 1 day after induction, and (3) *N(intra)*, 6-7 days after induction suggested that the three treatments constituted a phenotypic series of increasing severity. These three treatments are referred to as *caN* (for constitutively-active Notch)-*mild*, *caN-intermediate*, and *caN-strong*, respectively, throughout the remainder of this chapter.

In addition to varying lengths of stalk structures, the experimental procedures generated varying cellular morphology within the long stalks. The cellular morphology of *caN-mild* stalks resembled that of a wild-type stalk: a single row of aligned, disc-shaped cells (Appendix B, Fig. 2B, bracket and Fig. 1C, arrow). Cells in the *caN-intermediate* and *caN-strong* stalks were not aligned in a single row; the stalks appeared much thicker than wild-type stalks, and the cells were organized as several aligned columns of cells (Appendix B, Fig. 2C and D). This morphology is reminiscent of the cellular organization observed earlier in the germarium at the time of the pinching-off event, before the cells have begun to intercalate (Appendix B, Fig. 2A, arrowhead and Fig. 1C, arrowhead).

A STALK CELL FATE MARKER IS EXPRESSED IN *CAN-MILD* AND *CAN-INTERMEDIATE* STALKS, BUT NOT IN *CAN-STRONG* STALKS

To determine whether more cells had actually taken the fate of stalk cells, I examined the identity of the cells in the long stalks with *93F*, an enhancer trap line that is expressed in stalk cells (Appendix B, Fig. 3A; Ruohola *et al.*, 1991). Expression of *93F* was analyzed in *caN-mild*, *caN-intermediate*, and *caN-strong* backgrounds. In control ovaries, all stalk cells were *93F* positive (Appendix B, Fig. 3A, brackets). In the case of *caN-mild*, some long stalks were detected that consisted entirely of cells expressing the stalk cell marker (Appendix B, Fig. 3B), consistent with the prediction that more cells

became stalk cells with expression of *caN*. Nevertheless, the majority of the long stalks (43/54, 80%, Appendix B, Table 1) contained a group of cells that did not express the stalk cell marker, despite being located in the stalk structures (Appendix B, Fig. 3C).

In *caN-intermediate* ovarioles, the long stalks also contained more *93F* positive stalk cells than are contained in normal stalks, indicating that additional cells had taken the fate of stalk cells. Many *93F* negative cells, however, were also detected in these long stalks (Appendix B, Fig. 3D). The number of cells that did not stain with the stalk cell marker also increased in number from *caN-mild* to *caN-intermediate*. Strikingly, no *93F*-positive stalk cells were detected in the extremely long stalks induced by *caN-strong* (Appendix B, Fig. 3E, bracket), indicating that none of the cells in these "stalks" took the fate of stalk cells. Therefore, three different kinds of long stalk-like structures were generated by the expression of constitutively-active Notch: those which consist entirely of *93F*-positive stalk cells, those which consist of *93F*-positive and *93F*-negative cells, and those which contain only *93F*-negative cells.

A POLAR CELL MARKER IS EXPRESSED IN *CAN-MILD* AND *CAN-INTERMEDIATE* STALKS BUT NOT IN *CAN-STRONG* STALKS

To assess the identity of the cells in long stalks that did not stain with the stalk cell marker, I used a marker for a subpopulation of follicle cells that differentiates at the time of, or just prior to, stalk formation: the *A101* enhancer-trap line. This mutation is a P-element insertion in the *neuralized* gene and shows a β-galactosidase staining pattern similar to that seen in an *in situ* hybridization using a *neuralized* cDNA probe. β-galactosidase activity is first detected in some of the polar cell precursors at the time of stalk formation. By stage four of oogenesis, its expression is restricted to two anterior and two posterior polar follicle cells (Appendix B, Fig. 4A; Ruohola *et al.*, 1991).

In the case of *caN-mild*, some long stalks were detected that contain no *A101* positive cells (Appendix B, Fig. 4B). Many long stalks were found, however, that had some *A101* positive cells located within the stalk structures (Appendix B, Fig. 4C and Tables 1, 2). Based on the expressivity of the phenotype and the number of cells that expressed *A101*, it appears that the cells that express the polar cell marker likely correspond to those cells in the long stalks that do not express the stalk cell marker. There was no trend in the position of the *A101* positive cells in the long stalk. In most cases the normal number of polar cells was still found in the egg chambers surrounding the long stalks. However, in a few instances only one of the usual two polar cells was detected. *caN-intermediate* stalks exhibited more *A101* positive cells than *caN-mild* mutant stalks (Appendix B, Fig. 4D, bracket). Long stalks in *caN-strong* ovaries, however had no *A101* positive cells (Appendix B, Fig. 4D, bracket and Table 2). Based on the expression of *93F* and *A101*, three different kinds of long stalk-like structures can be generated by the expression of constitutively-active Notch: those that consist entirely of stalk cells, those which consist of stalk cells and polar cells, and those which contain neither of these two cell types.

One possibility for the polar cells located in long stalks is that Notch function is required both for the initiation of stalk cell fate as well as the maintenance of this fate. According to this hypothesis, transient expression of constitutively-active Notch, initiated stalk cell fate in a group that normally should become polar cells. However, when the transiently expressed Notch was degraded, the cells returned to their normal fate: polar cells. This scenario is unlikely since polar cells were detected in *caN-intermediate* stalks where N(intra) is expressed persistently. This hypothesis also predicts, in the case of transient expression of constitutively-active Notch, that the longer the time period after heat shock, the more long stalks with *A101* positive cells would be detected. In addition, each long stalk should have more *A101* positive cells the further they progress

through oogenesis. As neither prediction is upheld (Appendix B, Table 1), it seems unlikely that Notch is required for the maintenance of stalk cell fate.

THE NUMBER OF CELLS EXPRESSING A PRECURSOR MARKER INCREASES IN THE LONG STALKS AS THE SEVERITY OF THE PHENOTYPE INCREASES

As mentioned previously, I noted a morphology reminiscent of precursor cells in some of the long stalks. In addition, expression of 2 markers for more differentiated cells, *93F* for stalk cells and *A101* for polar cells, was not detected in the long stalk-like structures generated by *caN-strong* (Appendix B, Figs 3E, 4E). To address whether Notch may inhibit the differentiation of stalk cells, I examined the stalks with a marker that reflects the differentiation state of stalk cells: Big Brain (Bib) protein. In wild-type ovaries, Bib is expressed in the follicle cells that are going to intercalate to form a stalk (Appendix B, Fig. 5B, arrowhead) as well as fully developed stalk cells (Appendix B, Fig. 5B, arrow). Importantly, the subcellular localization of Bib differs in these two cell populations. In the precursors, Bib is detected predominantly at the apical tip of the cells as they constrict (Fig. 5B, arrowhead and inset 1). In the stalk cells that had aligned into a single row, the apical pattern is lost and Bib is detected along the lateral surface (Appendix B, Fig. 5B, arrowhead and inset 2). A complementary marker, Fasciclin III (Fas III) protein, is first detected in all follicle cells in region 2 of the germarium (Appendix B, Fig. 5A, bracket). Fas III expression begins to be restricted to the polar cell precursors by region 3 of the germarium, and is fully restricted when the chamber is completely pinched from the germarium (Appendix B, Fig. 5A, arrows).

In the long stalks generated by *caN-mild* and *caN-intermediate*, more cells were detected that stained with the Bib precursor pattern and early Fas III pattern than in wild-type ovaries, indicating that more precursors were present in these ovarioles (Appendix B, Fig. 5C-F). If these stalks were allowed to develop, however, the cells matured (or partially matured) to stalk cells or polar cells (Appendix B, Fig. 5E,F). In

caN-strong, more precursors were also detected; nevertheless, these cells did not mature into stalk cells or polar cells, but stayed in the precursor stage, based on Bib and FasIII staining (Appendix B, Fig. 5G,H). Thus, the strongest phenotype observed due to constitutively-active Notch expression was persistence of the Bib-positive precursor stage.

In conclusion, three different classes of long stalk-like structures can be generated by the expression of constitutively-active Notch: those which consist of too many stalk cells (I), stalk cells and polar cells (II), or precursor cells (III) (Appendix B, Figure 7). A transient excess of precursors was also detected in classes I and II, suggesting that the severity of the phenotype correlates with the length of the precursor stage, culminating in a complete block at the precursor stage (class III).

To address whether the precursor stage was also affected by the loss of Notch function, I stained $Notch^{ts}$ ovaries with Bib and Fas III antibodies. After 25 hours at the restrictive temperature, no Bib positive cells were detected in $Notch^{ts}$ germaria (Appendix B, Fig. 5J). This result indicates that no cells are in the precursor stage, either because they never develop to this stage or because this stage is transient and therefore not detected.

EXPRESSION OF CONSTITUTIVELY-ACTIVE NOTCH IN FOLLICLE CELLS RESULTS IN A DEFECT IN THE OOCYTE ANTERIOR-POSTERIOR AXIS

Based on the loss-of-function mutant phenotypes of *Notch* and *Delta*, it has been proposed previously that a signal from the posterior follicle cells is required for setting the proper A-P axis of the developing oocyte (Ruohola *et al.*, 1991; Clark *et al.*, 1994; Ruohola-Baker *et al.*, 1994). The nature of the signal and the posterior follicle cell type that may send the signal, are not known. To investigate this question further, I analyzed how expression of constitutively-active Notch in follicle cells affects the posterior follicle cells and the anterior-posterior axis of the underlying oocyte.

In wild-type egg chambers after stage 4, two Fas III positive cells are detected at the anterior and posterior poles of each egg chamber (Appendix B, Fig. 6A). After two heat shock inductions of ΔEN in a one day interval, egg chambers were observed in which no Fas III positive cells were detected (Appendix B, Fig. 6B-D). These egg chambers were not separated by a long stalk, indicating that in this case constitutively-active Notch acts in a cell-fate decision separate from stalk formation. The egg chambers that lacked Fas III positive cells did not develop further but instead became necrotic. Therefore, in order to determine the effects that expression of constitutively-active Notch in the follicle cells have on the underlying oocyte during the later stages of oogenesis, I expressed constitutively-active Notch either under *HS-GAL4* with milder heat shock conditions [*ΔEN(HS)*] or under the enhancer trap line *GAL4-109(3)9* [*ΔEN(109)*]. Neither of these conditions resulted in detectable follicle cell-fate defects; two Fas III positive cells were detected at each pole of the egg chambers, and there was no change in the domain of *pointed* expression, a posterior follicle cell marker (data not shown; Morimoto *et al.*, 1996). Nevertheless, I cannot rule out the possibility that further differentiation of the posterior follicle cells is defective. The A-P polarity of the oocyte was examined in these egg chambers using markers employed previously in analysis of $Notch^{ts}$ (Ruohola *et al.*, 1991). The effects of constitutively-active Notch on embryonic polarity could not be addressed because development was blocked prior to embryogenesis.

An early marker for anterior-posterior polarity, *oskar (osk)* mRNA is localized to the posterior pole of wild-type oocytes at stage 8 (Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991; Appendix B, Fig. 6D) and is essential for development of the abdomen and formation of the germ-line precursor cells at the posterior pole of the embryo (Lehmann and Nüsslein-Volhard, 1986; Ephrussi and Lehmann, 1994). Another marker for A-P polarity, Staufen (Stau) protein is localized to the posterior pole of the oocyte at stage 8 (Appendix B, Fig. 6G; St Johnston *et al.*, 1991). Whole mount *in situ* hybridization with an *osk* cDNA probe, as well as immunocytochemistry with Stau

antibody revealed that expression of constitutively-active Notch causes mislocalization of these posterior components: they are mislocalized in ~80% of ΔEN(HS) egg chambers and in ~60% of ΔEN(109) egg chambers (Appendix B, Table 3). Mislocalization was either to the center of the oocyte (Appendix B, Fig. 6F,I; Table 3) or to a streak from the posterior (Appendix B, Fig. 6E,H and Table 3). In addition, expression of activated Notch under the control of *HS-GAL4* also generated mislocalization of both *osk* and Stau in granules throughout the oocyte (Appendix B, Table 3 and data not shown).

bcd mRNA was localized to the anterior pole of the oocyte in wild-type egg chambers (Appendix B, Fig. 6M) and is necessary for the establishment of anterior-posterior polarity (Nüsslein-Volhard *et al.* 1987; St Johnston *et al.*, 1989; Berleth *et al.*, 1988). *In situ* hybridization indicated that *bcd* mRNA was localized properly to the anterior end of the oocyte of *ΔEN(HS)* ovaries (data not shown). In 15% (11/73) of the *ΔEN(109)* stage 8 egg chambers, however, *bicoid* was detected at both the anterior and posterior poles of the oocyte (Appendix B, Fig. 6N). This phenotype has been detected previously in *Notch*, *Delta*, *Protein Kinase A*, *gurken*, *torpedo*, and *cornichon* mutants, and reflects a defect in microtubule organization (Ruohola *et al.*, 1991; Lane and Kalderon, 1994; González-Reyes *et al.*, 1995; Roth *et al.*, 1995).

To analyze whether the microtubular structures of the oocyte were oriented properly, a strain that expresses a Kinesin-βgal fusion protein (Clark *et al.*, 1994) was crossed to both *ΔEN(HS)* and *ΔEN(109)*. This fusion protein is a reliable marker for microtubule organization (Clark *et al.*, 1994; Lane and Kalderon, 1994; González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Gillespie and Berg, 1995). In control egg chambers, Kinesin-βgal localized to the posterior of the oocyte (Appendix B, Fig. 6J), suggesting that at least a subset of microtubules were oriented with their plus ends directed towards the posterior. Kinesin-βgal was mislocalized in 89% of *ΔEN(HS)* and 81% of *ΔEN(109)* egg chambers (Appendix B, Table 3). These mislocalizations were either to the center of

the oocyte (Fig. 6L; Table 3), to a posterior streak (Appendix B, Fig. 6K; Table 3), or throughout the oocyte (Appendix B, Table 3), as described above for other posterior components.

CONSTITUTIVELY-ACTIVE NOTCH IN THE FOLLICLE CELLS INDUCES PREMATURE OOPLASMIC STREAMING

The defect in localization of Kinesin-βgal indicates a defect in the organization of the microtubules. Another indirect method for analyzing the organization of the microtubules is to assay whether ooplasmic streaming commences at the correct time. Ooplasmic streaming is a microtubule dependent process (Gutzeit, 1982). In wild-type egg chambers at stage 9-10, an anterior to posterior gradient of microtubules is present in the oocyte. At this stage, no ooplasmic streaming is detected (Appendix B, Figs 6O, 7B). A rearrangement of microtubules is observed at stage 10b that results in arrays of parallel microtubules adjacent to the oocyte cortex that are associated with ooplasmic streaming (Theurkauf *et al.*, 1992). Premature ooplasmic streaming was detected at stage 8 in 65% (13/20) of *ΔEN(109)* egg chambers (Appendix B, Fig. 6P), suggesting that the normal gradient of microtubules was replaced by subcortical microtubule bundles. In controls (*109(3)9 TM3*, 15 stage 8 egg chambers analyzed) no continuous circular swirling movements were detected, only random flushing movements.

DISCUSSION

To better define the mechanism of Notch action, I have investigated the effects of expressing constitutively-active Notch in oogenesis. Specifically, I have tested the hypothesis that Notch functions in follicle cell fate determination and that signaling from the posterior follicle cells is required for the establishment of the oocyte A-P axis (Ruohola *et al.*, 1991; Xu *et al.*, 1993).

I have shown that at two stages of oogenesis, constitutively-active Notch induces follicle cell-fate defects that are opposite to those generated by a loss-of-function allele of *Notch*: hyperplasia of stalk cells early in oogenesis and a loss of polar cells later. The most extreme phenotype obtained with constitutively-active Notch was the persistence of a precursor stage phenotype in the follicle cells required for pinching off the egg chamber. This observation suggests a novel function for Notch in differentiation of these follicle cells: holding them in a precursor stage of development.

I have also shown that expression of constitutively-active Notch in the follicle cells affects localization of the posterior components *oskar* and Staufen. In addition, mislocalization of a Kinesin-βgal fusion protein and premature onset of cytoplasmic streaming was seen, as well as mislocalization of the anterior component, *bicoid*. These phenotypes reflect a defect in the organization of the microtubules within the oocyte and further implicate information from the follicle cells in regulation of this organization.

MODELS FOR NOTCH ACTION IN OOGENESIS

Three models for Notch action have been proposed previously: instructive, permissive, and prohibitive (reviewed in: Greenwald, 1994; Muskavitch, 1994; Artavanis-Tsakonas, 1995). In the instructive model, Notch directs cells to adopt one of two alternative fates - a binary cell fate decision (Struhl *et al.*, 1993; Greenwald, 1994). In the permissive model Notch makes the cells competent to respond to other inductive signals (Muskavitch, 1994). In the prohibitive model, Notch maintains cells in an uncommitted state preventing them from responding to external differentiation signals (Fortini *et al.*, 1993; Coffman *et al.*, 1993; Muskavitch, 1994; Artavanis-Tsakonas, 1995). Therefore, in both the permissive and prohibitive models, Notch modulates the cells ability to respond to additional signals.

My data suggest that Notch is sufficient to keep cells in a specific precursor stage. This result is most consistent with the prohibitive model, which proposes that Notch holds cells in an uncommitted state. This fate could correspond to the Bib-positive precursor stage being retained due to expression of constitutively-active Notch. My data are also consistent with the binary cell fate decision model if a binary switch is assumed between precursor fate and a more differentiated cell fate (Greenwald, 1994).

THE ANTERIOR-POSTERIOR AXIS OF THE OOCYTE

The mislocalization of *bicoid* to both the anterior and posterior ends of the oocyte and the localization of posterior components to the middle of the oocyte are phenotypes reminiscent of those seen with ovaries from mutants in *Notch*, *Delta*, *Protein Kinase A*, *torpedo*, *gurken*, and *cornichon* (reviewed in Rongo and Lehmann, 1996). During stages 1-6 of oogenesis, a microtubule organizing center (MTOC) is located at the posterior pole of the oocyte and extends a microtubule network from the oocyte into the nurse cells. During mid-oogenesis, this cytoskeleton is reorganized so that an anterior to posterior gradient of microtubules is present in the oocyte, suggesting that the MTOC at the posterior degenerates and the microtubules are nucleated at the anterior of the oocyte. It has been postulated that in these mutants (*N*, *Dl*, *PKA*, *top*, *grk*, *cni*) the posterior MTOCs are not properly destroyed, resulting in oocytes with both anterior and posterior microtubule concentrations (Lane and Kalderon, 1994; Ruohola-Baker *et al.*, 1994). A mirror image axis duplication results (Ruohola *et al.*, 1991; Lane and Kalderon, 1994; González-Reyes *et al.*, 1995; Roth *et al.*, 1995). One possibility is that mislocalization of the posterior components as a streak from the posterior is a milder phenotype resulting from this type of defect in microtubule organization. In egg chambers that express constitutively-active Notch in the follicle cells, ooplasmic streaming in the oocyte commences early, beginning around stage 8 of oogenesis. This phenotype is presumably also a secondary consequence of defects in the microtubule reorganizations.

One surprising result is that expression of constitutively-active Notch under the control of either *GAL4-109(3)9* or *HS-GAL4* (mild heat shock) can lead to defects in the A-P axis of the oocyte without generating any obvious defects in the fate of the posterior follicle cells. By analogy to constitutively-active Notch action in the germarium it is possible, however, that the timing of posterior follicle cell differentiation is altered in the mutant situation. Isolation of markers specific for different stages of posterior follicle cell differentiation should shed light on this possibility. It is also possible that the Notch pathway is closely linked to the signaling pathway from the follicle cells to the oocyte, and expression of constitutively-active Notch affects signaling without affecting the fates of these cells. Further analysis of this subject requires a better understanding of the signaling pathway from the follicle cells to the oocyte.

Chapter 3: The requirement for Notch signaling in anterior and posterior follicle cells during *Drosophila* oogenesis

INTRODUCTION

In *Drosophila melanogaster*, the body axes are set up during oogenesis through a process that involves signaling between the oocyte and the somatic follicle cells that surround the egg chamber. The proper localization of morphogenetic determinants is required for establishment of the embryonic anterior-posterior (A-P) axis and depends upon proper polarization of the oocyte cytoskeleton. Recent data suggest that signaling from specialized follicle cells at the posterior end of the oocyte is required for this polarization (reviewed in Anderson, 1995). Thus, the establishment of the oocyte anterior-posterior axis requires: (1) differentiation of specialized posterior follicle cells, (2) signaling from these posterior follicle cells to the oocyte, and (3) modulation of the polarity of the oocyte cytoskeleton upon reception of the signal.

Three signaling pathways are known to be required for proper patterning of posterior follicle cells: the EGF-receptor pathway, the Notch pathway, and the Hedgehog (Hh) pathway (González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Ruohola *et al.*, 1991; Xu *et al*, 1992; Bender *et al.*, 1993; reviewed in Rongo and Lehmann, 1996; Forbes *et al.*, 1996). The Egfr pathway transmits inductive information from the oocyte to the follicle cells, while the Hh and Notch pathways act in communication between different subsets of the follicle cells. Studies of mutants in the Egfr and Notch pathways have led to the model that the anterior and posterior groups of follicle cells are initially identical (Rongo and Lehmann, 1996). These follicle cells are transformed to the posterior fate by Egfr signaling if they are located adjacent to the oocyte. If and how the Notch and Egfr

pathways interact in this process is unknown, but several models have been proposed (Rongo and Lehmann, 1996; see discussion).

Studies with temperature-sensitive alleles of Notch, as well as my work with constitutively-active Notch, have shown that Notch functions in determining at least two follicle cell populations: the terminal follicle cells and the stalk cells that separate the egg chambers from one another. The defects in the terminal follicle cells lead to aberrant oocyte anterior-posterior axis formation, but I was unable to discern how expression of constitutively-active Notch leads to these defects. Such an analysis was made difficult by the necrosis that resulted from expression of constitutively-active Notch in the follicle cells that surround the oocyte. For this reason, I carried out further analysis in these cells by over-expressing the Notch ligand, Delta. Similar phenotypes are generated by over-expression of Delta, but with less necrosis; I was therefore able to analyze the follicle cell defects more thoroughly. My studies with constitutively-active Notch in stalk formation suggested that the role of Notch signaling was to hold cells in an uncommitted state, leading me to ask whether Notch plays the same role in the follicle cells surrounding the oocyte, and which cells require Notch-signaling function.

RESULTS

NOTCH IS REQUIRED FOR ANTERIOR AND POSTERIOR FOLLICLE CELL FATE

Previous studies demonstrated that the Notch pathway is required for proper establishment of posterior follicle cell fate (Ruohola *et al.*, 1991; Larkin *et al.*, 1996 - Chapter 2). Because anterior and posterior follicle cells have been shown to be equivalent in the absence of a posterior-inducing signal (Gonzalez-Reyes *et al.*, 1995; Roth *et al.*, 1995; Reviewed in Lehmann, 1995; Rongo and Lehmann; 1996), I investigated whether the Notch pathway is also required for anterior follicle cell fate. To do this, I analyzed the expression of follicle cell markers specific to anterior fate, the

enhancer trap lines *L53b* and *5A7*, in N^{ts} egg chambers at the restrictive temperature. Loss of Notch function abolished the normal onset of both of these markers at stage 8 in the anterior terminal cells in 97% (56/58) and 92% (47/51) of egg chambers, respectively (Appendix C, Fig. 1A-F). Therefore, loss of Notch function results in defective anterior follicle cells, suggesting that the Notch pathway is also required for anterior follicle cell fate.

DELTA MUST BE TURNED OFF IN THE POSTERIOR FOLLICLE CELLS AT STAGE 6.

Based on the data presented, Notch acts in both anterior and posterior follicle cells. There is a difference, however, between Delta expression in anterior and posterior follicle cells. In wild-type ovarioles, Delta protein is detected in a specific pattern, as previously determined with antibodies to Delta (Bender *et al.*, 1993; Appendix C, Fig. 2). Delta protein is first detected in the germarium, and later accumulates strongly in the membranes of follicle cells (Appendix C, Fig. 2A). Delta disappears from the posterior follicle cells beginning around stage 6 of oogenesis (Bender *et al.*, 1993; Appendix C, Fig. 2B,C). This pattern of expression is consistent with the mRNA localization pattern detected by whole mount *in situ* hybridizations with a probe to *Delta* mRNA (Ruohola *et al.*, 1991).

To test whether this difference in expression has a functional relevance, an undergraduate and I prolonged Delta expression in the posterior follicle cells by using a *UAS-Delta* construct and the GAL4 line *109* (Appendix C, Fig. 2D,E) then analyzed the effects on the determination of the posterior follicle cells and establishment of the oocyte A-P axis. I analyzed posterior cell fate by monitoring the mRNA for a receptor tyrosine kinase, *derailed* (*drl*; Callahan *et al.*, 1995) as a marker for posterior follicle cell differentiation. In wild type, *drl* mRNA is expressed in posterior follicle cells from the germarium until about stage 6 of oogenesis (Clegg, N. and Ruohola-Baker, H., unpublished; Appendix C, Fig. 3A and Table 1). In the *UAS-Dl/+; GAL4-109/+* mutant

egg chambers the posterior *drl* expression was abnormal; *drl* was still detected at stage 8 in *UAS-Dl -; GAL4-109 -* egg chambers (Appendix C, Fig. 3B). Prolonged expression of *drl* was also detected in *UAS-Dl -; HS-GAL4 -* ovaries, one day after a 25 minute heat-shock induction at 37° (Appendix C, Table 1). These data indicate that prolonged expression of Delta induced prolonged expression of *drl* mRNA in posterior follicle cells, confirming that cessation of Delta expression in the posterior follicle cells is important for their normal development.

To investigate where and when ectopic Delta must be expressed to prolong *drl* expression, I analyzed egg chambers that expressed Delta under the control of the GAL4-lines *c709*, *107c1*, and *A62*, the help of an undergraduate (Appendix C, Fig. 2). The expression patterns of these lines was investigated by using them to express *UAS-lacZ* and staining with X-gal. *GAL4-A62* drives expression in the follicle cells in a cap over the oocyte, beginning in stage 6. *GAL4-c709* results in patchy X-gal expression throughout the follicle cell layer; expression is slightly later than that of *GAL4-109* in that it does not begin until early stage 7. *GAL4-107c1* drives expression in the border cells and nurse-cell associated cells. In *107c1*, no prolonged *drl* expression was detected, suggesting that ectopic Delta expression in anterior follicle cells cannot induce prolonged expression of *drl* in posterior follicle cells (data not shown). Prolonged expression of *drl* was also not detected when *UAS-Delta* was expressed under the control of *c709* or *A62*, suggesting that Delta expression must begin prior to *c709* or *A62* onset during stage 7. An alternative possibility is that Notch signaling requires differing levels of *Delta* in adjacent cells (Wilkenson *et al.*, 1994), and *A62* results in uniform expression (Appendix C, Fig. 2G).

To further investigate the defect in the posterior follicle cells, I analyzed an anterior marker, *L53b*, that is repressed in the posterior follicle cells by the Egfr pathway. In addition to normal anterior expression, *L53b* was also activated in the follicle cells over

the oocyte in 34% (26/76; Appendix C, Fig. 4) of stage 10 *UAS-Dl -; GAL4-109 L53b* egg chambers. Therefore, expression of Delta can induce anterior markers in the posterior follicle cells. This phenotype has been observed previously in mutants that compromise the activity of the Egfr pathway in posterior follicle cells (González-Reyes *et al.*,1995; Roth *et al.*,1995; Clegg *et al.*,1997; González-Reyes *et al.*, 1997).

Over-expression of Delta in the posterior follicle cells at stage 6 disturbs their development, presumably due to over-activation of the Notch pathway. Therefore, these data suggest that Delta expression is important for control of the Notch pathway. It is possible that this control could be at the transcriptional level (through a feedback loop) or by direct activation of the Notch receptor. Recent experiments have indicated that in some systems the Notch pathway is regulated by a feedback mechanism (reviewed in Kimble and Simpson, 1997). In an attempt to address this possibility, I looked at the expression of Notch protein in *UAS-Dl -; GAL4-109 -* ovaries. No obvious change in the protein expression pattern was detected (data not shown). This result suggests that in the follicle cells surrounding the oocyte, the level of Delta protein is controlling the Notch pathway through direct activation of the receptor. I cannot rule out, however, that a feedback loop generated a transient change in the Notch level which could not be detected in normal antibody stainings.

PROLONGED EXPRESSION OF DELTA IN POSTERIOR FOLLICLE CELLS GENERATES OOCYTES WITH TWO ANTERIOR ORGANIZING CENTERS

To address whether the posterior follicle cell defects caused by prolonged expression of Delta generate defects in the A-P axis of the oocyte, I expressed Delta in the follicle cells, either under the control of *HS-GAL4* or *GAL4-109*, and then analyzed the oocyte for localized components. Anterior components, such as *bicoid* mRNA (*bcd*; Berleth *et al.*, 1988; Appendix C, Fig. 5C), were mislocalized to both the anterior and posterior of the oocyte (Appendix C, Fig. 5D and Table 2), while posterior components,

oskar mRNA(*osk*; Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991; Appendix C, Fig. 5A) and Kinesin-βgal fusion protein (Fig. 5E), were mislocalized to the center of the oocyte (Appendix C, Fig. 5B,F and Table 2). The normally anterior oocyte nucleus was now found mislocalized to the posterior (Appendix C, Figure 5B and Table 2). In addition, premature ooplasmic streaming was detected in some egg chambers (Appendix C, Table 2). Combinations of these phenotypes have been observed previously in *Notch*, *Delta*, *Protein Kinase A*, *gurken*, *torpedo*, *cornichon*, *mago nashi*, and *maelstrom* mutants, as well as with expression of constitutively-active *Notch*, and reflect a defect in the microtubule reorganization that takes place in mid-oogenesis (Ruohola *et al.*, 1991; Lane and Kalderon, 1994; González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Larkin *et al.*, 1996; Micklem *et al.*, 1997; Newmark *et al.*, 1997; Clegg *et al.*, 1997). In wild type, a microtubule organizing center (MTOC) was initially localized to the posterior end of the oocyte by an unknown mechanism. During mid-oogenesis, the MTOC disappears from the posterior, and microtubules begin to nucleate from the anterior of the oocyte. No oocyte A-P axis defects were detected in lines that induce Delta expression in the anterior or lateral follicle cells (*107c1*, *c709*) or in the posterior follicle cells later in oogenesis (*A62*; data not shown).

THE DEFECTS IN THE A-P AXIS OF THE OOCYTE ARE DUE TO DEFECTS IN THE ORGANIZATION OF THE MICROTUBULES

The defects in the A-P axis of the oocyte observed with constitutively-active Notch (Larkin *et al.*, 1996), over-expression of *Delta*, and loss of function alleles of *Notch* and *Delta* (Ruohola *et al.*, 1991; Clark *et al.*, 1994) suggested abnormal organizations of the microtubule cytoskeleton. To investigate this possibility, I visualized the microtubules by staining them with an antibody to α-tubulin. To assay the functionality of the microtubules, the egg chambers were simultaneously stained with antibodies to Staufen (Stau), another marker for A-P polarity, which is localized to the posterior pole of the oocyte at stage 8 (St Johnston *et al.*, 1991). Expression of

constitutively-active Notch, over-expression of Delta, and loss of Notch or Delta led to a high concentration of microtubules in the posterior of the oocyte (Appendix C, Fig. 6). Stau was mislocalized to the center of the oocyte where there is a void detected in the concentration of microtubules. These data suggest retention of the posterior microtubule organizing center and that the defects in the A-P axis are due to defects in the organization of the microtubule cytoskeleton.

In summary, I conclude that prolonged activation of the Notch pathway disturbs the posterior follicle cells prior to the stage in which they are competent to signal to the oocyte, resulting in retention of the posterior microtubule organizing center and bipolar A-P axis. This result shows that sufficient signaling did not occur prior to stage 6.

ECTOPIC EXPRESSION OF DELTA IN THE GERMARIUM FOLLICLE CELLS GENERATES LONG STALK-LIKE STRUCTURES

Our previous studies showed that expression of constitutively-active Notch generates long stalks in the germarium and causes defects in the A-P axis of the oocyte (Larkin *et al.*, 1996). Since expression of UAS-Delta generates similar phenotypes in the oocyte, I looked to see whether it could also generate defects in stalk formation.

To express *UAS-Dl* in the germarium, I heat shocked *UAS-Dl: HS-GAL4* flies and analyzed them two days after the heat shock. Over-expression of Delta in the germarium lead to the formation of long stalk-like structures in 81% (119/147) of the ovarioles (Appendix C, Figs 7,8). In contrast to the normal wild-type stalk, which is 6-7 cells long, the average length of the long stalks was ~33 cells. Based on their morphology and the expression of markers, two classes of long stalks were observed, "intermediate" (30% of long stalks) and "extreme" (70% of long stalks). The cells in the intermediate long stalks were aligned as a single row and expressed either the stalk cell marker, *93F* (Appendix C, Fig. 7D; Ruohola *et al.*, 1991), or the polar cell marker, *A101* (Appendix C,

Fig. 7F). The extremely long stalks were several follicle cells thick, exhibiting the morphology of follicle cells that are ready to intercalate to form a stalk in the germarium, and do not contain differentiated *93F* or *A101* positive cells (Appendix C, Fig. 8B). Both classes of long stalks were detected previously with expression of constitutively-active Notch (Chapter 2; appendix B, Larkin *et al.* 1996).

THE CELLS IN THE LONG STALK-LIKE STRUCTURES ARE ARRESTED IN A PRECURSOR STAGE

Because the cells in the extremely long stalks did not express the differentiation markers *93F* or *A101*, I analyzed the expression of the precursor markers Big Brain (Bib; Larkin *et al.*, 1996; Doherty *et al.* 1997) and Fasciclin III (FasIII) proteins (Appendix C, Fig. 8D,F). In wild-type ovaries, Bib is expressed in the precursors for polar cells and the follicle cells intercalating to form a stalk, as well as in fully formed stalks. Importantly, the subcellular localization of Bib differs in these two cell populations. In the precursors, Bib is found predominantly in the apical tips of the cells where they meet as the cells are constricting (Appendix C, Fig. 8E, arrow). In the stalk cells that have become aligned as a single row, Bib is detected along the lateral surface (Appendix C, Fig. 8E, bracket). FasIII is detected in all of the follicle cells in region 2 of the germarium, but is restricted to polar cell precursors by the time the egg chamber leaves the germarium (Appendix C, Fig. 8C). In all of the extremely long stalks generated by over-expression of *Delta*, Bib was detected in a line along the apical tips of the cells (Fig. 8F), and FasIII was detected in all of the cells (Appendix C, Fig. 8D), the precursor patterns for these markers. These data, coupled with the fact that there was no expression of the differentiation markers, *93F* and *A101*, suggest that in the most extreme cases the cells in the long stalk-like structures were precursors for both the stalk cells and the polar cells. Recent mosaic analysis has also revealed that stalk cells and polar cells have a common precursor, separate from the precursors for lateral follicle cells (Tworoger, M., Bryant, Z., M. K. L., and H. R.-B., submitted). These data indicate that over-expression of Delta functions similarly to constitutive activation of the Notch receptor, forming long

stalk-like structures by prolonging the precursor stage. Therefore, expression of the Notch ligand, Delta, plays a critical role in controlling the activity of the Notch pathway in oogenesis.

UAS-DELTA ACTS THROUGH THE NOTCH RECEPTOR TO GENERATE LONG STALKS

To confirm that *UAS-Dl* generates long stalks by acting through the Notch receptor, I analyzed the phenotype generated by over-expression of Delta in a *Notch* loss-of-function background. If *UAS-Delta* acts through the Notch receptor, then no phenotype due to over-expression of Delta should be detected without functional *Notch*. N^{ts} N^{ts}; *UAS-Dl HS-GAL4* and control females were heat-shocked to induce Delta expression and then transferred to the restrictive temperature (See Materials and Methods). 94% of control N^{ts} N^{ts} ovaries exhibited a loss of stalks (61/65, Appendix C, Fig. 9C), while 69% of *UAS-Dl HS-GAL4* ovaries displayed long stalk-like structures, as expected (24/35, Appendix C, Fig. 9B). 98% of N^{ts} N^{ts}; *UAS-Dl HS-GAL4* ovaries displayed the loss of stalk defect (52/53, Appendix C, Fig. 9D), demonstrating that the over-expression of Delta requires functional Notch protein in order to generate long stalk-like structures.

DISCUSSION

I have shown that the Notch pathway is required in the anterior follicle cells for proper establishment of anterior fate. Moreover, prolonged activation of the Notch pathway in the posterior follicle cells leads to defects in the posterior follicle cells, including ectopic expression of anterior markers. Because over-activation of the Notch pathway and loss of the Egfr pathway result in the same phenotype - ectopic expression of anterior markers - it is possible that repression of the Notch pathway in the posterior is accomplished by the Egfr pathway. In the germarium expression of *UAS-Delta* generates

long stalk-like structures similar to those detected with expression of constitutively-active Notch, and these are formed by a block in the precursor stage. In addition, expression of *UAS-Delta* in the absence of Notch function leads to a loss of stalks, indicating that *UAS-Delta* acts through the Notch receptor.

OVER-ACTIVATION OF THE NOTCH PATHWAY DISTURBS THE POSTERIOR FOLLICLE CELLS PRIOR TO FOLLICLE CELL TO OOCYTE SIGNALING

In wild-type posterior follicle cells, the *derailed* staining pattern defines separate developmental stages. Before stage 6, the posterior follicle cells are *derailed* positive. From stage 6 on, *derailed* expression is no longer detected in the posterior of the egg chamber. Based on the expression patterns in mutant and wild-type situations, over-expression of Delta at stage 6 disturbs the posterior follicle cell development and causes a defect in oocyte A-P axis, presumably due to a defect in the follicle-cell-to-oocyte signaling. Over-expression of Delta after stage 6 does not affect the oocyte. Signaling from the follicle cells to the oocyte that is required for the proper oocyte A-P polarity must therefore occur (or continue to occur) during late stage 6.

THE NOTCH PATHWAY ACTS IN BOTH ANTERIOR AND POSTERIOR FOLLICLE CELLS

My data, in conjunction with previous studies, show that the Notch pathway is required in both the anterior and posterior follicle cells. An important difference, however, is that the Notch pathway must be inactivated in the posterior prior to stage 6. This inactivation involves cessation of Delta expression in the posterior follicle cells. If Delta is continues to be expressed, posterior markers such as *derailed* mRNA are defective, and markers that are usually expressed only in the anterior are inappropriately expressed in the posterior (*5A7* and *L53b*). These data are consistent with two models. In the first model, Notch signaling acts as an inducer. The Notch pathway can induce anterior fate in both the anterior and posterior follicle cells and must be turned off in the

posterior before the posterior follicle cells can take their appropriate fate. Both over-activation of the Notch pathway and loss of the Egfr pathway lead to similar phenotypes - namely expression of the anterior markers, *L53b* and *5A7*, in the posterior follicle cells. Therefore, a possible model is that the Notch pathway can induce anterior fates in both the anterior and posterior but is repressed in the posterior by the Egfr pathway.

An alternative interpretation is that, like its function in stalk formation, Notch signaling maintains the follicle cells around the egg chamber in a precursor stage. Loss of Notch function results in loss of the anterior markers because the anterior follicle cells have not yet reached the stage at which these markers are expressed. Prolonged Notch signaling in the posterior results in prolonged expression of *derailed* by blocking posterior follicle cells in the *derailed*-positive stage. Thus, Notch signaling is required in both anterior and posterior follicle cells early, then must stop in the posterior follicle cells around stage 6. The idea that Notch signaling maintains follicle cells in a precursor stage is attractive in that Notch signaling is not responsible for induction of particular fates (which is a difficult to idea to reconcile with the pleiotropy of Notch function), and may be analogous to Notch function in stalk formation.

Chapter 4: Conclusions

The work presented here, in conjunction with that from other laboratories, has demonstrated a requirement for Notch signaling in three distinct processes in oogenesis: germ-line mitosis, stalk formation, and determining of the fate of follicle cells that surround the egg chamber, and therefore oocyte anterior-posterior axis formation. Additionally, this work has helped define mechanisms of Notch action.

MODELS FOR THE ROLE OF NOTCH ACTION IN OOGENESIS

Three models for Notch action have been proposed previously: instructive, permissive, and prohibitive (reviewed by Greenwald, 1994; Muskavitch, 1994; Artavanis-Tsakonas, 1995; Blaumueller and Artavanis-Tsakonas, 1997). In the instructive model, Notch directs cells to adopt one of two alternative fates - a binary cell fate decision (Struhl *et al.*, 1993; Greenwald, 1994). In the permissive model Notch makes the cells competent to respond to inductive signals (Muskavitch, 1994). In the prohibitive model, Notch maintains cells in an uncommitted state, preventing them from responding to external differentiation signals (Fortini *et al.*, 1993; Coffman *et al.*, 1993; Muskavitch, 1994; Artavanis-Tsakonas, 1995). Therefore, in both the permissive and prohibitive models, Notch modulates the ability of cells to respond to additional signals. My data suggest that Notch may not always act by one mechanism in every process; the mechanism of Notch action appears to be context dependent. In *Drosophila* oogenesis, I have demonstrated diverse roles for Notch signaling in the three processes I have investigated. Proposals for the role of the Notch pathway in each of these processes are discussed below.

GERM-LINE MITOSIS

My work has shown that Notch signaling is required for the control of germ-line division in *Drosophila* oogenesis; loss of either of the neurogenic genes *Notch* or *neuralized* results in an extra round of germ-line mitosis. A similar role for the Notch pathway has been detected in other organisms, including *C. elegans* where mutations in the Notch pathway result in defects in germ-line division (Austin and Kimble, 1987; Kimble and White, 1981). It has been suggested that the function of the Notch pathway in control of cell division may be distinct from its role in induction or lateral specification (Go *et al.*, 1998; reviewed by Egan *et al.*, 1998); in the *Drosophila* wing disc, it was shown that activation of the Notch receptor induces expression of patterning genes and strong mitotic activity, but that the effect on cell proliferation was not a consequence of the upregulation of the patterning genes (Go *et al.*, 1998). In the control of *Drosophila* germ-line mitosis, it is possible, however, that Notch signaling is responsible for determining the fate of the follicle cells that signal to the germ line, or alternatively that being mitotically active or inactive is a cell-fate determination. This latter idea could explain how abnormal activation of Notch in mammals has been associated with neoplasias (reviewed by Gridley, 1997). In these cases, neoplastic cells could be maintained in a mitotically-active precursor stage by over-activation of the Notch pathway.

STALK FORMATION

My data related to stalk formation suggest that Notch signaling is sufficient to keep cells in a specific precursor stage. This result is most consistent with the prohibitive model. This model is particularly attractive because it proposes that Notch holds cells in an uncommitted state, which could correspond to the Bib-positive precursor stage being retained due to expression of constitutively-active Notch or over-expression of Delta. Furthermore, the prohibitive model invokes additional differentiation signals, one of which could be the recently cloned gene, *toucan* (Grammont *et al.*, 1997). Toucan is

expressed in the germ line, and germ-line over-expression of Toucan results in long stalks in which all of the cells express a stalk cell marker. One hypothesis that could explain these data is that Notch signaling maintains cells in an uncommitted state until they are induced to differentiate by a signal from the germ line, involving Toucan. This role for Notch signaling offers one explanation for how Notch signaling could be responsible for so many cell-fate decisions in so many organisms, being responsible for integration of other inductive signals.

Other studies utilizing constitutively-activated Notch constructs have also suggested that Notch signaling functions by maintaining cells in an uncommitted state (reviewed by Artavanis-Tsakonas *et al.*, 1995; Egan *et al.*, 1998). In *Xenopus*, activated Notch interferes with the differentiation of neural and mesodermal cell lineages (Coffman *et al.*, 1993). Transplantation experiments had previously shown that ectoderm loses the ability to respond to induction during gastrulation (Albers, 1987; Servetnick and Grainger, 1991). Coffman *et al.* (1991) cultured constitutively-active Notch injected ectodermal tissue in the presence of a potent inducer, Activin A, and isolated tissue before and after the start of gastrulation. The competence of this tissue to respond to Activin A was prolonged past the start of gastrulation. The authors suggested that cell determination was delayed, resulting in prolonged competence. This is consistent with the idea that Notch-signaling holds cells in an uncommitted state (this state being competent to respond to inductive signals) and parallels what I have found in *Drosophila* oogenesis, where the uncommitted state corresponds to the precursors for the stalk and polar cells.

Similar results have been found in the *Drosophila* embryo, where neuroblast segregations occur in temporal waves that are prevented by persistent expression of constitutively-active Notch (Struhl *et al.*, 1993). More telling, however, is the finding that these waves of segregation are delayed, but not prevented, by transient expression of constitutively-active Notch (Struhl *et al.*, 1993). This result strongly suggests that Notch signaling holds cells in an uncommitted state and is difficult to

reconcile with other models for Notch-signaling function, because the cells are only temporarily prevented from selecting their correct fate. This argues against the idea that Notch simply controls binary cell fate decisions, unless these decisions are reversible and do not result in commitment.

Results in *Xenopus* and the *Drosophila* with transient expression of constitutively-active Notch suggest that once Notch signaling activity has abated, cells may recover and respond to developmental cues; in some cases differentiating properly (as in *Drosophila* neurogenesis) and in other cases responding to alternative cues. This type mis-specification has been found in the *Drosophila* eye imaginal disc, where transient expression of activated Notch led cells to ignore normal inductive cues and adopt alternative fates (Fortini *et al.*, 1993). Mis-specification such as this explains the "polar cells" located within the long stalks generated by expression of constitutively active Notch or over-expression of Delta.

How does Notch signaling maintain cells in an undifferentiated state? Because the differentiation state of the cell is ultimately defined by the expression of various genes, such a differentiation delay would most likely be accomplished by repression of certain genes and the simultaneous induction of others. Indeed, the cytoplasmic domain of Notch has been hypothesized to act as a molecular scaffold that promotes the assembly of a transcriptional regulating complex on specific gene promoters (Egan *et al.*, 1998). Expression of Notch proteins in the nuclei of some cells, both *in vitro* and *in vivo*, has lead to the proposal that nuclear localization of Notch could be related to the differentiation state of the cell (Fortini *et al.*, 1993, Aster *et al.*, 1994; Kopan *et al.*, 1994; Ahmad *et al.*, 1995; Zagouras *et al.*, 1995; reviewed by Blaumueller and Artavanis-Tsakonas, 1997). Here it is thought that terminally differentiated cells express an "activated" form of Notch, which localizes primarily to the nucleus, and this makes the cells unresponsive to external ligands.

THE FOLLICLE CELLS SURROUNDING THE EGG CHAMBER

My data, in conjunction with previous studies, show that the Notch pathway is required in both the anterior and posterior follicle cells. An important difference, however, is that the Notch pathway must be inactivated in the posterior prior to stage 6. This change involves cessation of Delta expression in the posterior follicle cells. If Delta continues to be expressed, expression of an early posterior marker, *derailed* mRNA, is prolonged and markers that are usually expressed only in the anterior are inappropriately expressed in the posterior. These data are consistent with two models. In the first model, Notch signaling acts as an inducer. The Notch pathway can induce anterior fate in both the anterior and posterior follicle cells and must be turned off in the posterior before the posterior follicle cells can assume their appropriate fate. Both prolonged activation of the Notch pathway and loss of the Egfr pathway lead to similar phenotypes - namely expression of the anterior markers, *L53b* and *5A7*, in the posterior follicle cells. Therefore, a possible model is that the Notch pathway can induce anterior fates in both the anterior and posterior but is repressed in the posterior by the Egfr pathway.

An alternative interpretation is that, like its function in stalk formation, Notch signaling maintains the follicle cells around the egg chamber in a precursor stage. Loss of Notch function results in loss of the anterior markers because the anterior follicle cells have not yet reached the stage at which these markers are expressed. Prolonged Notch signaling in the posterior results in prolonged expression of *derailed* by blocking posterior follicle cells in the *derailed* positive stage. Thus, Notch signaling is required in both anterior and posterior follicle cells early, then must stop in the posterior follicle cells around stage 6. It is not unusual for the timing of Notch signaling to be regulated or for the Notch pathway to switch roles. In *Drosophila* eye development, for example, Notch signaling is required at one time point to inhibit expression of Atonal, but is responsible for maintaining its expression at a different time point (Baker and Yu, 1997). It is highly

possible that the Notch pathway is acting in an analogous manner in oogenesis, carefully regulating other signals. This model for Notch function is particularly attractive in that Notch signaling is not responsible for induction of particular fates (which is a difficult to idea to reconcile with the pleiotropy of Notch function in the development of many organisms), and it is analogous to the function I have detected for Notch signaling in stalk formation.

Thus, in *Drosophila* oogenesis, the Notch pathway acts in several different manners: to control cell-division, to inhibit cell differentiation, and possibly to induce cell fates. These studies indicate that Notch signaling can accomplish a wide range of effects and lend credence to the idea that the role of Notch signaling is context dependent.

INTERACTIONS BETWEEN THE SOMA AND GERM LINE

Another interesting aspect of the three processes investigated here is that interactions between the soma and germ line are required for each. Interactions between the somatic and germ cells are a common theme throughout development; such interactions have been found to be important during oogenesis in many organisms from flies and worms to mammals (reviewed by Buccione *et al.*, 1990; Anderson, 1995; Kimble and Simpson, 1997). The role of the somatic cell-germ cell interaction in each of the three processes studied is discussed below.

GERM-LINE DIVISION

My data have shown that Notch signaling is required for the control of germ-line mitosis. Furthermore, my clonal analysis has shown that *neuralized* is required in the germ line, while *Notch* is required in the follicle cells. These results suggest that interactions between the germ line and the soma are required for the regulation of germ-line mitosis. A similar situation has been encountered in *C. elegans*, where a somatic cell

called the distal tip cell signals to the neighboring germ line to control of cell-division (Kimble and White, 1981; reviewed by Kimble and Simpson, 1997). Similarly, *Drosophila* spermatogenesis requires *punt*, a transforming growth factor beta type II receptor, and *schnurri*, a transcription factor, in the somatic cyst cells that surround germ cells in order to restrict germ cell proliferation (Matunis *et al.*, 1997). Mammalian oogenesis also requires factors within the surrounding somatic cells to maintain the oocyte in meiotic arrest (Leibfried and First, 1980; Dekel, 1988; Racowsky and Baldwin, 1989). It is particularly interesting to note that Notch pathway homologues are involved in this interaction in *C. elegans* (Austin and Kimble, 1987, Priess *et al.*, 1987; Greenwald *et al.*, 1983; Ferguson and Horvitz, 1985).

Because it appears that Neuralized acts upstream of Notch in epistasis studies (de la Concha *et al.*, 1988; Lieber *et al.*, 1993), this suggests that there is a signal from the germ line to the soma. Since mutations in either *Notch* or *neuralized* result in a germ-line division defect, there must be a subsequent signal from the soma to the germ line to govern mitosis. Alternatively, this is one instance where Neuralized acts downstream of Notch or is independent of Notch.

STALK FORMATION

My work has shown convincingly that activation of the Notch pathway in the follicle cells of the germarium leads to a block in the precursor stage - leading to the formation of long stalk-like structures. More recent work has shown that mutations in the *toucan* gene lead to similar phenotypes in the germarium (Grammont *et al.*, 1997). Absence of stalks in flies transheterozygous for *toucan* and either *daughterless*, *Notch*, or *Delta* suggests that all of these genes participate in the same biological process. Furthermore, the requirement for *toucan* in the germ line and *Notch* and *Delta* in the follicle cells implies interactions between the germ line and the somatic follicle cells. One possible model is that Notch signaling holds the precursor follicle cells in an

undifferentiated state until Toucan signals them to differentiate. This could be one way that the germarium could ensure that germ-line cysts do not pinch-off and leave the germarium until they are at the appropriate stage of development. This could be tested by over-expressing Toucan in the germ line in a constitutively-active Notch background. One could then analyze whether Toucan can still generate long stalks that express a stalk cell marker. If Notch is mediating the Toucan signal, one would expect that the cells would remain blocked in the precursor stage despite the presence of the Toucan stalk-inducing signal.

FOLLICLE CELLS SURROUNDING THE EGG CHAMBER

The proper localization of morphogenetic determinants within the oocyte is required for establishment of the embryonic anterior-posterior (A-P) axis and depends upon proper polarization of the oocyte cytoskeleton. Recent data suggest that signaling from specialized follicle cells at the posterior end of the oocyte is required for this polarization (reviewed by Anderson, 1995). Furthermore, proper establishment of posterior fate in the posterior follicle cells requires a signal from the oocyte (González-Reyes *et al.*, 1995; Roth *et al.*, 1995). In short, the establishment of the oocyte anterior-posterior axis requires: (1) a signal from the oocyte to the posterior follicle cells, (2) differentiation of specialized posterior follicle cells, (3) signaling from these posterior follicle cells to the oocyte, and (4) modulation of the polarity of the oocyte cytoskeleton upon reception of the signal. Here, once more, interactions between the soma and germ line are vital to this process.

It is interesting to note that in the follicle cells surrounding the oocyte, as with the follicle cells involved in stalk formation, the Notch pathway is required in the follicle cells and other signaling molecules act in the actual soma/germ line interaction. This observation seems to contrast with the control of germ-line mitosis, where the Notch pathway appears to mediate the soma/germ line interaction and is required in both

(because I have seen that *Notch* is required in the follicle cells and *neuralized* is required in the germ line), once again illustrating that the Notch pathway is context-dependent. Moreover, it is becoming evident that the somatic cells and germ cells regulate each other closely and repeatedly during *Drosophila* oogenesis and that these interactions are essential for the differentiation of both cell types (reviewed by Anderson, 1995). The same has been found to be true during the development of many other organisms, including worms (Kimble and White, 1991; reviewed by Kimble and Simpson, 1997) and mammals, both during oogenesis and spermatogenesis (Bitgood *et al.*, 1996; reviewed by Buccione *et al.*, 1990; Eppig, 1991).

INTERPLAY BETWEEN THE EGFR AND NOTCH PATHWAYS IN POSTERIOR FOLLICLE CELL DEVELOPMENT

One of the major objectives in biology is to elucidate how multiple signals are integrated by cells. For example, both vulval development in *C. elegans* and axis formation in *Drosophila* oogenesis require the Notch and Egfr pathways for establishment of specific cell fates (reviewed in Duffy and Perrimon, 1996). But how these pathways are integrated inside a cell is not yet understood. In *Drosophila* oogenesis, several models have been proposed to explain the roles of these two pathways in the specification of posterior follicle cell fate and signaling (Rongo and Lehmann, 1996). Some models suggest that the Notch and Egfr pathways act independently, while others suggest that they interact. I have shown that prolonged expression of Delta results in failure of posterior follicle cells to receive the Grk signal, because a marker that is normally turned off in response to the Grk signal is not repressed. This result suggests that the pathways do not act independently because the Notch pathway can interfere with activity of the Egfr pathway. Our data can be explained is by a repression model in which high Notch activity is required for establishment of anterior fate and low Notch activity for posterior fate. In the posterior, the Notch pathway is depressed by the Egfr pathway, resulting in posterior

follicle cell fate. Prolonged expression of *Delta* partially overcomes the repression by the Egfr pathway, leading to the expression of an anterior marker in the posterior of the egg chamber. An alternate possibility is that prolonged Notch signaling in the posterior interferes with the proper developmental program in the posterior follicle cells (by retaining them in a "precursor" stage longer than usual), interfering with their ability to receive the Egfr signal - thus causing them to resort to the default anterior fate. It is also possible that both of these ideas are true, that Notch signaling is affecting the precursor stage, and is required for anterior fate.

IMPLICATIONS FOR FOLLICLE CELL TO OOCYTE SIGNALING

It was previously not known precisely when the follicle cells signal to the oocyte to establish the anterior-posterior axis. In wild-type posterior follicle cells, the *derailed* staining pattern defines separate developmental stages. Before stage 6 the posterior follicle cells express *derailed*, then it is turned off. Based on the expression patterns in mutant and wild-type situations, continued expression of Delta at stage 6 disturbs the posterior follicle cell development and causes a defect in oocyte A-P axis, presumably due to a defect in the follicle-cell-to-oocyte signaling. Continued expression of Delta after stage 6 does not affect the oocyte. Signaling from the follicle cells to the oocyte that is required for the proper oocyte A-P polarity must therefore occur (or continue to occur) during late stage 6.

Chapter 5: Future Directions

This research has furthered our understanding of the Notch signaling pathway, as well as several processes in *Drosophila* oogenesis. There are many gaps, however, to be filled in our comprehension of both. Here I discuss a few of the many possible experiments.

Our understanding of signal transduction by the Notch signaling cascade has grown dramatically over the last few years, yet it is apparent that only a fraction of the factors that participate in the Notch pathway have been uncovered, and we do not understand a great deal about how the pathway functions. Because of the extensive use of the Notch pathway during development for cell fate decisions, the characterization of molecules that interact with Notch have general interest. It would be very informative to find molecules cooperating with Notch during *Drosophila* oogenesis, both those already encountered in other processes and organisms, and new components. How does Notch signaling achieve such seemingly different effects in cell fate determinations and the control of germ-line mitosis? Does the Notch pathway function as a signaling "cassette", using the same components in all processes? Are there different downstream effectors, and if so how are they differentially controlled by the same pathway? Many questions could be answered by detection of other Notch pathway genes. Mutations in many of these genes would presumably lead to lethality or female sterility. It could therefore be useful to screen existing female sterile mutant collections for three types of *Notch*-like mutant phenotypes: germ-line cysts with more than 16 cells, arrest of oogenesis in the germarium, and mislocalization of components within the oocyte. The pinching-off defect in the germarium is easy to detect morphologically, and nuclear staining facilitates detection of egg chambers with more than the normal complement of germ-line cells. Anterior-posterior axis defects can be observed by many means, including *in situ* hybridization of *bicoid* or *oskar* mRNA, immunocytochemistry with antibodies to Staufen,

or crossing *kinesin-lacZ* into the mutant lines. Notch-signaling-pathway candidates could then be tested for interactions with known components, such as Notch or Delta. In addition to new components of the Notch pathway, this type of screen could possibly lead to other signaling pathways involved in the same processes. Additional screening could be carried out using the enhancer trap approach (O'Kane and Gehring, 1987) and search for genes with expression patterns that may suggest they are involved in one of the processes requiring Notch. This type of screening is useful because enhancer trap collections can include genes that result in lethality when mutated. The neurogenic genes were originally found to be involved in oogenesis through just such a screen (Ruohola *et al.*, 1991).

For the same reasons, it would also be informative, and possibly less time consuming, to analyze existing *Drosophila* mutations in components of the Notch pathway. For example, little is known about *neuralized*, and further characterization of its mutant phenotype in processes in which Notch and Delta are known to act, such as stalk formation and differentiation of the follicle cells surrounding the egg chamber, could shed light on whether *neuralized* always functions in Notch signaling events, and its role in these events. There are several other *Drosophila* mutations in genes that participate in Notch signaling, including: *bigbrain*, *deltex*, *Enhancer of split*, *groucho*, *mastermind*, *Suppressor of hairless*, *brainiac*, *canoe*, *kuzbanian*, *scabrous*, *shaggy*, and *strawberry notch* (Flybase). Examination of the role (if any) of *strawberry Notch* in these processes could prove informative. Flies mutant for of *strawberry Notch* exhibit phenotypes in those Notch-dependent processes that involve induction, but not in those that involve lateral inhibition, leading to the hypothesis that the specificity for inductive versus lateral signaling may be conferred by activation of Strawberry notch (Majumdar *et al.*, 1997). This could help discern the role of Notch signaling in the processes that I have discussed. Studies such as these could aid greatly in analysis of the mechanism of Notch signaling in oogenesis.

There is still much to be learned about the Notch pathway in the control of germ-line division, and it is particularly interesting since it appears that Notch may act by a different mechanism than induction or lateral specification. Moreover, it appears either that there is a signal from the germ line to the soma and a subsequent a signal from the soma to the germ line, or that Neuralized is acting downstream of Notch. Careful dissection of the components involved in this process and the manner in which they interact could elucidate whether the Notch pathway is acting by a novel mechanism in the control of germ-line division in *Drosophila* (and perhaps other organisms), and how the soma and germ line interact in this process. To begin with, I would generate marked *Notch* clones to determine which follicle cells require the Notch receptor for this process, to learn which follicle cells interact with the germ line in this process. I would also analyze the follicle cells in Notch pathway mutants with different follicle cell markers, such as markers for the terminal filament cells, the tip cells, or the inner sheath cells, to determine whether mutations in the Notch pathway result in any cell fate changes as found in other Notch mutant situations. I would next investigate the requirement for Delta and Serrate, the two known ligands for Notch in *Drosophila* development. Preliminary experiments suggest that Delta is not required in this process. There has been no reported requirement for Serrate during *Drosophila* oogenesis, so it could prove worthwhile to investigate the role of the ligands in this process. We could find either that Serrate is required during oogenesis, or alternatively that some other ligand is involved in this process. One could also investigate whether Delta and Serrate are redundant during oogenesis. In addition, determination of which cells require the ligand, germ line or somatic follicle cells, could help determine the direction that the pathway is signaling.

It is interesting that over-expression of the ligand can generate the same phenotype as expression of constitutively-active Notch in formation of the stalk. Is the ligand the limiting factor in stalk formation, or is Delta affecting the expression of Notch protein through a feedback loop - as has been suggested by other studies (Heitzler and

Simpson, 1991; Huppert *et al.*, 1997)? I was unable to determine with antibody stainings whether over-expression of Delta was generating a change in the expression of Notch. It may be possible to detect a difference in the level of Notch expression in a Western blot, particularly if only the early stages of oogenesis are tested.

One of the issues remaining in cell signaling and pattern formation is how different signaling pathways involved in specification events interact and if so to what level. There is interest in the interplay between the Notch and Egfr pathways since both are involved in so many specification events and are highly conserved (reviewed by Duffy and Perrimon, 1996). Both pathways are required in *Drosophila* oogenesis for specification of follicle cells and establishment of the oocyte anterior-posterior axis. Our studies with the Notch pathway have suggested two possible models for how the two pathways interact in these follicle cells. In the first model, Notch signaling is required for anterior fate and is repressed in the posterior follicle cells by the Egfr pathway, resulting in posterior fate. The second model suggests that prolonged Notch signaling in the posterior interferes with the proper developmental program in the posterior follicle cells (by retaining them in a "precursor" stage longer than usual), interfering with their ability to receive the Egfr signal - thus causing them to resort to the default anterior fate. Differentiating between these two models would not only shed light on the determination of follicle cells fates required for polarity of the oocyte but could possibly indicate a general mechanism for the interaction of these two pathways. Given the ease of genetic manipulations in *Drosophila*, it seems simplest to test this interaction genetically.

In the early stages of oogenesis, a microtubule organizing center (MTOC) is located in the posterior end of the oocyte and extends microtubules into the nurse cells. Later in oogenesis, the MTOC disappears from the posterior and microtubules begin to nucleate from the anterior of the oocyte. In mutants in which there is a defect in the patterning of the posterior follicle cells, MTOCs are detected at both the anterior and posterior ends of the oocyte. It is currently unknown whether a signal from the posterior

follicle cells is required in the oocyte to stabilize the posterior MTOC until stage 6-7, or destabilize the MTOC after stage 6-7. Future experiments could investigate whether the signal is involved in stabilization or destabilization. This type of analysis might prove difficult in *Notch* or *Delta* mutants. If the role of the Notch pathway is indeed the same in the later stages of oogenesis as it is during stalk formation, the defects in the anterior-posterior axis would most likely be the result of retaining the posterior follicle cells in an early stage of differentiation. If the signal from the follicle cells stabilizes MTOCs and must cease for the posterior MTOC to disintegrate, the immature cells may continue to signal, resulting in retention of the posterior MTOC. If the signal is primarily destabilizing, the cells may not be mature enough to signal and the posterior MTOC would once again be retained. Mutants that generate ectopic polar cells afford an excellent way to distinguish between these two models; ectopic expression of either *hedgehog* or *neuralized* results in ectopic polar cells. Double staining, with antibodies to Fasciclin III and Tubulin, could be carried out in these egg chambers. Fasciclin III antibody specifically recognizes polar cells, while tubulin recognizes microtubules, primarily recognizing high concentrations: the MTOCs. If the signal from the follicle cells is involved in destabilization of the MTOC, then one would predict microtubule deficits abutting the ectopic polar cells, while if the signal is involved in stabilization of the MTOC, microtubular concentrations are expected. Preliminary experiments suggest that there are high concentrations of microtubules abutting ectopic polar cells.

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APPENDIX A: Materials and Methods

STOCKS

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar medium at 25°C or at 18°C. The following fly strains were used: *Oregon R*, w^-, *GAL4(112A1) FM6*, *GAL4 109(3)9* (generated in the Jan laboratory), *HS-GAL4 CyO*, *UASlacZ CyO*, *UASlacZ FM6* (Brand and Perrimon, 1993), *UAStau-GFP(5.8)* on the second chromosome (Brand, 1995), *93F TM3* (Ruohola *et al.*, 1991), *KL503 TM3* (Clark *et al.*, 1994), *A101 TM3* (Bier *et al.*, 1989), *UASΔEN*-lines: *B33c-3 TM3*, *B2a-2 CyO* and *B2a-3 TM3* (for details of the construct see Fuerstenberg and Giniger, in preparation), *Act5C>y⁻>Notch(intra)#2 and#3* (*=N(intra)*) and a line carrying *P[ry⁻ hsp70-flp]* on the X-chromosome (generous gifts from Gary Struhl; Struhl *et al.*, 1993), *GAL4-107c1* (generated in the Jan laboratory), *GAL4-A62* (Gustafson and Boulianne, 1995), *GAL4-c709* (a gift from L. Manseau), w^-Notch^{ts1}, $Delta^{vial}$, $Delta^{6B37}$, *L53b* (González-Reyes *et al.*, 1995), *UAS-Delta*^{20} *CyO*, *UAS-Delta*^{33} *FM6* (a gift from E. Giniger; Doherty *et al.*, 1996), *5A7*, *BB127* (Roth *et al.*, 1995), neu^{KV9} *TM3*, neu^{9B9} *TM3* (L. and Y. Jan), *yw; P[ry⁻, hs-neo, FRT]*^{82B}, neu^{KV9} *TM3*, *yw; P[ry⁻, hs-neo, FRT]*^{82B}, neu^{9B9} *TM3*, *yw; P[ry⁻, hs-neo, FRT]*^{82B}, ovo^{D1} *3R TM3*, *P[ry⁻, hs-neo, FRT]*^{82B}, *tubulinFRTlacZ*^{84} *TM3* (Harrison and Perrimon, 1993), $hsFLP^{1369}$ *FM7*, neu^{R121} *TM3* (generated in the Jan lab), *XX; y w f/ ovo*^{D1}*v P[ry⁻, hs-neo, FRT]*^{101}w^-; FLP^{38} FLP^{38}, w^9N^{55e11} *P[ry⁻, hs-neo, FRT]*^{101}w^- *FM6,y w ct B/ wY 0*, *y w v P[ry⁻, hs-neo, FRT]*^{101}, $bam^{\Delta 86}$ *TM3* (McKearin and Ohlstein, 1995).

HEAT SHOCK TREATMENT

To induce constitutively-active Notch expression from the *UASΔEN* construct, *UASΔEN(33c-3)* was crossed to *HS-GAL4*, progeny were raised at 18°C and heat shocked to induce expression of GAL4 by the hsp70-promoter. For adult heat shocks, glass vials containing 3-4 day old *HS-GAL4 -;UASΔEN(33c-3) -* animals were incubated in a 39°C water bath for 15 minutes (or 37°C for 30 minutes). After heat shocks, the animals were returned to 18°C for the appropriate times.

To induce expression of the intracellular form of N (*N(intra)*, a FRT>y^+>FRT cassette was excised between an actin promoter and the *N(intra)* construct by heat shock induced expression of the *flipase* gene from the hsp70 promoter. To achieve this, Ni^{ts2} Ni^{ts2};Ni^{ts3} Ni^{ts3} was crossed to the *hsp70flp hsp70flp* animals and the progeny were raised at 25°C. The progeny were heat shocked at 37°C for 30 min (adults) or at 39°C for one hour (late pupae). After heat shocks the animals were returned to 25°C for the appropriate times. Both adult and pupae-heat shock paradigms will result in a *caN-intermediate* phenotype at early time points (1-2 days) and a *caN-strong* phenotype at late time points (6-7 days).

To induce Delta expression from the *UAS-Delta* construct, *UAS-*Dl^{33} was crossed to *HS-GAL4* and progeny were raised at 25°C and heat shocked to induce expression of GAL4 by the hsp70-promoter. To generate long stalks, glass vials containing 1-2 day old *UAS-*$Delta^{33}$*; HS-GAL4* animals were incubated in a 37°C water bath for 15 minutes on day one, 15 minutes on day two, and dissected on day three or day four. A higher percentage of extreme long stalks (70% as compared to 22%; Fig. 8) was generated by dissecting on day four. To generate defects in the A-P axis, flies were incubated in a 37°C water bath for 25 minutes, and dissected the next day.

To test whether over-expression of other proteins using the GAL4/UAS system with our protocols leads to the formation of long stalks, we analyzed ovarioles over-expressing Pointed P2 or Derailed using the same GAL4-system and heatshock conditions. Over-expression of these proteins does not generate long stalk-like structures.

IMMUNOCYTOCHEMISTRY, *IN SITU* HYBRIDIZATION AND OTHER STAINING PROCEDURES

Immunocytochemistry, *in situ* hybridization, and X-Gal staining procedures were performed as described earlier (Ruohola *et al.*, 1991), with the following minor modifications. For immunocytochemistry and *in situ* hybridizations the ovaries were dissected in PBS, fixed for one hour in 4% paraformaldehyde/PBS/0.1% DMSO, dehydrated and stored at -20°C. For X-Gal staining the ovaries were dissected in PBS, fixed in 2% glutaraldehyde/PBS for 5-7 min, rinsed with PBS and incubated in 0.2% X-Gal solution in Fe/NaP buffer at 37°C overnight. Samples were washed in PBS, fixed for 20 minutes in 4% paraformaldehyde/PBS, and mounted in glycerol.

The antibodies used in this study were Mab22C10 (Zipursky *et al.*, 1984), anti-Fasciclin III (1:15 dilution, Ruohola *et al.*, 1991), anti-65F (1:4000 dilution), anti-Big Brain (1:1500; D. Doherty, 1995, thesis, UCSF and Doherty *et al.*, 1997), anti-Notch (intracellular domain, 1:1000), anti-α–tubulin (1:250), anti-Delta (1:3000; Bender *et al.*, 1993), and anti-Staufen (1:3000; St Johnston *et al.*, 1991; a gift from Daniel St Johnston). The DNA plasmids used for *in situ* hybridization probes were pGEM3bcd (Driever *et al.*, 1990), *BSK-derailed* (Callahan *et al.*, 1995), and BSKS-osk (Ephrussi *et al.*, 1991).

PHALLOIDIN/PROPIDIUM IODIDE STAINING

Ovaries were dissected into 1X PBS. They were then fixed in 4% paraformaldehyde/PBS for 20 minutes to one hour. After going through an ethanol series, ovaries were frozen at -20°C until ready to use. Ethanol was replaced with 1X PBS, and then phalloidin-flourescein was added at 1:40, and incubated overnight at 4°C. Ovaries were then washed 3X in PBT, then RNase A was added at 0.5 mg/ml for 30 minutes at room temperature. After 3 washes with PBT at 5 minutes each, propidium iodide was added at 1 μg/ml for 10 minutes. Ovaries were then washed 3X, 5 minutes with PBS and mounted in 70% glycerol.

UAS-DELTA WITH LOSS OF *NOTCH* FUNCTION

To examine the effects of *UAS-Delta* expression in a loss of *Notch* function background, the flies (N^{ts} N^{ts};*UAS-Dl*^{20} *HS-GAL4*) were heatshocked at 39°C for 15 minutes, allowed to recover at room temperature for thirty minutes, placed at 32°C for 16 hours, and allowed to develop at room temperature for 32 hours before dissection.

MICROTUBULE/STAUFEN IMMUNOCYTOCHEMISTRY

Ovaries were dissected in 1xRobbs and then transferred immediately to 100% methanol (room temperature). To fix the ovaries, methanol was exchanged for -20°C methanol and the samples were kept at -20°C for 10 minutes. Ovaries were rinsed twice with Phosphate Buffered Saline (PBS) and incubated in PBS with 1% saponin at room temperature for 2 hours. After a wash in PBSS (PBS and 0.1% saponin) the samples were incubated with anti-α-tubulin (1:250, Sigma) and anti-Staufen (1:3000) in PBSS for 48 hours at 4°C. After rinsing 3X 15min with PBS, the samples were incubated with Texas Red goat anti-mouse and FITC BODIPY goat anti-rabbit secondary antibodies

(each at 1:200; Molecular Probes) in PBSS overnight at 4°C. After rinsing 4X 15 minutes in PBSS, the samples were mounted in glycerol with 2% N-propyl gallate.

CUTICLE PREPARATION

Embryo cuticles were prepared by washing them thoroughly with water, then placing them in 50% bleach for 3 minutes. Bleach was removed by washing several times with a solution of 0.8% NaCl, 0.1% Triton X-100. Embryos were then transferred to a tube containing 700 µl of PEMFA (100mM Pipes [pH 6.5], 2 mM EGTA, 5 mM $MgSO^4$, 1% formaldehyde) and 700 µl of heptane and rotated for 30 minutes at room temperature. The lower phase was removed, 700 µl of methanol was added, and the mixture was vortexed for 15 seconds. The overlying liquid was then removed, and the embryos were washed 3 times with 700 µl methanol. The methanol was replaced with 4:1 acetic acid:glycerol, and the tube was incubated at 65°C o/n. The solution was then diluted 1:1 with Hoyer's mountant, transferred to a microscope slide, covered with a cover slip, and left on a warming tray o/n. Alternatively, embryo cuticles were prepared without devitellization. They were prepared by cleaning them thoroughly with water, placing them on a slide with 1:1 Hoyer's : lactic acid and incubating at 65°C o/n.

WESTERN BLOT ANALYSIS

The ovary samples were prepared as described earlier (Markussen *et al.*, 1995) and run on 7% SDS-PAGE gels with 3.5% stacking gels. Proteins were transferred to a 0.45µm Protran nitrocellulose membrane (Schleicher and Schuell) and probed with polyclonal antibody to the intracellular domain of Notch (1:3000 dilution). Antibodies were visualized by chemiluminescence using a goat-anti-rabbit Ig(H+L) secondary antibody conjugated to alkaline phosphatase and detecting with the chemiluminescent substrate, Lumigen PPD (Boehringer Mannheim).

MICROSCOPY

Microscopy was performed on a Leitz DMRB with Nomarski differential interference contrast. Confocal images were collected using the MRC 600 laser scanning confocal microscope system (Bio-Rad Microsciences Division) using the 488 and 568 wavelengths of a krypton argon laser.

CYTOPLASMIC STREAMING ASSAYS

Bulk cytoplasmic movements within living oocytes were assayed as follows: Adult females of genotype *B2A3/109(3)9* were transferred to a slide and covered with halocarbon oil (700). Ovaries were removed, and dissected into separate ovarioles under oil. The slide was then transferred to a confocal microscope and yolk granules were imaged using the fluorescein filter set. Single frame images were collected in 10 second intervals for 100 seconds (10 images total). Time lapse images were created by projecting the time series into a single image. For the time-lapse video microscopy the egg chambers were viewed on a Leitz DMRB microscope with Nomarski optics and recorded with a AIMS CV-252 CCD camera, a Boeckeler IMG-100 image processor and a Mitsubishi HS-S5600U time lapse video recorder at the rate of one frame every 8 seconds. To analyze the streaming movement the images were projected on a screen (Sony PVM-135) at 30 frames per second (240x).

APPENDIX B: Expression of Constitutively-active Notch Arrests Follicle Cells at a Precursor Stage During *Drosophila* Oogenesis and Disrupts the Anterior-Posterior Axis of the Oocyte.

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Expression of constitutively active Notch arrests follicle cells at a precursor stage during *Drosophila* oogenesis and disrupts the anterior-posterior axis of the oocyte

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SUMMARY

During early development, there are numerous instances where a bipotent progenitor divides to give rise to two progeny cells with different fates. The *Notch* gene of *Drosophila* and its homologues in other metazoans have been implicated in many of these cell fate decisions. It has been argued that the role of Notch in such instances may be to maintain cells in a precursor state susceptible to specific differentiating signals. This has been difficult to prove, however, due to a lack of definitive markers for precursor identity. We here perform molecular and morphological analyses of the roles of Notch in ovarian follicle cells during *Drosophila* oogenesis. These studies show directly that constitutively active Notch arrests cells at a precursor stage, while the loss of Notch function eliminates this stage. Expression of moderate levels of activated Notch leads to partial transformation of cell fates, as found in other systems, and we show that this milder phenotype correlates with a prolonged, but still transient, precursor stage. We also find that expression of constitutively active Notch in follicle cells at later stages leads to a defect in the anterior-posterior axis of the oocyte.

Key words: constitutively active Notch, precursor, cell fate, oogenesis, axis, *Drosophila*

INTRODUCTION

In *Drosophila*, the establishment of both the anterior-posterior (A-P) and dorsal-ventral (D-V) body axes takes place during oogenesis. The establishment of this polarity requires signaling between the oocyte and the somatic follicle cell layer that surrounds it. In particular, the asymmetric localization of morphogens in the oocyte depends on interactions between the oocyte and subpopulations of specialized follicle cells. The discrimination of cell types among the somatic follicle cells is thus a committed event leading to the specification of embryonic polarity.

The follicle cell subpopulation that is necessary for establishment of the A-P axis is the posterior follicle cell group (Ruohola et al., 1991; Gonzalez-Reyes et al., 1995; Roth et al., 1995; reviewed in Rongo and Lehman, 1996). Recent experiments have shown that signaling from the posterior follicle cells is required for polarization of the oocyte microtubule cytoskeleton (Clark et al., 1994; Lane and Kalderon, 1994). Polarization of this cytoskeleton is necessary for proper localization of anterior and posterior morphogens (Pocrywka and Stephenson, 1991; Clark et al., 1994; Lane and Kalderon, 1994). It is therefore important to determine how the posterior follicle cells are defined.

Three signaling pathways are known to be required for differentiation of subgroups of follicle cells: the EGF receptor pathway, the Notch pathway and the Hedgehog pathway (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Ruohola et al., 1991; reviewed in Rongo and Lehman, 1996; Forbes et al., 1996). The EGF receptor pathway transmits inductive information from the oocyte to the follicle cells, while the Hedgehog and Notch pathways act in communication among the follicle cells. Notch functions in determining two follicle cell populations: the posterior follicle cells and the stalk cells which separate the egg chambers from one another and from the germarium, the structure in which the germ-line divisions take place. In Notch loss-of-function mutants, both expansion of the posterior follicle cell field and loss of stalk cells are detected. The expansion of the posterior follicle cell field correlates with a defect in the establishment of the A-P polarity of the oocyte.

Despite the wealth of information about the loss-of-function phenotype of Notch in oogenesis, the precise role of Notch activity remains obscure. Notch is a transmembrane receptor which functions in a wide variety of cell fate specification events in multiple species. The mechanism by which Notch signaling mediates these cell fate choices is under investigation. We have addressed this question by studying the effects

produced by activation of Notch function in follicle cells during oogenesis. For this purpose, we have taken advantage of Notch derivatives that uncouple intracellular signal transduction from ligand binding. Specifically, recent studies have shown that a fragment of Notch lacking the extracellular domain is constitutively active, regardless of the presence or absence of ligand. Persistent or transient ubiquitous expression of activated Notch in *Drosophila* causes an overproduction of epidermis at the expense of neural structures (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993), a phenotype opposite to that seen with the loss-of-function allele of *Notch* (Poulson, 1940; Lehmann et al., 1983; Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991). Activated Notch causes defects in developmental processes in *C. elegans* (Roehl and Kimble, 1993), *Xenopus* (Coffman et al., 1993), chicken (Austin et al., 1995) and humans (Ellisen et al., 1991), as well as *Drosophila* eye development (Fortini et al., 1993). Absence or delay of normal differentiation in some of these cases has led to the proposal that ongoing Notch activity prevents competent cells from undergoing differentiation in vivo. However, this has been hard to prove due to the lack of definitive molecular markers for the precursor stage.

Here we show directly by molecular and morphological markers that, in early oogenesis, constitutively active Notch arrests follicle cells in a specific precursor stage blocking the development of both stalk cells and polar cells. Loss of Notch function eliminates this precursor stage. In a later stage of oogenesis, activation of Notch in follicle cells leads to aberrant anterior-posterior axis formation.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar medium at 25°C or at 18°C. The following fly strains were used: *Oregon R*, *w*; *GAL4(112A1)/FM6*, *GAL4 109(3)9* (generated in the Jan laboratory), *HS-GAL4/CyO*, *UASLacZ/CyO*, *UASLacZ/FM6* (Brand and Perrimon, 1993), *UAStau-GFP(5.8)* on the second chromosome (Brand, 1995), *93F/TM3* (Ruohola et al., 1991), *KL503/TM3* (Clark et al., 1994), *A101/TM3* (Bier et al., 1989), *UAS-ΔEN* lines: *B33c-3/TM3*, *B2a-2/CyO* and *B2a-3/TM3* (for details of the construct see Fuerstenberg and Giniger, in preparation), *Act5C>y+>Notch(intra)#2* and *#3* (=*N(intra)*) and a line carrying *P[ry+ hsp70-flp]* on the X-chromosome (generous gifts from Gary Struhl, Struhl et al., 1993).

To construct *UAS.ΔEN* lines, *ΔEN* was placed immediately downstream of the GAL4 upstream activating sequence (*UAS*, Brand and Perrimon, 1993) and introduced into flies using P-element-mediated transformation. To verify that *ΔEN* acts similarly to other published extracellular deletion constructs, we investigated the effect of *ΔEN* expression in neurogenesis. *UAS.ΔEN* was crossed to a line that expresses GAL4 protein in embryonic epidermis (*GAL4(112)*). The embryos from this cross were stained with mAb22C10 antibody to visualize the neurons (Zipursky et al., 1984). Fewer neurons were detected in *ΔEN* embryos than in control embryos (compare Fig. 1B to 1A). Since the phenotype of loss-of-function *N* mutations is hyperplasia of neurons, *ΔEN* behaves as expected for a gain-of-function mutation in neurogenesis. Western blotting analysis showed that this construct produced a truncated form of Notch in ovaries (data not shown).

Heat-shock treatment

To induce constitutively active Notch expression from the *UAS.ΔEN* construct, *UAS.ΔEN(33c-3)* was crossed to *HS-GAL4*, progeny were raised at 18°C and heat shocked to induce expression of GAL4 by the hsp70-promoter. For adult heat shocks, glass vials containing 3-4 day old *HS-GAL4/+*; *UASΔEN(33c-3)/+* animals were incubated in a 39°C water bath for 15 minutes (or 37°C for 30 minutes). After heat shocks, the animals were returned to 18°C for the appropriate times.

To induce expression of the intracellular form of N (*N(intra)*), a FRT>y+>FRT cassette was excised between an actin promoter and the *N(intra)* construct by heat-shock-induced expression of the *flipase* gene from the hsp70 promoter. To achieve this, Ni^{ts2}/Ni^{ts2}; Ni^{ts1}/Ni^{ts1} was crossed to the *hsp70flp/hsp70flp* animals and the progeny were raised at 25°C. The progeny were heat shocked at 37°C for 30 minutes (adults) or at 39°C for 1 hour (late pupae). After heat shocks the animals were returned to 25°C for the appropriate times. Both adult and pupal heat-shock paradigms will result in a *caN-intermediate* phenotype at early time points (1-2 days) and a *caN-strong* phenotype at late time points (6-7 days).

Immunocytochemistry, in situ hybridization and other staining procedures

Immunocytochemistry, in situ hybridization and X-Gal-staining procedures were performed as described earlier (Ruohola et al., 1991), with the following minor modifications. For immunocytochemistry and in situ hybridizations, the ovaries were dissected in PBS, fixed for 1 hour in 4% paraformaldehyde/PBS/0.1% DMSO, dehydrated and stored at −20°C. For X-Gal staining, the ovaries were dissected in PBS, fixed in 2% glutaraldehyde/PBS for 5-7 minutes, rinsed with PBS and incubated in 0.2% X-Gal solution in Fe/NaP buffer at 37°C overnight. Samples were washed in PBS, fixed for 20 minutes in 4% paraformaldehyde/PBS and mounted in glycerol.

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with a AIMS CV 252 CCD camera, a Boeckeler IMG-100 image processor and a Mitsubishi HS-S5600U time lapse video-recorder at the rate of one frame every 8 seconds. To analyze the streaming movement, the images were projected on a screen (Sony PVM-135) at 30 frames per second (240×).

RESULTS

Constitutively active Notch constructs and experimental design

Two constructs of *Notch* (*N*) were used in these studies in order to express constitutively active N either persistently or transiently: *Ntmtra)* and *ΔEN. Ntmtra)* consists of the intracellular domain of N, separated from an actin promoter by a cassette which is surrounded by two Flipase recognition target (FRT) sites and contains a stop codon. Flies carrying this construct were crossed to flies carrying flipase (flp) downstream of an hsp70 promoter. Progeny were heat shocked, resulting in a pulse of flp expression which excises the cassette and allows Ntmtra) to be expressed under the control of the actin promoter (for details see Experimental Procedure and Struhl et al., 1993). This construct has been shown to act like a gain-of-function mutation of *N* (Struhl et al., 1993).

ΔEN expression under the control of GAL4 upstream activating sequence (UAS, Brand and Perrimon, 1994) leads to production of a protein fragment comprising the last four amino acids of the extracellular domain of Notch, the transmembrane domain, and the amino-terminal half of the intracellular domain, including the SuH) interacting domain and the CDC10/ankyrin repeats. This construct is expected to behave as a constitutively active form of Notch based on earlier studies (Lyman and Young, 1993; Coffman et al., 1993; Fortini et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Roehl and Kimble, 1993; Kopan et al., 1996), and this has been verified by analyzing its phenotype in embryonic neurogenesis (Fig. 1A,B).

Functionally, the significant difference between *ΔEN* and *Ntmtra)* is that excision of the FRT cassette from

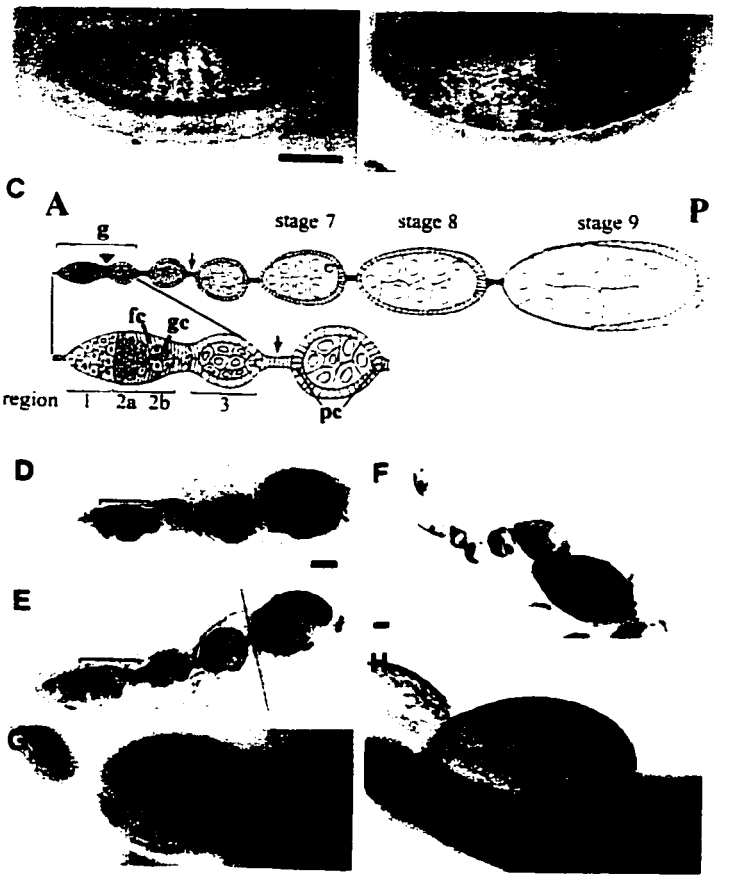

Fig. 1. (A,B) *UAS.ΔENotch* construct behaves as a gain-of-function mutation in neurogenesis. The embryonic neurons are visualized by mAb22C10 antibody staining in (A) control *GAL4-112A* or *+* or *Y*) and (B) mutant *GAL4-112A* or *Y; UAS-ΔEN33c-3A* embryos. Fewer neurons are detected in the embryos expressing ΔEN due to GAL4 expression in embryonic epidermis by the GAL4-line *112A* than in the control embryos indicative of an anti-neurogenic phenotype. (C) Developmental sequence of oogenesis: diagram of an adult wild-type ovariole. *Drosophila* oogenesis is divided into 14 stages (stages 7, 8 and 9 indicated). The anterior-most structure, the germarium (g), contains the stem cells for germ line and the somatic follicle cells. The germarium is divided into four regions: in region 1 germ-line cells divide four times to produce a cyst of 16 germ cells (gc), 15 nurse cells and one oocyte. When the cyst has reached region 2, follicle cells (fc) migrate between two successive egg chambers and begin to intercalate, causing the region 3 egg chamber to 'pinch off' from the germarium (arrowhead). These cells then take the fate of either stalk cells (arrows) or follicle cells that surround the egg chamber, including the polar cells (pc). (D-F) *HS-GAL4* and (G-H) *GAL4-109(3)9* induce the expression of the target gene in follicle cells. One day after heat-shock induction (30 minutes, 37 C) β-gal expression was detected by X-Gal staining starting in region 2 in germaria of *HS-GAL4/UASLacZ* flies (D,E, brackets). Some variability in the staining pattern is detected which might account for variability in the penetrance of the phenotype. From the stage 7-8 on the expression of β-gal increased considerably (F). Patchy GAL4 expression detected in ovaries of *GAL4-109(3)9/UAS-LacZ* flies is first detected around stage 6 increasing by stage 9 in all of the follicle cells (G,H). Scale bars are equivalent to 20 μm. A and B are of the same magnification, D,E,G and H are of the same magnification.

between *N(intra)* and the actin promoter results in persistent expression of constitutively active N, while *ΔEN* is only transiently expressed as long as GAL4 is present.

To induce *ΔEN* expression in oogenesis, the flies carrying the *UASΔEN* construct were crossed either to flies expressing GAL4 under heat-shock control (*HS-GAL4*) or to the enhancer trap GAL4 line, *109(3)9*. The GAL4 expression patterns in ovaries for both of these lines were examined by crossing them to a line carrying *UAS-lacZ* and staining the progeny with X-Gal, or by crossing them to *UAS-tau-GFP* and analyzing the GFP pattern by fluorescence microscopy. *HS-GAL4* induced *UAS-LacZ* expression in follicle cells beginning in region two of the germarium (Fig. 1D,E), whereas *109(3)9* induced follicle cell expression is not detected prior to stage 6 in oogenesis (Fig. 1G,H). No germ-line expression of GAL4 was detected (Fig. 1G-H; data not shown).

Expression of constitutively active Notch in the germarium follicle cells generates long stalk structures

Ovaries contain egg chambers in a developmental order. In the anterior-most region of the germarium, region 1, the germ-line stem cells produce cystocytes, which divide through incomplete cytokinesis to form 16 cell cysts. The cysts are surrounded by a follicle cell layer and released from the germarium by 'pinching-off' (Fig. 1C, arrowhead). This process results in the formation of two kinds of specialized follicle cells: stalk cells, which intercalate between each other to form the stalk that separates two successive egg chambers (Fig. 1C, arrows), and polar cells, which form a cap at the anterior end of the older egg chamber and the posterior end of the younger egg chamber (Fig. 1C, pc). In loss-of-function N^{ts} mutant ovaries, no stalk cells are detected at the restrictive temperature and the egg chambers never leave the germarium (Fig. 2E; Ruohola et al., 1991; Xu et al., 1992).

To address whether expression of constitutively active N could induce long stalks between the egg chambers, a phenotype opposite to the loss of stalks observed with N^{ts}, we expressed constitutively active N transiently in the germarial follicle cells using *HS-GAL4* induced expression of ΔEN. This leads to a dramatic phenotype in the early stages of oogenesis. Instead of a wild-type stalk of 6-8 cells (Fig. 2A, bracket), long stalk structures consisting of an average of 15 cells were detected (Fig. 2B, bracket).

The sequential nature of oogenesis allows us to study whether ovarioles that include abnormally long stalks continue to develop, and at what stage of development the length of both wild-type and long stalks is determined. If expression of ΔEN is induced by a 15 minute heat shock at 39°C and the flies are then allowed to develop for 24 hours at 18°C, long stalks are first detected between stages one and two (Fig. 2B, bracket). At this timepoint, long stalks are not observed between the later stages (Fig. 2B, arrowhead), indicating that the length of fully formed stalks is not altered. Previous studies have indicated

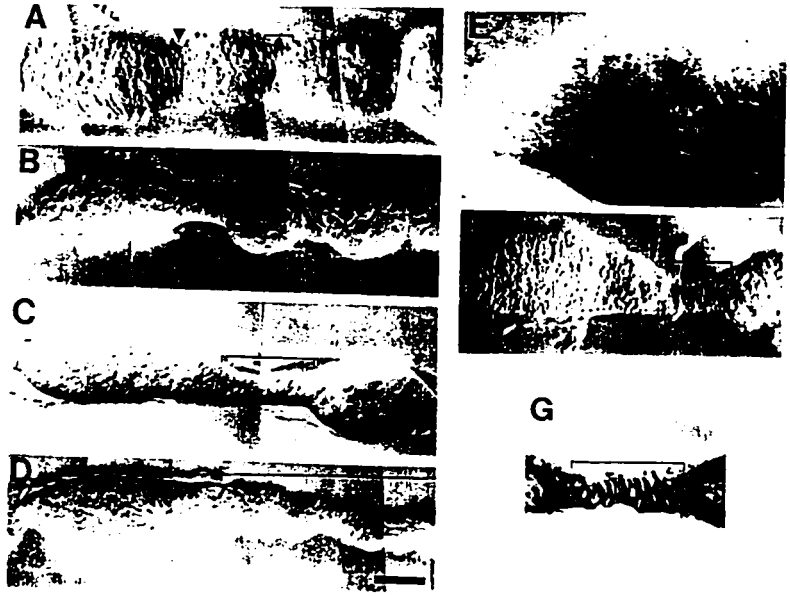

Fig. 2. Expression of constitutively active Notch generates long stalk-like structures; phenotypic series. (A) In wild-type ovary, a stalk forms in the germarium (arrowhead). A fully formed stalk consisting of 6-7 cells is detected between each egg chamber (bracket). (B) Transient expression of constitutively active N (*caN-mild*, 39°C, 15 min) caused long stalk formations two days (at 18°C) after induction (15.3 cells/stalk, bracket). A fully formed stalk was not affected (arrowhead). (C) Persistent expression of constitutively active N lead to even longer stalks when analyzed 24 hours (at 25°C) after the induction of expression (*caN-intermediate*, 22 cells, bracket). (D) Extreme stalks were detected after induction of constitutively active N when analyzed 6 days after heat shock (*caN-strong*, 101 cells, bracket). (E) No stalks are formed in N^{ts} (N^{ts}) ovaries allowed to develop at the restrictive temperature (one day at 32°C). (F) N^{ts}/N^{ts}; *UASΔEN/HS-GAL4*. The N^{ts} phenotype is suppressed by the expression of constitutively active N (*caN-mild*), suggesting that constitutively active N acts without endogenous N and that both mutations affect the same pinching-off event. (G) Constitutively active N (*UASΔEN*) was induced by HS-GAL4 expression due to two heat-shock pulses (39°C, 15 minutes and 39°C, 20 minutes) in a one day interval. The ovaries were dissected 2 days after the last induction and stained with Fasciclin III (Fas III). Fas III marks all the follicle cells during the pinching-off event and becomes restricted to the polar cells around stage 3 in oogenesis. The long stalk consists of 22 cells, indicating that longer stalks are detected with the *UASΔEN* construct after multiple heat-shock inductions. Scale bar is equivalent to 20 μm.

Table 1. Stalk is determined in the germarium

caN-mild	Position of long stalk	Total cells in stalk	% long stalks with 93F+ cells	% long stalks with A101+ cells	% A101-positive cells
39°C 15 minutes, 2 days	stages 2-3	15.3 (n=17)	77 (10/13)	79 (34/43)	18.3
39°C 15 minutes, 3 days	stages 3-6	16.5 (n=20)	79 (19/24)	73 (22/30)	15.5
39°C 15 minutes, 4 days	stages 6-8	16.5 (n=10)	82 (14/17)	79 (49/62)	11.5
37°C 30 minutes, 2 days	stages 2-3	11.8 (n=4)	NA	64 (7/11)	8.5
37°C 30 minutes, 3 days	stages 3-6	12.3 (n=10)	NA	64 (16/25)	11.3

NA= not analyzed

that a new egg chamber pinches off every 24 hours at this temperature (10-12 hours at 25°C, King, 1970; Margolis and Spradling, 1995). Since long stalks are detected between stages 1 and 2 only 24 hours after induction of ΔEN, this suggests that constitutively active N acts during or just before the beginning of the pinching-off event and has no effect on fully formed stalks. The expression pattern of *HS-GAL4* under these conditions indicates that it induces target gene expression in the follicle cells at regions 2 and 3 of the germarium (Fig. 1D-F), the region where pinching-off occurs. 48 hours after the induction of constitutively active N, the long stalks have progressed and are present between stage 2 and 3 egg chambers (Fig. 3B) and, after three days, the long stalk has moved from the germarium region to the region between stages 3 and 6 in oogenesis (Fig. 4B). The long stalks detected in the later stages are of the same average length as those seen earlier, indicating that the final number of cells in these long stalks is also determined in the germarium (Table 1).

To address whether constitutively active N affects the same developmental process as the loss-of-function mutation, we determined the phenotype produced when activated Notch is expressed in the background of N^{ts}. Only the long stalk phenotype of constitutively active N was detected in these ovaries (Fig. 2F, bracket). This suggests that, in stalk formation, activated Notch affects the same process as the wild-type protein and, further, that constitutively active N can act without endogenous N. Similar results have been seen in other systems, such as the nervous system (Struhl et al., 1993).

Long stalk structures were also observed when the flp-FRT system was used to drive expression of constitutively active N (Struhl et al., 1993). 24 hours after heat-shock induction of flipase, long stalk structures were detected (Fig. 2C, bracket). The long stalks observed one day after heat-shock induction resembled the long stalks detected after GAL4-induced expression. However, the penetrance of the phenotype was higher and the stalks were longer (Table 2), indicating a stronger phenotype either due to the different *N* construct or the experimental procedure. Multiple heat-shock inductions of *ΔEN*, in order to avoid the transient nature of GAL4 expression, result in longer stalks, which resemble those generated by *N(intra)* (Fig. 2G, bracket). This indicates that longer stalks can be generated by longer expression of *ΔEN* and the stronger phenotype of *N(intra)* is most likely due to the fact that it is expressed persistently.

To examine whether the long stalks generated by *N(intra)* also progressed through oogenesis, we looked at ovaries at later time points. 2 days after heat shock, the long stalk had progressed to between stages 3 and 6, and was usually followed by a second long stalk. 6 to 7 days after heat shock, extremely long stalk structures were detected (Table 2; Fig. 2D, bracket), which have presumably continued to grow for 3 days since the heat shock. Since they are detected at a later time point, these extremely long stalks could be due to a stem cell carrying *N(intra)* under the control of the actin promoter, leading to progeny that all express constitutively active N. The lengths of stalks generated in (1) *ΔEN* (37°C, 30 min or 39°C, 15 min), 2 days after induction, (2) *N(intra)*, 1 day after induction and (3) *N(intra)*, 6-7 days after induction suggest that the three treatments constitute a phenotypic series of increasing severity. These three treatments are referred to as *caN* (for constitutively active Notch)-*mild*, *caN-intermediate* and *caN-strong*, respectively, throughout the remainder of the paper.

In addition to varying lengths of stalk structures, the experimental procedures generated varying cellular morphology within the long stalks. The cellular morphology of *caN-mild* stalks resembles that of a wild-type stalk: a single row of aligned, disc-shaped cells (Fig. 2B, bracket; and Fig. 1C, arrow). Cells in the *caN-intermediate* and *caN-strong* stalks are not aligned in a single row, the stalks appear much thicker than wild-type stalks and the cells are organized as several aligned columns of cells (Fig. 2C,D). This morphology is reminiscent of the cellular organization observed earlier in the germarium

Table 2. Expression of constitutively active Notch: phenotypic series

Constit. active N construct	Heat shock conditions and survival time	Title	Penetrance (% long stalks)	Average length of stalk	Percent of stalks with A101+ cells	Percent of A101+ cells in stalk
-	-	Control	0	6 cells	0	0
ΔEN	37°C 30 minutes, 2 days at 18°C	*caN-mild*	69 (11/16)	12 cells (4)	64 (7/11)	8.5
ΔEN	39°C 15 minutes, 2 days at 18°C	*caN mild*	81 (43/55)	15 cells (17)	79 (34/43)	18
N(intra)	37°C 30 minutes, 1 day at 25°C	*caN intermediate*	94 (44/47)	22 cells (17)	98 (43/44)	45
N(intra)	39°C 1 hour, 6 days at 25°C	*caN strong*	100 (48/48)	101 cells (25)**	-	-
				38 cells (25)	0	0

*The controls were heat shocked under the same conditions as the constitutively active Notch constructs.

**The full length of these stalks is an average of 101 cells. A101 positive cells are found at the posterior end of some stalks, so the average length of stalk which does not contain any A101-positive cells is 38. In the most severe cases, none of the cells are A101 positive.

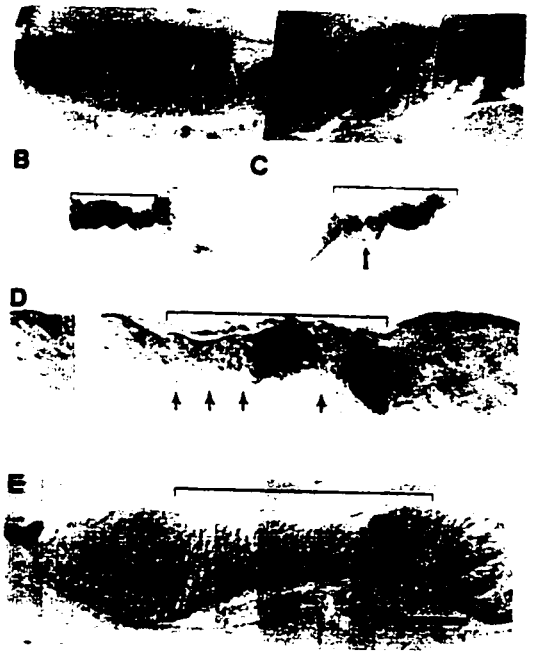

Fig. 3. A stalk cell fate marker is expressed in *caN-mild* and *caN-intermediate* stalks, but not in *caN-strong* stalks. (A) The *93F* enhancer trap line stains the terminal filament cells and stalks (brackets). (B,C) *caN-mild* three days (at 18 °C) after the heat shock (39 °C, 15 minutes), two groups of long stalks were detected (brackets). 21% of the long stalks consisted entirely of *93F*-positive cells (B, bracket), indicating that all of the cells in long stalks had taken the stalk cell fate. However, 79% of the long stalks consisted of *93F*-positive (C, bracket) and *93F*-negative cells (C, arrow). (D) *caN-intermediate*: extra *93F*-positive and *93F*-negative (arrows) cells are detected in long stalks (bracket). (E) *caN-strong*: the extremely long stalks (bracket) do not show *93F* staining. In some cases, a few *93F*-positive cells are seen at the very posterior end of the long stalk in the cells adjacent to the egg chamber (data not shown). Scale bar is equivalent to 20 μm.

at the time of the pinching-off event, before the cells have begun to intercalate (Fig. 2A, arrowhead; Fig. 1C, arrowhead).

A stalk cell fate marker is expressed in *caN-mild* and *caN-intermediate* stalks, but not in *caN-strong* stalks

To determine whether more cells had actually taken the fate of stalk cells, we examined the identity of the cells in the long stalks with *93F*, an enhancer trap line that is expressed in stalk cells (Fig. 3A; Ruohola et al., 1991). *93F* expression was analyzed in *caN-mild*, *caN-intermediate* and *caN-strong* backgrounds. In control ovaries, all stalk cells were *93F* positive (Fig. 3A, brackets). In the case of *caN-mild*, some long stalks were detected that consisted entirely of cells expressing the stalk cell marker (Fig. 3B), consistent with the prediction that more cells become stalk cells with expression of *caN*. However, the majority of the long stalks (mean value 43/54, 80%, Table 1) contained a group of cells that did not express the stalk cell marker, despite being located in the stalk structures (Fig. 3C).

In *caN-intermediate*, the long stalks also contained more *93F*-positive stalk cells than are contained in normal stalks, indicating that additional cells had taken the fate of stalk cells. However, many *93F*-negative cells were also detected in these long stalks (Fig. 3D). The number of cells that did not stain with the stalk cell marker also increased in number from *caN-mild* to *caN-intermediate*. Strikingly, no *93F*-positive stalk cells were detected in the extreme long stalks induced by *caN-strong* (Fig. 3E, bracket), indicating that none of the cells in these 'stalks' had taken the fate of stalk cells. Therefore, three different kinds of long stalk-like structures can be generated by the expression of constitutively active N: those that consist entirely of *93F*-positive stalk cells, those that consist of *93F*-positive and *93F*-negative cells and those that contain only *93F*-negative cells.

A polar cell marker is expressed in *caN-mild* and *caN-intermediate* stalks but not in *caN-strong* stalks

To assess the identity of the cells in long stalks that did not stain with the stalk cell marker, we used a marker for a subpopulation of follicle cells that differentiates at the time of, or just prior to, stalk formation: the *A101* enhancer trap line. This mutation is a P-element insertion in the *neuralized* gene and shows a β-galactosidase-staining pattern similar to that seen in an in situ hybridization using a *neuralized* cDNA probe. β-galactosidase activity is first detected in some of the polar cell precursors at the time of stalk formation. By stage four of oogenesis, its expression is restricted to two anterior and two posterior polar follicle cells (Fig. 4A; Ruohola et al., 1991).

In the case of *caN-mild*, some long stalks were detected that contained no *A101*-positive cells (Fig. 4B). However, a large group of long stalks were detected that did have some *A101*-positive cells located within the stalk structures (Fig. 4C, Tables 1, 2). Based on the penetrance of the phenotype and the number of cells that express *A101*, it appears that the cells that express the polar cell marker likely correspond to those cells in the long stalks that did not express the stalk cell marker. There was no trend in the position of the *A101*-positive cells in the long stalk. In most cases, the normal number of polar cells was still found in the egg chambers surrounding the long stalks. However, in a few instances only one of the usual two polar cells was detected. *caN-intermediate* stalks had more *A101*-positive cells than *caN-mild* mutant stalks (Fig. 4D, bracket indicates stalk). However, long stalks in *caN-strong* ovaries had no *A101*-positive cells (Fig. 4D, bracket; Table 2). Based on the expression of *93F* and *A101*, three different kinds of long stalk-like structures can be generated by the expression of constitutively active N: those that consist entirely of stalk cells, those that consist of stalk cells and polar cells and those that do not contain either of these two cell types.

One possibility for the polar cells located in long stalks is that N function is required both for the initiation of stalk cell fate as well as the maintenance of this fate. According to this model, with transient expression of constitutively active N, the initiation of the stalk cell fate was induced in a group that normally should become polar cells. However, when the transiently expressed N was degraded, the cells return to their normal fate: polar cells. This is unlikely since polar cells are

detected in *caN-intermediate* stalks where N(intra) is expressed persistently. This model also predicts, in the case of transient expression of constitutively active N, that the longer the time period after heat shock, the more long stalks that have *A101*-positive cells would be detected. In addition, each long stalk should have more *A101*-positive cells the further they progress through oogenesis. As neither prediction is upheld (Table 1), it seems unlikely that Notch is required for the maintenance of stalk cell fate.

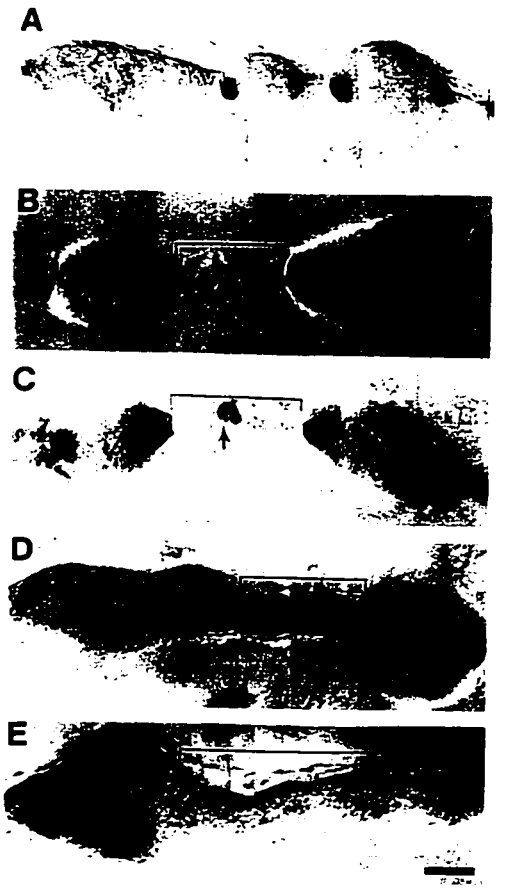

Fig. 4. A polar cell marker is expressed in *caN-mild* and *caN-intermediate* stalks but not in *caN-strong* stalks. (A) The *A101* enhancer trap line is a P-element insertion in the *neuralized* gene and induces β-gal expression in the follicle cells at the anterior tip of germarium, in polar cell precursors (5-6 cells at the anterior and posterior pole of the stage 1-3 egg chambers) and polar cells (two cells at the anterior and posterior pole of egg chambers). (B,C) Two groups of long stalks were detected in the *caN-mild* mutant ovaries 4 days after the heat-shock induction (39 C, 15 minutes) 21% of the long stalks did not stain with a polar cell marker *A101* (B, bracket). However, 79% of long stalks had 1-5 *A101*-positive cells in the stalk structures (C, arrow). (D) *caN-intermed*, 98% of long stalks contain cells that are *A101* positive. (E) *caN-strong*. The most severe long stalks do not contain any *A101*-positive cells. The stalk shown in E consists of 151 cells which do not express the marker *A101* (bracket). Scale bar is equivalent to 20 μm.

The number of cells expressing a precursor marker increases in the long stalks as the severity of the phenotype increases

As mentioned previously, we noted a morphology reminiscent of precursor cells in some of the long stalks. In addition, expression of two markers for more differentiated cells, *93F* for stalk cells and *A101* for polar cells, was not detected in the long stalk-like structures generated by *caN-strong* (Figs 3E, 4E). To address whether N may inhibit the differentiation of stalk cells, we examined the stalks with a marker that reflects the differentiation state of stalk cells: Big Brain (Bib) protein. In wild-type ovaries, Bib is expressed in the follicle cells that are going to intercalate to form a stalk (Fig. 5B, arrowhead) as well as fully developed stalk cells (Fig. 5B, arrow). Importantly, the subcellular localization of Bib differs in these two cell populations. In the precursors, Bib is detected predominantly at the apical tip of the cells as they constrict (Fig. 5B, arrowhead and inset 1). In the stalk cells that have aligned into a single row, the apical pattern is lost and Bib is detected along the lateral surface (Fig. 5B, arrowhead and inset 2). Another marker, Fasciclin III (Fas III) protein is first detected in all follicle cells in region 2 of the germarium (Fig. 5A, bracket). It begins to restrict to the polar cell precursors by region 3 of the germarium, and is fully restricted once the chamber is completely pinched from the germarium (Fig. 5A, arrows).

In the long stalks generated by *caN-mild* and *caN-intermediate*, more cells were detected that stained with the Bib precursor pattern and early Fas III pattern than in wild-type ovaries, indicating that more precursors are present in these ovarioles (Fig. 5C-F). However, if these stalks were allowed to develop, the cells matured (or partially matured) to stalk cells or polar cells (Fig. 5E,F). In *caN-strong*, more precursors were also detected; however, these cells did not mature into stalk cells or polar cells, but stayed in the precursor stage, based on Bib and FasIII staining (Fig. 5G,H). Therefore, the strongest phenotype observed due to constitutively active N expression is persistence of the Bib-positive precursor stage.

Therefore, three different classes of long stalk-like structures can be generated by the expression of constitutively active N: those that consist of too many stalk cells (I), stalk cells and polar cells ("confusion", II), or precursor cells (III) (Fig. 7). A transient excess of precursors was also detected in classes I and II suggesting that the severity of the phenotype correlates with the length of the precursor stage, culminating in a complete block at the precursor stage (class III).

In order to address whether the precursor stage was also affected by the loss of N function, we stained N^{ts} ovaries with Bib and Fas III antibodies. After 25 hours at the restrictive temperature, no Bib-positive cells were detected in N^{ts} germaria (Fig. 5J). This indicated that no cells were in the precursor stage, either because they never develop to this stage or because this stage is transient and therefore not detected.

Expression of constitutively active Notch in follicle cells results in a defect in the oocyte anterior-posterior axis

Based on the loss-of-function mutant phenotypes of *Notch* and *Delta*, it has previously been proposed that a signal from the posterior follicle cells is required for setting the proper A-P axis of the developing oocyte (Ruohola et al., 1991; Clark et al., 1994; Ruohola-Baker et al., 1994). However, the nature of the signal and the posterior follicle cell type which may send the signal are not known. To further investigate this, we have analyzed how expression of constitutively active N in follicle cells affects the posterior follicle cells and the anterior-posterior axis of the underlying oocyte.

In wild-type egg chambers after stage 4, two Fas III-positive cells are detected at the anterior and posterior poles of each egg chamber (Fig. 6A). After two heat-shock inductions of ΔEN in a one day interval, egg chambers were observed in which no Fas III-positive cells are detected (Fig. 6B-D). These egg chambers were not separated by a long stalk, indicating that, in this case, constitutively active N acts in a cell fate decision separate from stalk formation. The egg chambers that lack Fas III-positive cells did not develop further but instead became necrotic. Therefore, in order to address the effects that expression of constitutively active N in the follicle cells has on the underlying oocyte during the later stages of oogenesis, we expressed constitutively active N either under *HS-GAL4* with milder heat-shock conditions [*ΔEN(HS)*] or under *GAL4-109(3)9* [*ΔEN(109)*]. Neither of these results in detectable follicle cell fate defects; two Fas III-positive cells are detected at each pole of the egg chambers, and there is no change in the domain of *pointed*, a posterior follicle cell marker (data not shown; Morimoto et al., 1996). However, we cannot rule out the possibility that further differentiation of the posterior follicle cells is defective. The A-P polarity of the oocyte was examined in these egg chambers using markers used in analysis of N^{ts} (Ruohola et al., 1991). The effects of constitutively active Notch on embryonic polarity could not be addressed because development was blocked prior to embryogenesis.

An early marker for anterior-posterior polarity, *oskar (osk)* mRNA, is localized to the posterior pole of wild-type oocytes at stage 8 (Ephrussi et al., 1991; Kim-Ha et al., 1991; Fig. 6D) and is essential for development of the abdomen and formation of the germ-line precursor cells at the posterior pole of the embryo (Lehmann and Nüsslein-Volhard, 1986; Ephrussi and Lehmann, 1994). Another marker for A-P polarity, Staufen (Stau) protein is localized to the posterior pole of the oocyte at

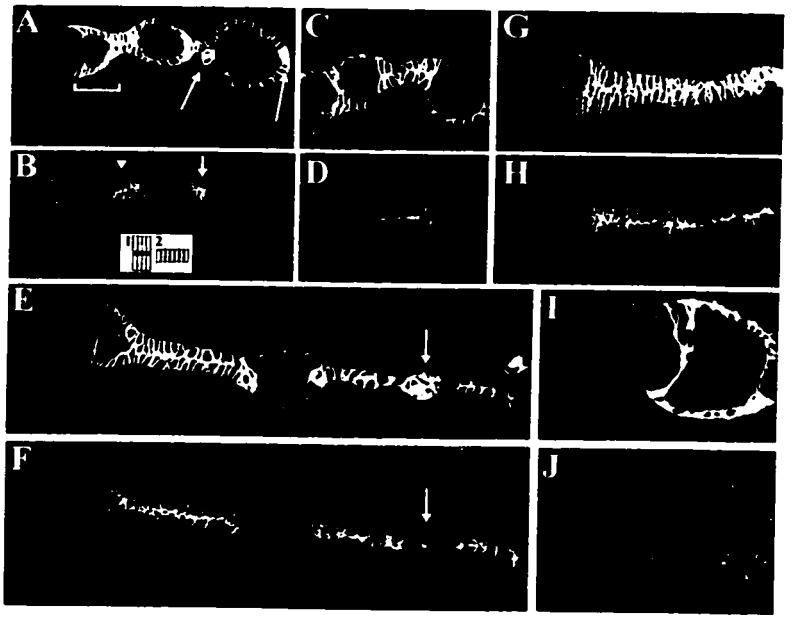

Fig. 5. The number of cells expressing a precursor marker increases in the long stalks as the severity of the constitutively active Notch long stalk phenotype increases. Wild-type (A,B) and mutant (C-J) ovaries are double stained with Fas III and Bib antibodies and visualized by confocal microscopy. (A) Fas III is first detected in all follicle cells in region 2 of the germarium (bracket). It becomes restricted to the polar cell precursors by region 3 of the germarium and is fully restricted once the chamber is completely pinched from the germarium (arrows). (B) Stalk cell precursors (arrowhead) and mature stalk cells (arrow) are stained with Big brain (Bib) antibody. Bib is first expressed in the apical tip of stalk precursors as they meet each other (arrowhead; inset 1). As the stalk matures and the cells align in a single row, this staining pattern ceases and Bib is detected as a line along the lateral surface of the cells (arrow; inset 2). (C,D) *caN-mild*: 2 days after the heat shock, more precursors are detected in the stalk between the germarium and the first egg chamber than in the wild type, based on precursor Bib (D) and Fas III (C) staining. However, these cells developed to stalk cells or polar cells later in oogenesis since older stalks express *93F* and *A101* markers (Figs 4B,C, 3B,C). (E,F) *caN-intermediate*: 48 hours after the heat-shock induction, the long stalks between the germarium and the most anterior egg chamber (Fas III staining, E) consists of precursor cells (Bib precursor staining, F). Stalks removed from the germarium begin to include a small subset of cells which show more intense Fas III staining (E, arrow) and less intense Bib staining (F, arrow) or begin to show the apical staining pattern and morphology of a mature stalk. (G,H) *caN-strong*: The extremely long stalk-like structures (FasIII staining, G) consist of precursor cells (Bib precursor pattern, H). (I,J) N^{ts}: after 24 hours at the restrictive temperature, Fas III (I) but no Bib expression (J) is detected in the follicle cells which have failed to form a pinch.

Table 3. Posterior components are mislocalized in constitutively active Notch mutant egg chambers

ΔEN(109); oskar	12% (7/65)	54% (35/65)	5% (3/65)	0%	0%
Staufen	38% (47/125)	54% (68/125)	8% (10/125)	0%	0%
K-βgal	19% (17/91)	46% (42/91)	35% (32/91)	0%	0%
ΔEN(HS); oskar	17% (18/108)	63% (68/108)	8% (9/108)	0%	12% (13/108)
Staufen	23% (11/47)	38% (18/47)	21% (10/47)	0%	17% (8/47)
K-βgal	11% (3/28)	54% (15/28)	0%	36% (10/28)	0%

stage 8 (Fig. 6G; St Johnston et al., 1991). Whole-mount in situ hybridization with an *osk* cDNA probe, as well as immunocytochemistry with Stau antibody revealed that expression of constitutively active N caused mislocalization of these posterior components: they are mislocalized in ~80% of ΔEN(HS) egg chambers and in ~60% of ΔEN(109) egg chambers (Table 3). Mislocalization was either to the center of the oocyte (Fig. 6F,I; Table 3) or to a streak from the posterior (Fig. 6E,H; Table 3). In addition, expression of activated N under the control of *HS-GAL4* also generated mislocalization of both *osk* and Stau in granules throughout the oocyte (Table 3 and data not shown).

bcd mRNA is localized to the anterior pole of the oocyte in wild-type egg chambers (Fig. 6M) and is also crucial in the establishment of anterior-posterior polarity (Nüsslein-Volhard et al. 1987; St Johnston et al., 1989; Berleth et al., 1988). In situ hybridization indicated that *bcd* mRNA was localized properly to the anterior end of the oocyte of *ΔEN(HS)* ovaries (data not shown). However, in 15% (11/73) of the *ΔEN(109)* stage 8 egg chambers, *bicoid* was detected at both the anterior and posterior poles of the oocyte (Fig. 6N). This phenotype has been detected previously in *N, Delta, Protein Kinase A, gurken, torpedo* and *cornichon* mutants, and reflects a defect in microtubule reorganization that takes place between stages 4 and 7 of oogenesis (Ruohola et al., 1991; Lane and Kalderon, 1994; Gonzales-Reyes et al., 1995; Roth et al., 1995).

To analyze whether the microtubular structures of the oocyte are oriented properly, a strain that expresses a Kinesin-βgal fusion protein (Clark et al., 1994) was crossed to both *ΔEN(HS)* and *ΔEN(109)*. This fusion protein has been used previously as a reliable marker for microtubule organization (Clark et al., 1994; Lane and Kalderon, 1994; Gonzales-Reyes et al., 1995; Roth et al., 1995; Gillespie and Berg, 1995). In control egg chambers, Kinesin-βgal localizes to the posterior of the oocyte (Fig. 6J), suggesting that at least a subset of microtubules are oriented with their plus ends directed towards the posterior. Kinesin-βgal was mislocalized in 89% of *ΔEN(HS)* and 81% of *ΔEN(109)* egg chambers (Table 3). These mislocalizations were either to the center of the oocyte (Fig. 6L; Table 3), to a posterior streak (Fig. 6K; Table 3), or throughout the oocyte (Table 3), as described above for other posterior components.

Constitutively active Notch in the follicle cells induces premature ooplasmic streaming

The defect in localization of Kinesin-βgal indicated a defect in the organization of the microtubules. Another indirect method for analyzing the organization of the microtubules is to assay whether ooplasmic streaming commences at the correct time. Ooplasmic streaming is a microtubule-dependent process (Gutzeit and Koppa, 1982). In wild-type egg chambers at stage 8, an anterior-to-posterior gradient of microtubules is present in the oocyte. At this stage, no ooplasmic streaming is detected (Fig 6O). A rearrangement of microtubules is observed at stage 10b, which results in arrays of parallel microtubules adjacent to the oocyte cortex that are associated with ooplasmic streaming (Theurkauf et al., 1992). Premature ooplasmic streaming was detected at stage 8 in 65% (13/20) of *ΔEN(109)* egg chambers (Fig. 6P), suggesting that the normal gradient of microtubules is replaced by subcortical microtubule bundles. In controls (*109(3)9/TM3*, 15 stage 8 egg chambers analyzed) random flushing movements were detected, but no continuous circular swirling movements.

DISCUSSION

To better define the mechanism of Notch action, we have investigated the effects of expressing constitutively active N in oogenesis. Specifically, we have tested the hypothesis that N functions in follicle cell fate determination and that signaling from the posterior follicle cells is required for the establishment of the oocyte A-P axis (Ruohola et al., 1991; Xu et al., 1992).

We have shown that, at two stages of oogenesis, constitutively active Notch induces follicle cell fate defects that are opposite to those generated by a loss-of-function allele of *N*: hyperplasia of stalk cells early in oogenesis and later, a loss of polar cells. The most extreme phenotype obtained with constitutively active Notch was the persistence of a precursor stage phenotype in the follicle cells required for pinching-off the egg chamber. This observation suggests a novel function for N in oogenesis in differentiation of these follicle cells: holding them in a precursor stage of development.

We have also shown that expression of constitutively active Notch in the follicle cells affects localization of the posterior components *oskar* and Staufen. In addition, mislocalization of a Kinesin-βgal fusion protein and premature onset of cytoplasmic streaming was seen, as well as mislocalization of the anterior component, *bicoid*. These phenotypes reflect a defect in the organization of the microtubules within the oocyte and implicates information from the follicle cells in regulation of this organization.

Models for Notch action in oogenesis

Three models for Notch action have been proposed previously: instructive, permissive and prohibitive (reviewed in:

Greenwald, 1994; Muskavitch, 1994; Artavanis-Tsakonas, 1995). In the instructive model, Notch directs cells to adopt one of two alternative fates. This binary cell fate decision can be a choice between two differentiated cell fates or between remaining a precursor and achieving a more differentiated fate (Struhl et al., 1993; Greenwald, 1994). In the permissive model, Notch makes the cells competent to respond to inductive signals (Muskavitch, 1994). In the prohibitive model, Notch maintains cells in an uncommitted state preventing them from responding to external differentiation signals (Fortini et al., 1993; Coffman et al., 1993; Muskavitch, 1994; Artavanis-Tsakonas, 1995). Therefore, in both the permissive and prohibitive models, Notch modulates the cells' ability to accept additional signals.

Our data suggest that Notch is sufficient to keep cells in a specific precursor stage. This is consistent with either the prohibitive model or the instructive model, but is difficult to reconcile with the permissive model. The prohibitive model proposes that Notch holds cells in an uncommitted state, which could correspond to the Bib-positive precursor stage retained by expression of constitutively active Notch. However, the prohibitive model invokes additional differentiation signals for which there is no evidence thus far. Recent results have shown that Hedgehog acts as an inductive signal affecting follicle cell fate around region 2B of the germarium, however, it appears to affect a decision prior to that controlled by Notch (Forbes et al., 1996). Our data are also consistent with the binary cell fate decision model if a

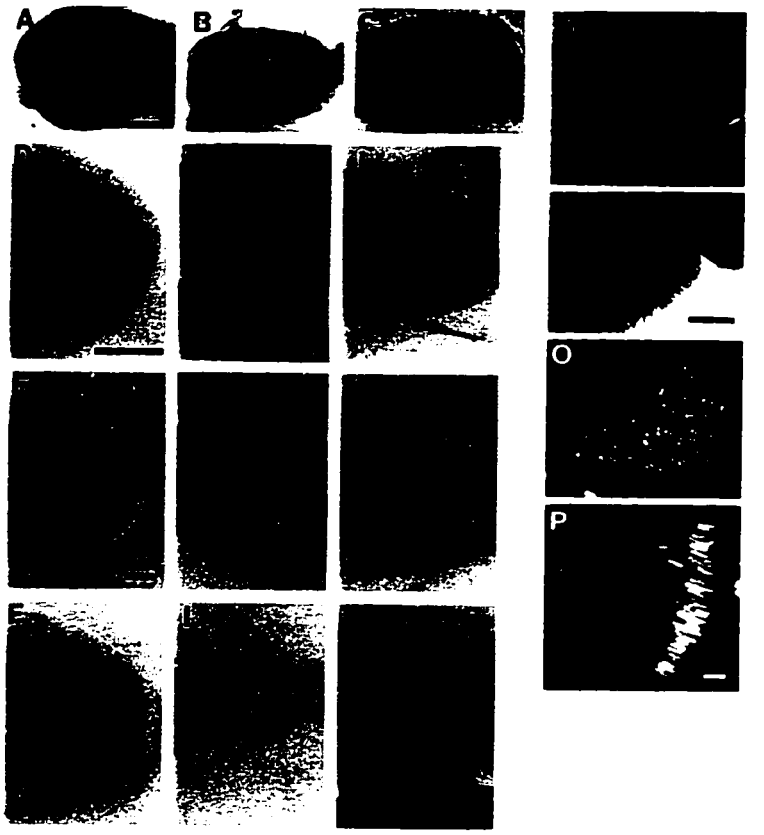

Fig. 6. Effects of *caN* expression in the follicle cells later in oogenesis. Expression of constitutively active Notch induces the loss of polar cells (A-C). Wild-type and mutant egg chambers are stained with antibodies against Fas III and 65F proteins. Normal locations of polar cells are indicated by arrowheads; the 65F-positive oocyte nucleus by an arrow. (A) In wild-type egg chamber, two Fas III-positive polar cells are detected at the anterior and posterior ends (arrowheads). (B,C) Constitutively active N was induced by *HS-GAL4* expression due to two heat-shock pulses (39 C, 15 minutes and 39 C, 20 minutes) in a one day interval. In mutant ovaries dissected two days after the last induction, egg chambers are detected which lack either posterior polar cells (B) or both anterior and posterior polar cells (C). Expression of constitutively active Notch (ΔEN) in follicle cells results in a defect in oocyte anterior-posterior axis (D-L). Posterior components and a Kinesin-βgal fusion protein are mislocalized. (D-F) Localization of *oskar* mRNA in wild-type and *ΔEN* egg chambers. (G-I) Localization of Staufen (Stau) protein in wild-type and ΔEN egg chambers. (J-L) Localization of Kinesin-βgal in wild-type and ΔEN egg chambers. In wild-type egg chambers, the posterior components, *osk* and Stau, are localized tightly to the posterior pole of the oocyte (D,G). A Kinesin-βgal fusion protein is also detected at the posterior pole (J). After expression of constitutively active N in the follicle cells by *GAL4-109(3)9*, posterior components and Kinesin-βgal are detected either diffusing from the posterior (E,H,K), or in the center of the oocyte (F,I,L). (M,N) The anterior morphogen, *bicoid*, is mislocalized. (M) In wild-type egg chambers, *bicoid* mRNA is localized as a ring at the anterior margin of the oocyte. (N) In *GAL4-109(3)9* egg chambers, *bicoid* is detected at both the anterior and posterior poles in 15% (11/73) of stage 8 mutant egg chambers (arrows indicate posterior pole of oocyte). (O,P) Cytoplasmic streaming commences early. Yolk granules within the oocyte are detected by autofluorescence. (O) In wild-type egg chambers at stage 8, no cytoplasmic movement is detected within the oocyte. Ooplasmic streaming does not begin until stage 10b of oogenesis. (P) In *GAL4-109(3)9*, premature ooplasmic streaming is detected in 55% (10/18) of the stage 8 oocytes (O). (A-D, M-N) 100×, (E-L, O-P) 40×, (E-F, J-L, O-P; not dehydrated). All scale bars are equivalent to 20 μm.

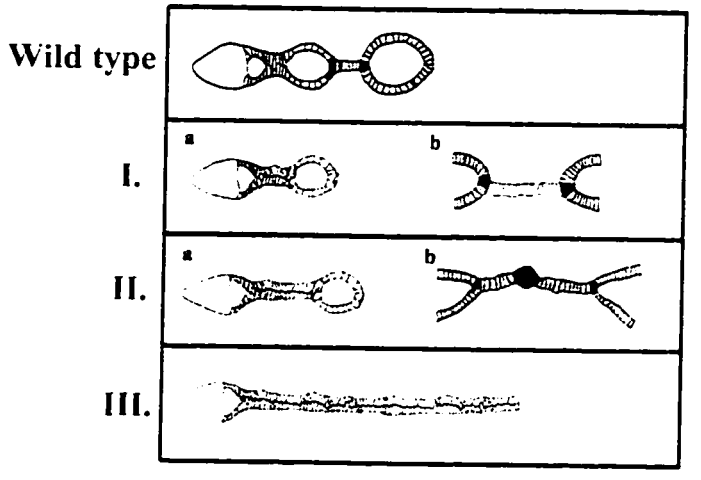

Fig. 7. Summary: three different types of long stalks are generated by expression of constitutively active Notch. Big brain (Bib) expression is shown in red, *A101* expression in blue and *93F* expression in green. (Wild type) In wild-type ovarioles, Bib is expressed in the precursor pattern in the cells which are going to constrict to form a stalk. *93F* is expressed in a in the fully formed stalks and *A101* is expressed in the polar cells at both poles of the egg chambers. Expression of constitutively active N generates three types of long stalks. (I) In the germarium, too many precursors are formed (a) and these further differentiate to form a long stalk which consists entirely of stalk cells (b). (II) Too many precursors are formed in the germarium (a), but these further differentiate into a long stalk which consists of stalk cells and polar cells (b). (III) A long stalk-like structure is formed in which all of the cells are in the precursor stage and they remain in this stage throughout development.

binary switch is assumed between precursor fate and a more differentiated cell fate (Greenwald, 1994).

The anterior-posterior axis of the oocyte

The mislocalization of *bicoid* both to the anterior and posterior ends of the oocyte (Fig. 6N) and the localization of posterior components to the middle of the oocyte (Fig. 6E-F, H-I, K-L) are phenotypes reminiscent of those seen with ovaries from mutants in *N, Delta, Protein Kinase A, torpedo, gurken* and *cornichon* (reviewed in Rongo and Lehmann, 1996). During stages 1-6 of oogenesis, a microtubule-organizing center (MTOC) is located at the posterior pole of the oocyte and extends a microtubule network from the oocyte into the nurse cells. During midoogenesis, this cytoskeleton is reorganized so that an anterior-to-posterior gradient of microtubules is present in the oocyte, suggesting that the MTOC at the posterior degenerates and the microtubules are nucleated at the anterior of the oocyte. It has been postulated that, in these mutants (*N, Dl, PKA, top, grk, cni*), the posterior MTOCs are not properly destroyed at stage 4-7 of oogenesis, resulting in oocytes with both anterior and posterior microtubule concentrations (Lane and Kalderon, 1994; Ruohola-Baker et al., 1994). A mirror-image axis duplication results (Ruohola et al., 1991; Lane and Kalderon, 1994; Gonzalez-Reyes et al., 1995; Roth et al., 1995). One possibility is that mislocalization of the posterior components as a streak from the posterior is a milder phenotype resulting from this type of defect in microtubule organization. In egg chambers that express constitutively active Notch in the follicle cells, ooplasmic streaming in the oocyte commences early, beginning around stage 8 of oogenesis. This is presumably also a secondary consequence of defects in the microtubule reorganizations.

One surprising result is that expression of constitutively active N either under the control of *GAL4-109(3)9* or *HS-GAL4* (mild heat shock) can lead to defects in the A-P axis of the oocyte without generating any obvious defects in the fate of the posterior follicle cells. By analogy to *caN* action in the germarium, it is possible, however, that the timing of posterior follicle cell differentiation is altered in the mutant situation. Isolation of markers specific for different stages of posterior follicle cell differentiation should shed light on this question. It is also possible that the Notch pathway is closely linked to the signaling pathway from the follicle cells to the oocyte and expression of constitutively active Notch has affected the signaling without affecting the fates of these cells. Further analysis of this subject requires a better understanding of the signaling pathway from the follicle cells to the oocyte.

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APPENDIX C: Notch signaling is required for anterior follicle cell fate in *Drosophila* oogenesis

Notch signaling is required for anterior follicle cell fate in *Drosophila* oogenesis

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Running Title: Notch signaling induces anterior cell fate

Abstract

During *Drosophila* oogenesis, the developing oocyte is surrounded by a layer of somatic follicle cells. A key event in the establishment of oocyte anterior-posterior polarity is the differential patterning of the two terminal follicle cell groups. The Notch and EGF receptor pathways are known to regulate this patterning. To understand how the Notch pathway acts in this process we have analyzed the expression of anterior markers with the loss of Notch function and with ectopic expression of Delta, one of the ligands for the Notch pathway. We find that the expression of anterior markers in anterior follicle cells is dependent upon functional Notch and that anterior markers are activated in posterior follicle cells in response to ectopic Delta expression. This suggests that anterior follicle cells are specified by high levels of Notch and posterior follicles cells by low levels of Notch and also raises the possibility that repression of Notch pathway in posterior is accomplished by activated EGF receptor pathway.

Key words:

Delta, Notch, follicle cells, cell fate, oogenesis, polarity, axis, Drosophila, EGF receptor

INTRODUCTION

In *Drosophila melanogaster*, the body axes are set up during oogenesis through a process that involves signaling between the oocyte and the somatic follicle cells that surround the egg chamber. The proper localization of morphogenetic determinants is required for establishment of the embryonic anterior-posterior (A-P) axis and depends upon proper polarization of the oocyte cytoskeleton. Recent data suggest that signaling from specialized follicle cells at the posterior end of the oocyte is required for this polarization (reviewed in Anderson, 1995). Thus, the establishment of the oocyte anterior-posterior axis requires: (1) differentiation of specialized posterior follicle cells, (2) signaling from these posterior follicle cells to the oocyte, and (3) modulation of the polarity of the oocyte cytoskeleton upon reception of the signal.

Three signaling pathways are known to be required for proper patterning of posterior follicle cells: the EGF-receptor (EGFr) pathway, the Notch (N) pathway, and the Hedgehog (Hh) pathway (González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Ruohola *et al.*, 1991; Xu *et al*, 1992; Bender *et al.*, 1993; reviewed in Rongo and Lehmann, 1996; Forbes *et al.*, 1996). The EGF receptor pathway transmits inductive information from the oocyte to the follicle cells, while the Hh and Notch pathways act in communication between different subsets of the follicle cells. Studies of mutants in the EGF receptor and Notch pathways have led to the model that the anterior and posterior groups of follicle cells are initially identical (Rongo and Lehmann, 1996). These prepatterned follicle cells are transformed to the posterior fate by EGF receptor signaling if they are located adjacent to the oocyte. If and how the Notch and EGF receptor pathways interact in this process is unknown, but several models have been proposed (Rongo and Lehmann, 1996; see discussion).

Notch functions in determining at least two follicle cell populations: the terminal follicle cells and the stalk cells that separate the egg chambers from one another. Studies with constitutively active Notch have shown that Notch suppresses differentiation in oogenesis. Constitutively active Notch arrests follicle cells in a specific precursor stage early in oogenesis, blocking the development of both stalk cells and terminal follicle cells (Larkin *et al.*, 1996). Loss of Notch function eliminates this precursor stage. Interestingly, studies with *toucan* mutants have suggested that a signal from the germ line is required for stalk formation (Grammont *et al.*, 1997). At a later stage of oogenesis loss of Notch function, as well as constitutive activation of Notch, in follicle cells leads to aberrant oocyte anterior-posterior axis formation.

In this study we show that the Notch pathway is required in the anterior follicle cells for proper establishment of anterior fate. Moreover, we find that prolonged activation of the Notch pathway in the posterior follicle cells leads to defects in the posterior follicle cells, including expression of anterior markers that are regulated by the EGF receptor pathway. Our data is consistent with Notch acting at both termini as an anterior inducer that is selectively repressed by the EGF receptor pathway in the posterior follicle cells. Furthermore, defects in the anterior posterior axis of the oocyte with prolonged expression of Delta suggest that signaling from the follicle cells to the oocyte that is required for the proper oocyte A/P polarity must occur during late stage 6. In the germarium, we demonstrate that expression of UAS-Delta generates long stalk-like structures similar to those detected with expression of constitutively active Notch, and that these are also due to a prolonged precursor stage.

Materials and Methods

Stocks

Drosophila melanogaster stocks were raised on standard cornmeal-yeast-agar medium at 25°C or at 18°C. The following fly strains were used: *Oregon R*, w^-, *GAL4-109*, *GAL4-107c1* (generated in the Jan laboratory), *HS-GAL4 CyO*, *UAS-LacZ CyO*, *UAS-LacZ FM6* (Brand and Perrimon, 1993), *UAS-tau-GFP* (Brand, 1995), *93F TM3* (Ruohola *et al.*, 1991), *KL503 TM3* (Clark *et al.*, 1994), *A101 TM3* (Bier *et al.*, 1989), *GAL4-A62* (Gustafson and Boulianne, 1995), *GAL4-c709* (a gift from L. Manseau), w^- $Notch^{ts1}$, $Delta^{vial}$, $Delta^{6B37}$, *L53b* (González-Reyes *et al.*, 1995), *UAS-Delta*^{20} *CyO*, *UAS-Delta*^{33} *FM6* (a gift from E. Giniger; Doherty *et al.*, 1996), *5A7*, *BB127* (Roth *et al.*, 1995).

Over-expression of Delta

Using the GAL4 system (Brand and Perrimon, 1993), we ectopically expressed a *UAS-Delta* transgene in the somatic follicle cells during oogenesis. Flies carrying the *UAS-Delta* construct (*UAS-Dl*^{33}) were crossed either to flies expressing GAL4 under heat-shock control (*HS-GAL4*) or to one of four enhancer trap GAL4-lines: *109*, *A62*, *107c1*, or *c709*. The GAL4 expression patterns of these different lines were determined by crossing the GAL4-lines to a line carrying *UAS-LacZ* and staining the progeny with X-GAL, or by crossing them to a *UAS-tau-GFP* line and analyzing the GFP pattern by fluorescence microscopy. *HS-GAL4* induced *UAS-LacZ* expression in follicle cells beginning in region two of the germarium (Fig. 7B), while the GAL4 enhancer trap lines induced *UAS-LacZ* expression only after stage 5 of oogenesis (Fig. 2). Therefore, the *HS-*

GAL4 line was used to study the effects of *UAS-Dl* expression in the germarium, while the GAL4 enhancer trap lines served as excellent tools to study the function of *Delta* later in oogenesis.

Heat Shock Treatments

To induce Delta expression from the *UAS-Delta* construct, *UAS-Dl*^{33} was crossed to *HS-GAL4* and progeny were raised at 25°C and heat shocked to induce expression of GAL4 by the hsp70-promoter. To generate long stalks, glass vials containing 1-2 day old *UAS-Delta*^{33}; *HS-GAL4* animals were incubated in a 37°C water bath for 15 minutes on day one, 15 minutes on day two, and dissected on day three or day four. A higher percentage of extreme long stalks (70% as compared to 22%; Fig. 8) was generated by dissecting on day four. To generate defects in the A-P axis, flies were incubated in a 37°C water bath for 25 minutes, and dissected the next day.

To test whether over-expression of other proteins using the GAL4/UAS system with our protocols leads to the formation of long stalks, we analyzed ovarioles over-expressing Pointed P2 or Derailed using the same GAL4-system and heatshock conditions. Over-expression of these proteins does not generate long stalk-like structures.

***UAS-Delta* with loss of *Notch* function**

To examine the effects of *UAS-Delta* expression in a loss of *Notch* function background, the flies (*N*^{ts}*N*^{ts};*UAS-Dl*^{20}/*HS-GAL4*) were heatshocked at 39°C for 15 minutes, allowed to recover at room temperature for thirty minutes, placed at 32°C for 16 hours, and allowed to develop at room temperature for 32 hours before dissection.

Immunocytochemistry, in situ hybridization and other staining procedures.

Immunocytochemistry, in situ hybridization, and X-GAL staining procedures were performed as described earlier (Larkin *et al.*, 1996).

The antibodies used in this study were anti-Fasciclin III (1:15 dilution, Ruohola *et al.*, 1991), anti-Big Brain (1:1500; Doherty *et al.*, 1997), anti-α-tubulin (1:250), anti-Delta (1:3000; Bender *et al.*, 1993) and anti-Staufen (1:3000; St Johnston *et al.*, 1991; a gift from Daniel St Johnston). The DNA plasmids used for in situ hybridization probes were *BSK-derailed* (Callahan *et al.*, 1995), *pGEM3bcd* (Driever *et al.*, 1990), and *BSKS-osk* (Ephrussi *et al.*, 1991).

Microtubule/Staufen immunocytochemistry

Ovaries were dissected in 1xRobbs and then transferred immediately to 100% methanol (room temperature). To fix the ovaries, methanol was exchanged for -20°C methanol and the samples were kept at -20°C for 10 minutes. Ovaries were rinsed twice with Phosphate Buffered Saline (PBS) and incubated in PBS with 1% saponin at room temperature for 2 hours. After a wash in PBSS (PBS and 0.1% saponin) the samples were incubated with anti-α-tubulin (1:250, Sigma) and anti-Staufen (1:3000) in PBSS for 48 hours at 4°C. After rinsing 3X 15min with PBS, the samples were incubated with Texas Red goat anti-mouse and FITC BODIPY goat anti-rabbit secondary antibodies (each at 1:200; Molecular Probes) in PBSS overnight at 4°C. After rinsing 4X 15 minutes in PBSS, the samples were mounted in glycerol with 2% N-propyl gallate.

Microscopy

Microscopy was performed on a Leitz DMRB with Nomarski differential interference contrast. Confocal images were collected using the MRC 600 laser scanning confocal microscope system (Bio-Rad Microsciences Division) using the 488 and 568 wavelengths of a krypton argon laser.

RESULTS

Notch is required for anterior and posterior follicle cell fate

Previous studies demonstrated that the Notch pathway is required for proper establishment of posterior follicle cell fate; loss of the Notch pathway results in hyperplasia of the posterior polar cells (Ruohola *et al.*, 1991). Since anterior and posterior follicle cells have been shown to be initially equivalent (Gonzalez-Reyes *et al.*, 1995; Roth *et al.*, 1995; Reviewed in Lehmann, 1995; Rongo and Lehmann; 1996), we decided to investigate whether the Notch pathway is also required for anterior follicle cell fate. To do this, we analyzed the expression of follicle cell markers specific to the anterior, the enhancer trap lines *L53b* and *5A7*, in N^{ts} egg chambers at the restrictive temperature. Surprisingly, loss of Notch function abolishes the normal onset of both of these markers at stage 8 in the anterior terminal cells in 97% (56/58) and 92% (47/51) of egg chambers, respectively (Fig. 1A-F). Therefore, loss of Notch function results in defective anterior follicle cells, suggesting that Notch is also required for anterior follicle cell fate.

Delta has to be turned off in the posterior follicle cells at stage 6.

Based on the data presented, Notch acts in both anterior and posterior follicle cells. Interestingly, there is a difference between Delta expression in anterior and posterior follicle cells. In wild type ovarioles, Delta protein is detected in a specific pattern, as previously determined with antibodies to Delta (Bender *et al.*, 1993; Fig. 2). Delta protein is first detected in the germarium, and later accumulates strongly in the membranes of follicle cells (Fig. 2A). Delta disappears from the posterior follicle cells beginning around stage 6 in oogenesis (Bender *et al.*, 1993; Fig. 2B,C). This pattern of expression is

consistent with the mRNA localization pattern detected by whole mount in situ hybridizations with a probe to *Delta* mRNA (Ruohola *et al.*, 1991).

We decided to test whether this difference in expression has a functional relevance by prolonging Delta expression in the posterior follicle cells. This was accomplished by using a *UAS-Delta* construct and the GAL4 line *109* (Fig. 2D,E) and analyzing the effects on the determination of the posterior follicle cells and establishment of the oocyte A-P axis due to prolonged expression of Delta. We analyzed posterior cell fate by monitoring a receptor tyrosine kinase *derailed* (*drl*; Callahan *et al.*, 1995) as a marker for posterior follicle cell differentiation. In wild type, *drl* mRNA is expressed in posterior follicle cells from the germarium until about stage 6 of oogenesis (Clegg, N. and Ruohola-Baker, H., unpublished; Fig. 3A; Table 1). In the *Dl, 109* mutant egg chambers the posterior *drl* expression was abnormal; *drl* was still detected at stage 8 in *UAS-Dl; GAL4-109* egg chambers (Fig. 3B). Prolonged expression of *drl* was also detected in *UAS-Dl; HS-GAL4* ovaries, one day after induction (Table 1). These data indicate that prolonged expression of Delta induces prolonged expression of *drl* mRNA in posterior follicle cells, confirming that cessation of Delta expression in the posterior follicle cells is important for their normal development.

To investigate where ectopic Delta must be expressed in order to prolong *drl* expression, we analyzed egg chambers that expressed Delta under the control of the GAL4-lines *c709*, *107c1*, and *A62* (Fig. 2). In *c709* or *107c1* , no prolonged *drl* expression was detected, suggesting that ectopic Delta expression in anterior follicle cells cannot induce prolonged expression of *drl* in posterior follicle cells. Prolonged expression of *drl* was not detected when *UAS-Delta* was expressed under the control of *A62* either, suggesting that Delta expression has to begin prior to *A62* onset during stage 7. An

alternative possibility is that Notch signaling requires differing levels of *Delta* in adjacent cells (Wilkenson *et al.*, 1994), and *A62* results in uniform expression (Fig. 2G).

To further investigate the defect in the posterior follicle cells, we analyzed an anterior marker that is repressed in the posterior follicle cells by the EGF receptor pathway, *L53b*. In addition to normal anterior expression, *L53b* was also activated in the follicle cells over the oocyte in 34% (26/76; Fig. 4) of stage 10 *UAS-Dl; GAL4-109* egg chambers. Therefore expression of Delta can induce anterior markers in the posterior follicle cells. This phenotype has been observed previously in mutants which compromise the activity of the EGF receptor pathway in posterior follicle cells (González-Reyes *et al.*,1995; Roth *et al.*,1995; Clegg *et al.*,1997; González-Reyes *et al.*, 1997).

Over-expression of Delta in the posterior follicle cells at stage 6 disturbs their development, presumably due to over-activation of the Notch pathway. Therefore, these data suggest that Delta expression is important for control of the Notch pathway. It is possible that this control could be at the transcriptional level - through a feedback loop, or by direct activation of the Notch receptor. Recent experiments have indicated that in some systems the Notch pathway is regulated by a feedback mechanism (reviewed in Kimble and Simpson, 1997). In an attempt to analyze this, we looked at the expression of Notch protein in *UAS-Dl; GAL4-109* ovaries. No obvious change in the protein expression pattern was detected (data not shown). This suggests that in the follicle cells surrounding the oocyte, the level of Delta protein is controlling the Notch pathway through direct activation of the receptor. However, we cannot rule out that a feedback loop generated a transient change in the Notch level which would not be detected in normal antibody stainings.

Prolonged expression of Delta in posterior follicle cells generates oocytes with two anterior organizing centers

To address whether the posterior follicle cell defects caused by prolonged expression of Delta generate defects in the A-P axis of the oocyte, we expressed Delta in the follicle cells, either under the control of *HS-GAL4* or *GAL4-109* and then analyzed the oocyte for localized components. Anterior components, such as *bicoid* mRNA (*bcd*; Berleth *et al.*, 1988; Fig. 5C), were mislocalized to both the anterior and posterior of the oocyte (Fig. 5D; Table 2), while posterior components, *oskar* mRNA (*osk*; Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991; Fig. 5A) and Kinesin-βgal fusion protein (Fig. 5E), were mislocalized to the center of the oocyte (Fig. 5B,F; Table 2). The normally anterior oocyte nucleus was now found mislocalized to the posterior (Figure 5B, Table 2). In addition, premature ooplasmic streaming was detected in some egg chambers (Table 2). Combinations of these phenotypes have previously been observed in *Notch*, *Delta*, *Protein Kinase A*, *gurken*, *torpedo*, *cornichon*, *mago nashi*, and *maelstrom* mutants as well as with expression of constitutively active *Notch*, and reflect a defect in the microtubule reorganization that takes place in mid-oogenesis (Ruohola *et al.*, 1991; Lane and Kalderon, 1994; González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Larkin *et al.*, 1996; Micklem *et al.*, 1997; Newmark *et al.*, 1997; Clegg *et al.*, 1997). In wild type, a microtubule organizing center (MTOC) is initially localized to the posterior end of the oocyte by an unknown mechanism. During mid-oogenesis, the MTOC disappears from the posterior, and microtubules begin to nucleate from the anterior of the oocyte. No oocyte A-P axis defects were detected in lines that induce Delta expression in the anterior or lateral follicle cells (*107c1*, *c709*) or in the posterior follicle cells later in oogenesis (*A62*).

The defects in the A-P axis of the oocyte are due to defects in the organization of the microtubules

The defects in the A-P axis of the oocyte with constitutively active Notch (Larkin *et al.*, 1996), over-expression of *Delta*, and loss of function alleles of *Notch* and *Delta* (Ruohola *et al.*, 1991; Clark *et al.*, 1994) suggested abnormal organizations of the microtubule cytoskeleton. To investigate this possibility, we visualized the microtubules by staining them with an antibody to α-tubulin. To assay the functionality of the microtubules, the egg chambers were simultaneously stained with antibodies to Staufen (Stau), another marker for A-P polarity, which is localized to the posterior pole of the oocyte at stage 8 (St Johnston *et al.*, 1991). Expression of constitutively active Notch, over-expression of Delta, and loss of Notch or Delta lead to a high concentration of microtubules in the posterior of the oocyte (Fig. 6). The posterior component Stau was mislocalized to the center of the oocyte where there was a void detected in the concentration of microtubules. These data suggest retention of the posterior microtubule organizing center, and that the defects in the A-P axis are due to defects in the organization of the microtubule cytoskeleton.

In summary, we conclude that prolonged activation of the Notch pathway disturbs the posterior follicle cells prior to the stage in which they are competent to signal to the oocyte, resulting in retention of the posterior microtubule organizing center and bipolar A-P axis. This shows that sufficient signaling did not occur prior to stage 6.

Ectopic expression of Delta in the germarium follicle cells generates long stalk-like structures

The Drosophila ovary consists of 15-20 ovarioles: strings of egg chambers aligned in developmental order. At the anterior end of each ovariole lies the germarium, where the germ line stem cells divide to form 16 cell cysts. These cysts are enveloped by a somatic follicle cell layer and released from the germarium as a subset of follicle cells intercalates to form an interfollicular stalk (Fig. 7A). Our previous studies showed that expression of constitutively active Notch generates long stalks in the germarium by virtue of holding the stalk cells and polar cells in a precursor stage and causes defects in the A-P axis of the oocyte (Larkin *et al.*, 1996). Since expression of UAS-Delta generates similar phenotypes in the oocyte, we looked to see whether it could also generate defects in stalk formation.

To express *UAS-Dl* in the germarium, we heat shocked *UAS-Dl; HS-GAL4* flies and analyzed them two days after the heat shock. Over-expression of *Delta* in the germarium leads to the formation of long stalk-like structures in 81% (119/147) of the ovarioles (Figs. 7,8). In contrast to the normal wild type stalk, which is 6-7 cells long, the average length of the long stalks was ~33 cells. Based on their morphology and the expression of markers, two classes of long stalks were observed, "intermediate" (30% of long stalks) and "extreme" (70% of long stalks). The cells in the intermediate long stalks were aligned as a single row and expressed either the stalk cell marker, *93F* (Fig. 7D; Ruohola *et al.*, 1991), or the polar cell marker, *A101* (Fig. 7F). The extreme long stalks were several follicle cells thick, exhibiting the morphology of follicle cells that are ready to intercalate to form a stalk in the germarium, and did not contain differentiated *93F* or *A101* positive cells (Fig. 8B). Both classes of long stalks have been detected previously with expression of constitutively active Notch (Larkin *et al.* 1996).

The cells in the long stalk-like structures are arrested in a precursor stage

Since the cells in the extreme long stalks did not express the differentiation markers *93F* or *A101*, we analyzed the expression of precursor markers Big Brain (Bib; Larkin *et al.*, 1996; Doherty *et al.* 1997) and Fasciclin III (FasIII) proteins (Fig. 8D,F). In wild type ovaries, Bib is expressed in the precursors for polar cells and the follicle cells intercalating to form a stalk, as well as in fully formed stalks. However, the subcellular localization of Bib differs in these two cell populations. In the precursors, Bib is found predominantly in the apical tips of the cells where they meet as the cells are constricting (Fig. 8E, arrow). In the stalk cells that have aligned as a single row, Bib is detected along the lateral surface (Fig. 8E, bracket). FasIII is detected in all of the follicle cells in region 2 of the germarium, but is restricted to polar cell precursors by the time the egg chamber leaves the germarium (Fig. 8C). In all of the extreme long stalks generated by over-expression of *Delta*, Bib is detected in a line along the apical tips of the cells (Fig. 8F), and FasIII is detected in all of the cells (Fig. 8D), the precursor patterns for these markers. This data, coupled with the fact that there is no expression of the differentiation markers, *93F* and *A101*, suggests that in the most extreme cases the cells in the long stalk-like structures are precursors for both the stalk cells and the polar cells. Recent mosaic analysis has also revealed that stalk cells and polar cells have a common precursor, separate from the precursors for lateral follicle cells (Tworoger, M., Bryant, Z., M. K. L., and H. R.-B., submitted). These data indicate that over-expression of Delta functions similarly to constitutive activation of the Notch receptor, forming long stalk-like structures by prolonging the precursor stage. Therefore, expression of the ligand, Delta, plays a critical role in controlling the activity of the Notch pathway in oogenesis.

UAS-Delta acts through the Notch receptor to generate long stalks

To confirm that *UAS-Dl* generates long stalks by acting through the Notch receptor, we analyzed the phenotype generated by over-expression of Delta in a *Notch* loss-of-function background. If *UAS-Delta* acts through the Notch receptor, no phenotype due to over-expression of Delta should be detected without functional *Notch*. $N^{ts}N^{ts}$; *Dl HS* and control females were heat shocked to induce Delta expression and thereafter transferred to the restrictive temperature (See Materials and Methods). 94% of control $N^{ts}N^{ts}$ ovaries exhibited a loss of stalks (61/65, Fig. 9C), while 69% of *Dl HS* ovaries displayed long stalk-like structures, as expected (24/35, Fig. 9B). 98% of $N^{ts}N^{ts}$; *Dl HS* ovaries displayed the loss of stalk defect (52/53, Fig. 9D), demonstrating that the over-expression of Delta requires functional Notch protein in order to generate long stalk-like structures.

DISCUSSION

We have shown that the Notch pathway is required in the anterior follicle cells for proper establishment of anterior fate. Moreover, we have shown that prolonged activation of the Notch pathway in the posterior follicle cells leads to defects in the posterior follicle cells, including ectopic expression of anterior markers. Since over-activation of the Notch pathway and loss of the EGFr pathway result in the same phenotype - ectopic expression of anterior markers - it is possible that repression of the Notch pathway in the posterior is accomplished by the EGFr pathway. In the germarium, we have demonstrated that expression of UAS-Delta generates long stalk-like structures similar to those detected with expression of constitutively active Notch, and that these are formed by a block in the precursor stage. In addition, expression of UAS-Delta in the absence of Notch function leads to a loss of stalks, indicating that UAS-Delta acts through the Notch receptor.

The Notch pathway acts in both anterior and posterior follicle cells

Our data, in conjunction with previous studies, has shown that the Notch pathway is required in both the anterior and posterior follicle cells. An important difference, however, is that the Notch pathway has to be inactivated in the posterior prior to stage 6. This is normally accomplished by cessation of Delta expression in the posterior follicle cells. If Delta is over expressed, posterior markers such as *derailed* mRNA are defective, and markers that are usually expressed only in the anterior are mis-expressed in the posterior (*5A7* and *L53b*). These data are consistent with a model in which the Notch pathway can induce anterior fate in both the anterior and posterior follicle cells and must be turned off in the posterior before the posterior follicle cells can take their appropriate fate. Moreover, both over-activation of the Notch pathway and loss of the EGF receptor pathway lead to similar phenotypes - namely expression of the anterior markers, *L53b* and *5A7*, in the posterior follicle cells. Therefore, the simplest model is one in which the Notch pathway can induce anterior fates in both the anterior and posterior but is repressed in the posterior by the EGF receptor pathway.

Interplay between the EGF receptor and Notch pathways in posterior follicle cell development

One of the major objectives in modern biology is to elucidate how multiple signals are integrated inside a cell. For example, vulval development in C. elegans and axis formation in Drosophila oogenesis require the Notch and EGF receptor pathways for specific cell fates (reviewed in Duffy and Perrimon, 1996). However, if and how these pathways are integrated inside a cell is not yet understood. In Drosophila oogenesis, several models

have been proposed to explain the roles of these two pathways in the specification of posterior follicle cell fate and signaling (Rongo and Lehmann, 1996). Some models suggest that the Notch and EGF receptor pathways act independently, while others suggest that they interact in some capacity. We have shown that prolonged expression of Delta results in failure of posterior follicle cells to receive the Grk signal, since a marker that is normally turned off in response to the Grk signal is not repressed. This suggests that the pathways do not act independently because the Notch pathway can interfere with reception of the EGF receptor pathway. Our data can be explained by a derepression model in which high Notch activity is required for establishment of anterior fate and low Notch activity for posterior fate. In the posterior, the Notch pathway is depressed by the EGF receptor pathway, resulting in posterior follicle cell fate. Loss of Notch function results in defective terminal follicle cells in both poles of the egg chamber. At stage 7, when *grk* is no longer located at the posterior of the oocyte, inhibition of the Notch pathway is accomplished by cessation of *Delta* expression in the posterior follicle cells. Over-expression of *Delta* partially overcomes the repression by the EGF receptor pathway, leading to the expression of an anterior marker in the posterior of the egg chamber (Fig. 10).

Over-activation of the Notch pathway disturbs the posterior follicle cells prior to follicle cell to oocyte signaling

In wild type posterior follicle cells, the *derailed* staining pattern defines separate developmental stages. Before stage 6 the posterior follicle cells are *derailed* positive. From stage 6 on, *derailed* expression is no longer detected in the posterior of the egg chamber. Based on the expression patterns in mutant and wild type situations, over-

expression of Delta at stage 6 disturbs the posterior follicle cell development and causes a defect in oocyte A-P axis, presumably due to a defect in the follicle-cell-to-oocyte signaling. Over-expression of Delta after stage 6 does not affect the oocyte. Signaling from the follicle cells to the oocyte that is required for the proper oocyte A-P polarity must therefore occur (or continue to occur) during late stage 6.

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Tables

Table 1: *derailed* expression in posterior follicle cells

	stage 4	stage 5	stage 6	stage 7
control	100% (27/27)	100% (34/34)	36% (12/33)	0% (0/24)
UAS-Dl;GAL4-109	100% (35/35)	100% (32/32)	93% (54/58)	46% (20/44)
UAS-Dl;HS-GAL4	100% (32/32)	100% (30/30)	67% (53/79)	29% (23/79)

Table 2: Axis defects generated by prolonged expression of Delta protein in the follicle cells

	oskar - central	*bicoid* - ant.&post.	Kinβgal - central	nucleus - posterior	premature swirling
UAS-Dl; HS-GAL4	81% (42/52)	11% (5/46)	90% (66/73)	NA	NA
UAS-Dl; GAL4-109	93% (70/75)	68% (42/62)	91% (63/69)	12% (14/117)	56% (15/27)

NA = not analyzed

Figure legends

Figure 1. The effects of the loss of the Notch pathway on anterior markers that are repressed in the posterior follicle cells by the EGF receptor pathway.

(A) The enhancer trap line *L53b* is expressed in anterior follicle cells: the border cells, nurse cell associated cells, and centripetally migrating cells. (B,C) Loss of Notch pathway function (N^{ts}) leads to loss of *L53b* expression. (D) The enhancer trap line *5A7* is expressed in the border cells. (E,F) Loss of Notch pathway function (N^{ts}) leads to loss of *5A7* expression.

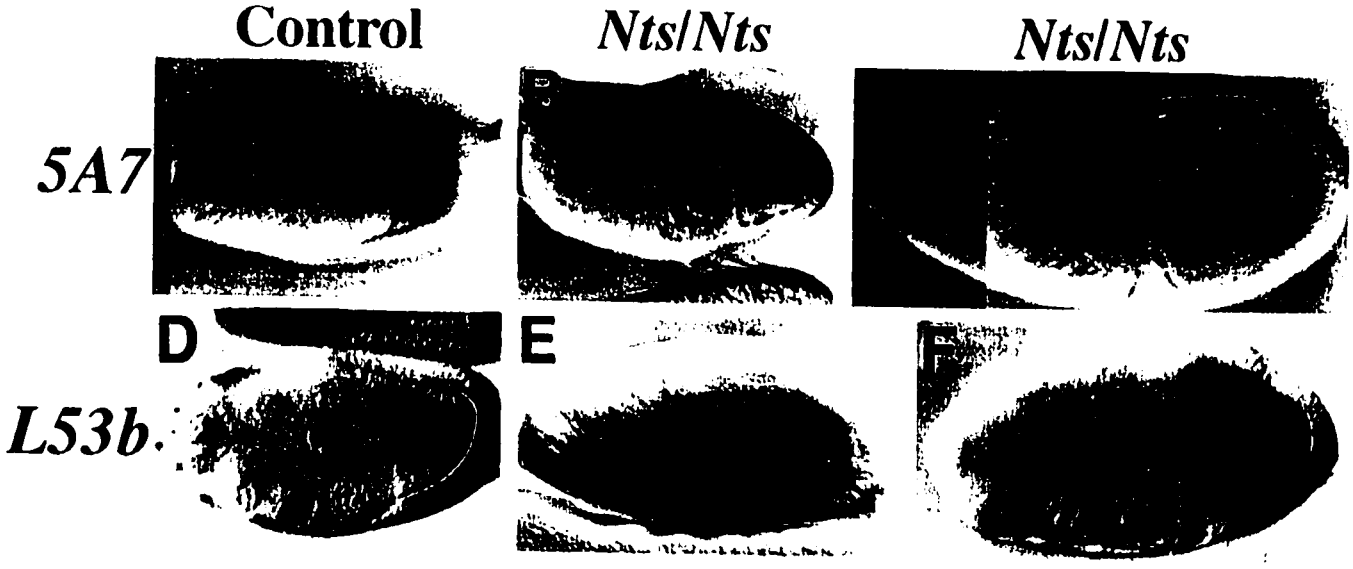

Control
Nts/Nts
Nts/Nts
5A7
D
E
F
L53b

Figure 2. Delta protein expression. (A-C) Delta protein expression in wild type ovarioles. Intense expression is detected in the membranes of follicle cells early (A), but begins to disappear from the posterior follicle cells around stage 6 (B). It is completely gone by stage 7 (C), and this pattern continues through the later stages of oogenesis. (D-G) Brackets indicate posterior follicle cells. (D,E) In *UAS-Dl; GAL4-109* egg chambers, a very high level of Delta protein is detected in various follicle cells, including posterior cells, from stage 6 (D) onward (E). No Delta protein is detected in *Dl; A62* in posterior follicle cells at stage 6 (F) but high levels are detected after stage 6 (G). *LacZ;c709* does not exhibits βgal expression during stage 6 (H), but has patchy expression in the anterior follicle cells during stage 7 (I), and then patchy expression throughout the follicle cell layer later. (J,K) *LacZ 107c1* expresses βgal in the border cells and the nurse cell associated cells, starting around stage 6.

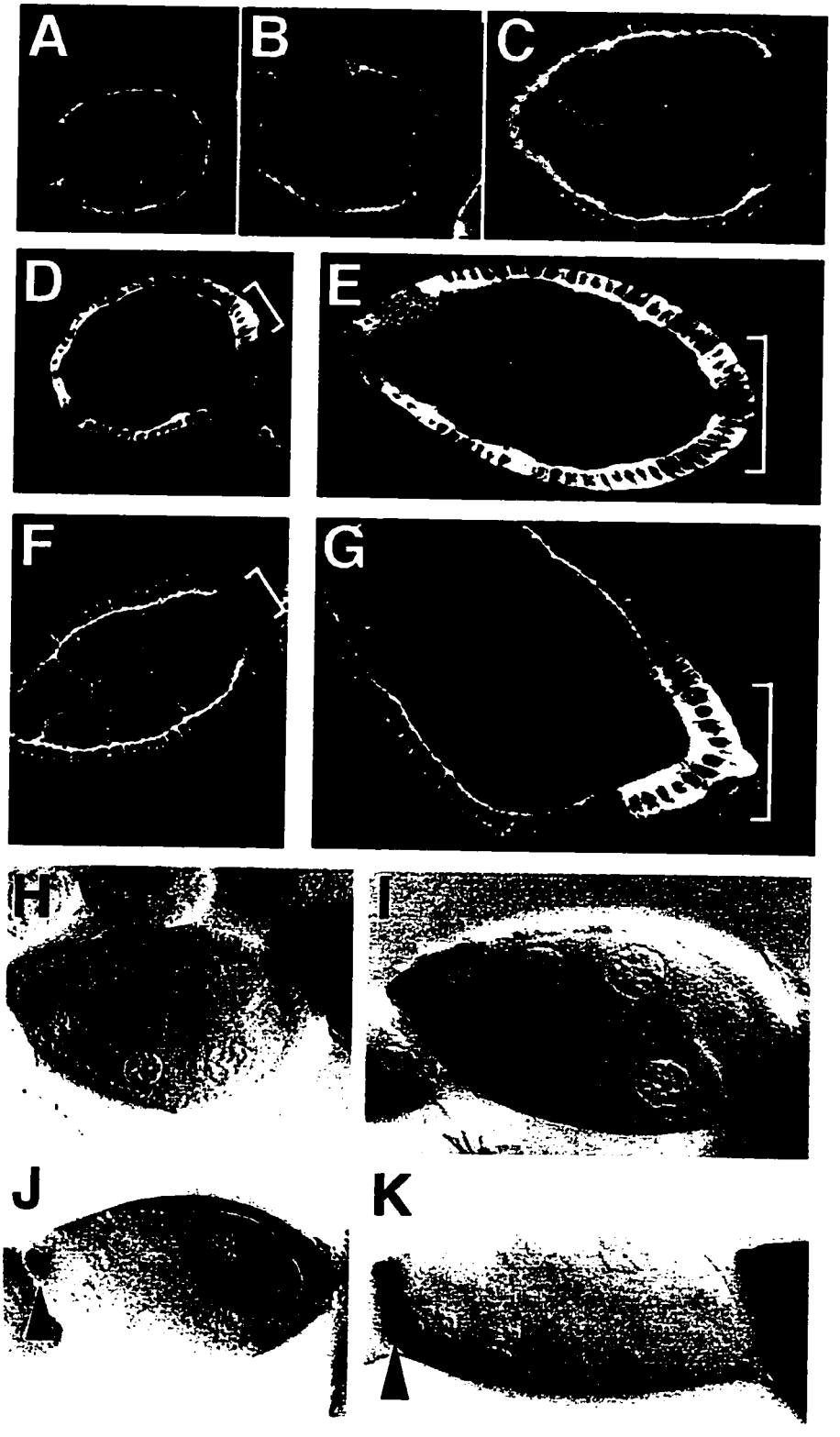

A
B
C
D
E
F
G
H
I
J
K

Figure 3. Prolonged expression of Delta disturbs posterior follicle cells. (A-C) In wild type ovarioles, *drl* mRNA is expressed in the posterior follicle cells until stage 6. *derailed* mRNA expression is prolonged in *UAS-Dl; GAL4-109* egg chambers (D-F).


A
B

Figure 4. Anterior markers are activated in posterior follicle cells in response to specific over-expression of Delta.

(A) The enhancer trap line *L53b* is expressed in anterior follicle cells: the border cells, nurse cell associated cells, and centripetally migrating cells. (B) *UAS-Dl; GAL4-109*. Prolonged expression of Delta leads to ectopic expression of *L53b* in posterior follicle cells in 34% of the egg chambers.

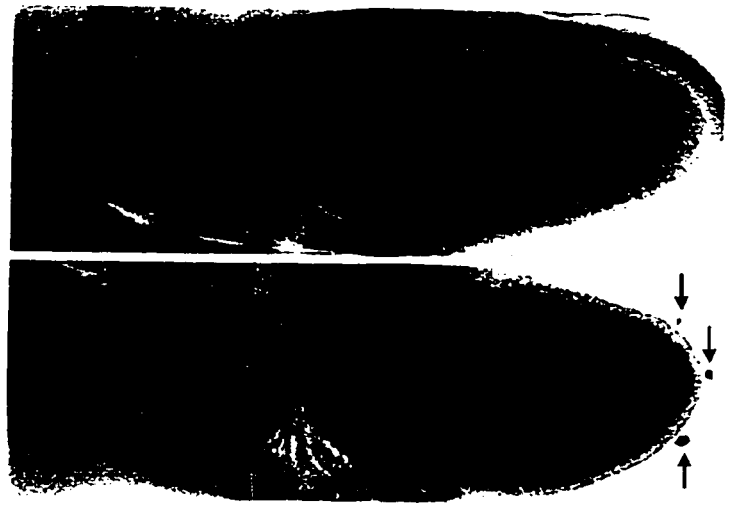


Figure 5. Prolonged expression of Delta in the follicle cells results in defects in the anterior-posterior axis of the oocyte. (A,C,E) wild type (B,D,F) *UAS-Dl; GAL4-109*. (A) *oskar* mRNA is tightly localized to the posterior pole of wild type oocytes. (B) Prolonged expression of Delta results in mislocalization of *oskar* to the center of the oocyte. (C) *bicoid* mRNA is found along the anterior of wild type oocytes. (D) With prolonged expression of Delta, *bicoid* is found at both the anterior and posterior end of the oocyte. (E) In wild type oocytes, a Kinesin-βgal fusion protein is found tightly localized to the posterior pole. (F) With prolonged expression of Delta, Kinesin-βgal is mislocalized to the center of the oocytes.

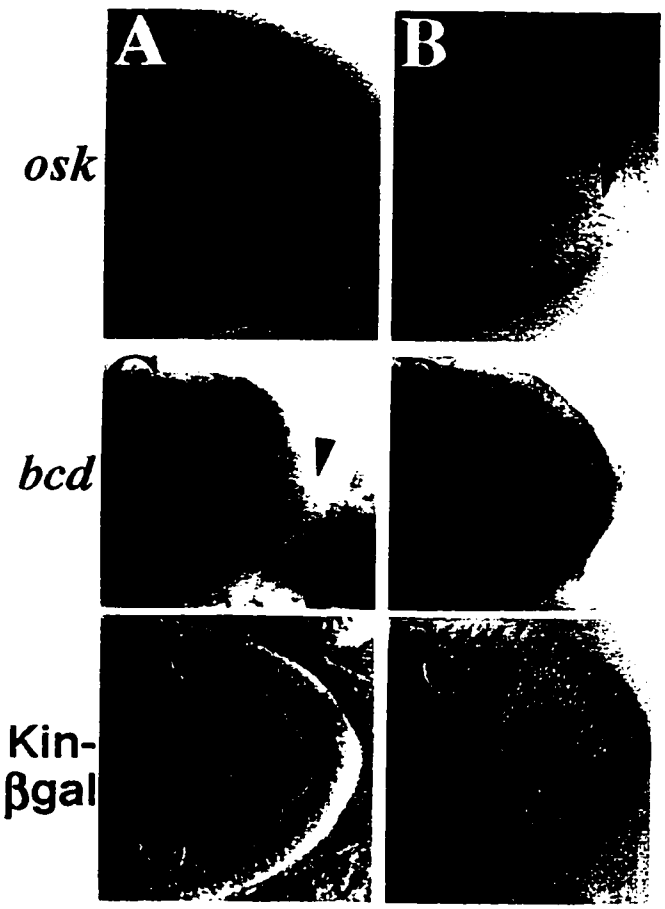

A
B
osk
bcd
Kin-
βgal

Figure 6. The defects in the anterior-posterior axis are the result of defects in the polarization of the microtubule cytoskeleton. Chambers are double stained; the left column is stained with antibodies to α-tubulin while the right column is stained with antibodies to Staufen. (A-B) wild type egg chambers. Microtubules are detected along the anterior edge of the oocyte, while Staufen protein is found at the posterior pole. (C-J) Mutant egg chambers. In all of these mutants, microtubules are detected at both the anterior and posterior of the oocytes, while Staufen is in a more central location. Occasionally the microtubules were detected as large "bundles" at the cortex of the oocyte. (C-D) *UAS-Dl; HS-GAL4*, 76.9% (20/26) have microtubule and Staufen phenotypes. (E-F) *UAS-Dl; GAL4-109*, 69.4% (25/36) have microtubule and Staufen phenotypes. (G-H) Dl^{vial} Dl^{6B37}, 48 hours at restrictive temperature with a 4 hour recovery at room temperature, 100% (19/19) have microtubule and Staufen phenotypes. (I-J) N^{ts}, 24 hours at restrictive temperature with a 4 hour recovery at room temperature, 100% (25/25) have microtubule and Staufen phenotypes.

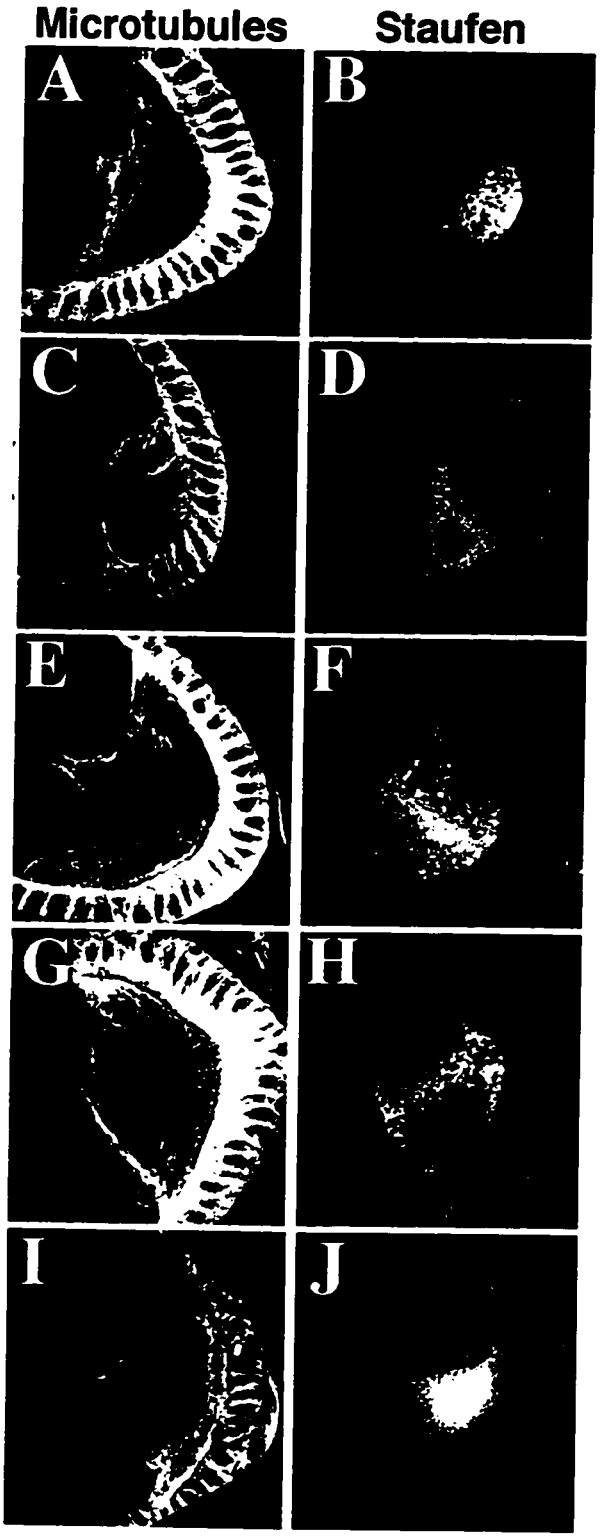

Microtubules
Staufen
A
B
C
D
E
F
G
H
I
J

Figure 7. Intermediate long stalks are composed of stalk cells and polar cells. (A) Diagram of the germarium. gc = germ cells, fc = follicle cells, bracket = precursor cells, red = stalk cells, blue = polar cells. (B) *HS-GAL4* induces the expression of the target gene in the follicle cells of the germarium. Six hours after heat shock induction, β-gal expression is detected by X-gal staining, starting in region 2 of the germaria of *HS-GAL4/UAS-LacZ* flies. Some variability in the staining pattern is detected, which might account for the incomplete penetrance of the phenotype. (C-F) Brackets = length of stalks (C) *93F* enhancer trap expression in control ovarioles. *93F* is expressed in the cells of the terminal filament and in all cells of the mature stalks. (D) 91% (20/22) of the intermediate long stalks generated by over-expression of Dl (*UAS-Dl; HS-GAL4*) contained at least some cells that did not express *93F* (in addition to *93F* positive cells). (E) *A101* enhancer trap expression in control ovarioles. *A101* is expressed in the polar cells starting in the germarium; no expression is detected in the stalks. (F) 78% (35/45) of the intermediate long stalks generated by over-expression of Dl contained *A101* positive cells.

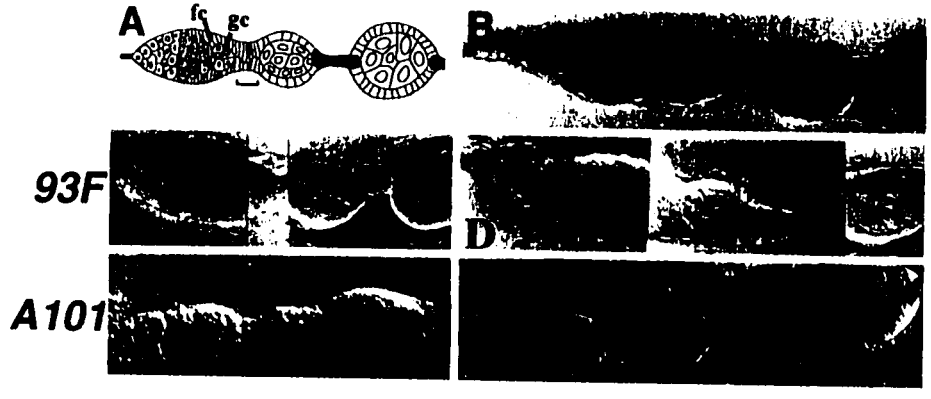

A
fc
gc
B
93F
D
A101

Figure 8. Extreme long stalks are composed of cells which are in the precursor stage. (A) In a control ovariole, *93F* is expressed in all mature stalk cells. (B) *93F* is not expressed in extreme long stalks generated by over-expression of *Delta* (*UAS-Dl; HS-GAL4*). Wild type (C,E) and *UAS-Dl; HS-GAL4* (D,F) ovarioles are double stained with FasIII and Big brain antibodies and visualized by confocal microscopy. (C) FasIII expression in a control ovariole is first detected in all follicle cells in region 2 in the germarium and becomes restricted to polar cell precursors by region 3. (D) In a *UAS-Dl; HS-GAL4* ovariole, all cells in the extreme long stalks are positive for FasIII. (E) Stalk cell precursors (arrow) and mature stalk cells (bracket) are stained with Bib antibody. Bib is first expressed in the apical tips of stalk precursor as they meet each other. As the stalk matures and the cells align as a single row, this staining pattern ceases and Bib is detected as a line along the lateral surface of the cells. (F) In *UAS-Dl; HS-GAL4* ovarioles, the cells in the extreme long stalks all stain with the Bib pattern detected in precursor cells, in 100% (41/41) of long stalks.

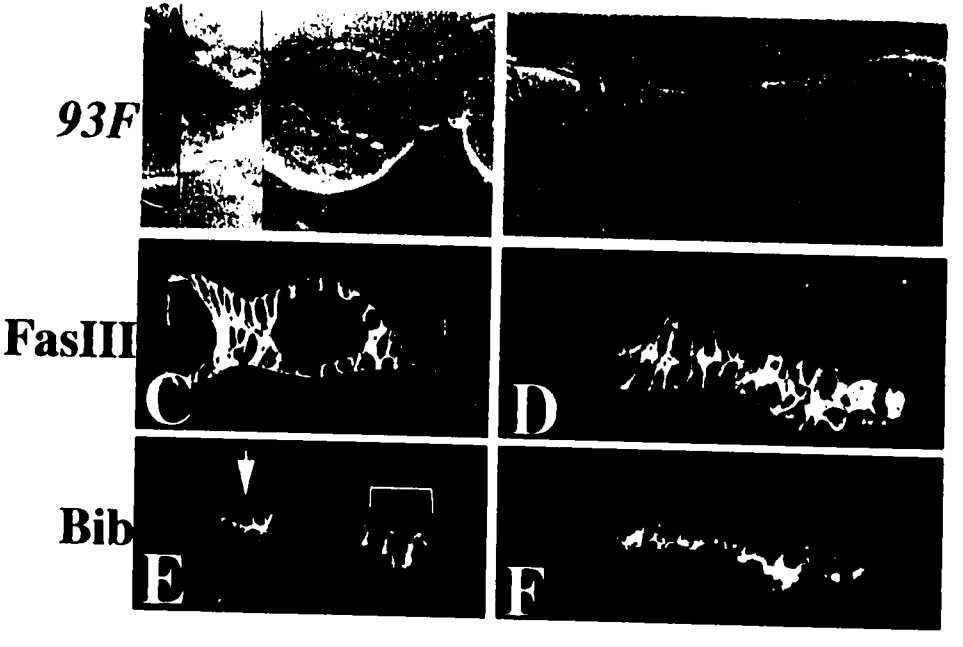
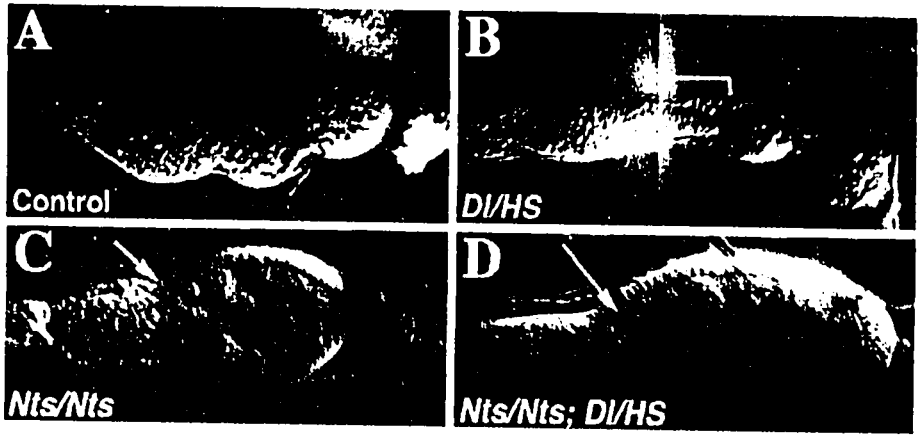

93F
FasIII
Bib
C
D
E
F

Figure 9. Delta acts through the Notch receptor to generate long stalks. Brackets = stalks. (A) Control ovariole. (B) Over-expression of *Delta* (*UAS-Dl; HS-GAL4*) results in a long stalk. (C) No stalk cells are detected in a N^{ts}/N^{ts} ovariole; arrow indicates follicle cells that have failed to form a stalk. (D) In N^{ts}/N^{ts}; *Dl/HS* ovariole no stalk cells are detected; arrow indicates follicle cells that have failed to form a stalk. The phenotype is the same as that found in N^{ts}/N^{ts} ovarioles.


A
Control
B
Dl/HS
C
Nts/Nts
D
Nts/Nts; Dl/HS

VITA

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1998

Michele Keller Larkin graduated with top honors from Western Washington University with a Bachelor of Science Degree in Biochemistry in 1993. She entered graduate school in the Department of Biochemistry in September of 1994 and received a predoctoral fellowship from Cytotherapeutics.

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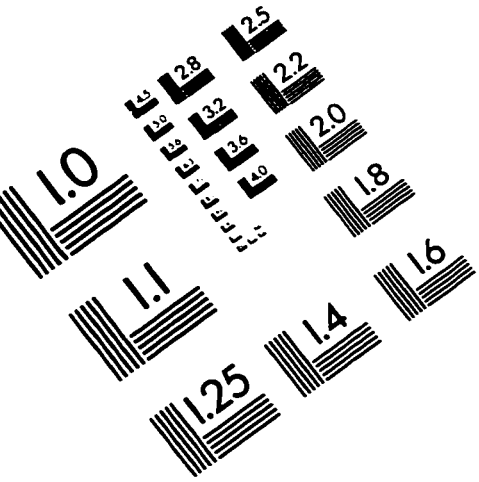
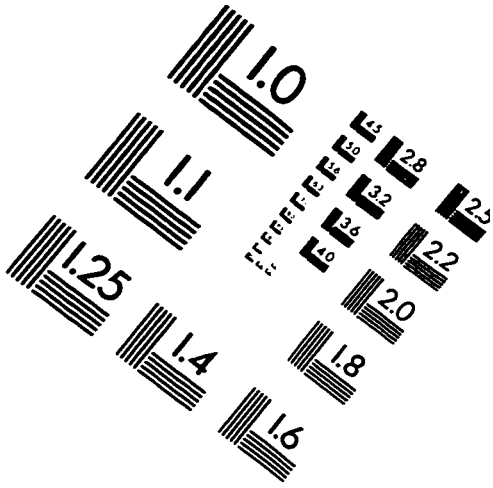
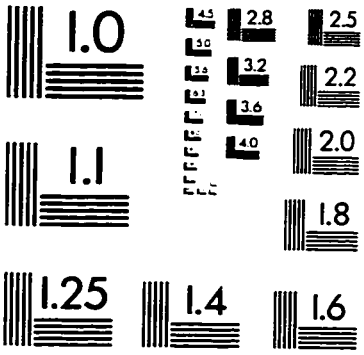
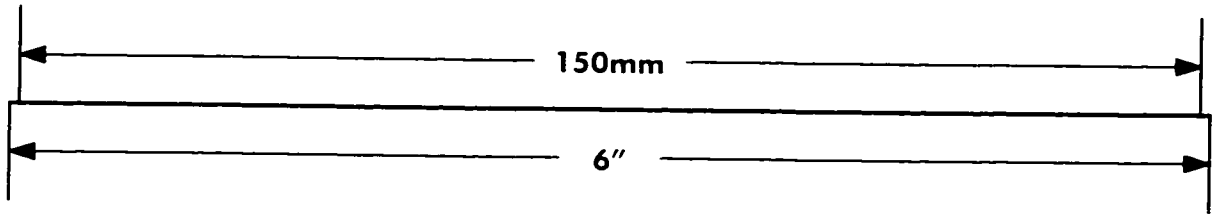
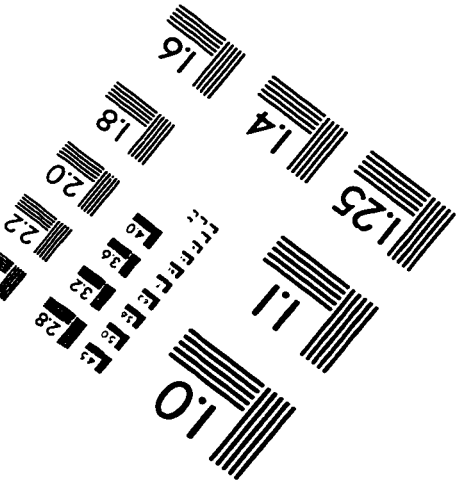
IMAGE EVALUATION TEST TARGET (QA-3)

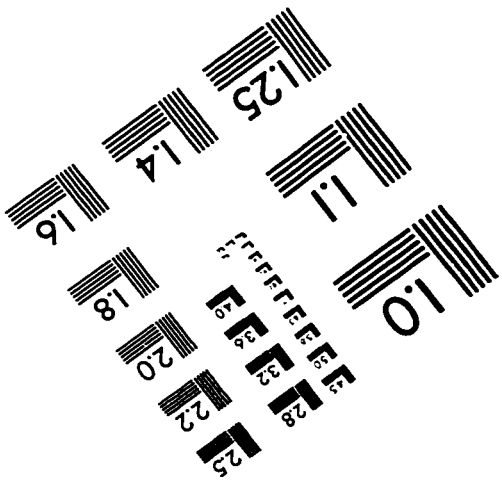












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