

The involvement of the *Notch* locus in *Drosophila* oogenesis

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Summary

The *Notch* gene in *Drosophila* encodes a transmembrane protein with homology to EGF that, in a variety of tissues, appears to mediate cell interactions necessary for cell fate choices. Here we demonstrate that oogenesis and spermatogenesis depend on *Notch*. We examine the phenotypes of the temperature-sensitive *Notch* allele, *N^{ts1}*, and, using a monoclonal antibody, determine the cellular and subcellular distribution of Notch protein during oogenesis. We show that *N^{ts1}* is associated with a missense mutation in the extracellular, EGF homologous region of *Notch* and that at non-permissive tem-

peratures oogenesis is blocked and the subcellular distribution of the protein is altered. In wild-type ovaries, Notch protein is found on the apical surface of somatically derived follicle cells, while in the germline-derived cells the protein is not polarized. These findings are discussed in view of the hypothesis that *Notch* acts as a multifunctional receptor to mediate developmentally important cell interactions.

Key words: *Drosophila* oogenesis, Notch protein, cell-cell interactions.

Introduction

The pioneering work of Nüsslein-Volhard and Wieschaus (1980), as well as a multitude of more recent molecular and genetic studies (reviewed, e.g. by Akam, 1987), has revealed groups of genes that act hierarchically to build the fly embryo. While some aspects of *Drosophila* morphogenesis depend on lineage relationships, the choice of cell fate within a particular tissue often depends on regulative events between neighboring cells. The *Notch* gene has been shown to play a central role in cell interactions necessary for regulative decisions throughout development (Artavanis-Tsakonas and Simpson, 1991).

Notch encodes an $\sim 300 \times 10^3 M_r$ transmembrane protein (we refer to this protein as "Notch") that spans the cell membrane a single time (Wharton et al., 1985; Kidd et al., 1986). The 1,700 amino acid-long extracellular domain of Notch contains 36 tandemly arrayed epidermal growth factor [EGF]-like repeats, as well as three cysteine-rich repeats - also found in two nematode genes (Yochem and Greenwald, 1989) - that are adjacent to the transmembrane domain. The remaining 1,000 amino acids define the intracellular domain, which displays sequence motifs found in other proteins; most notable are an array of repeats also occurring in the membrane cytoskeletal protein ankyrin (Lux et al., 1990) and certain transcription factors (e.g. Thompson et al., 1991).

Genetic and molecular analyses of *Notch* suggest that its gene product interacts with other proteins and is involved

in a cell interaction mechanism that mediates the differentiation of the embryonic nervous system (Poulson, 1940; Wharton et al., 1985; Fehon et al., 1990). The phenotypic analysis of mutants (Welshons, 1965), as well as expression studies (Johansen et al., 1989; Kidd et al., 1989; Fehon et al., 1991), indicate that wild-type *Notch* activity is necessary not only for the first steps of neural development but also for other embryonic and postembryonic developmental events that depend on cell interactions.

The present study describes the involvement of *Notch* in oogenesis which, as in neural development, requires extensive cell-cell interactions. Within the ovary, three different cell types contribute to the production of the egg: the oocyte, the nurse cells, and the follicle cells (King, 1970; Mahowald and Kambyzellis, 1980; Spradling, 1991). In the developing egg chamber, the follicle cells originate from somatic mesoderm and surround the oocyte-nurse cell complex that derives from the germline. These three cell types both provide the raw materials necessary to support the beginning of embryogenesis and play an important role in defining the spatial organization of the embryo. The germline and the somatic components of the ovary communicate with each other to determine the pattern of the somatically derived eggshell and the germline-derived embryo (Schüpbach, 1987).

In this paper, we describe the cellular and subcellular expression pattern of *Notch* during the development of the oocyte. We show that *Notch* is required in oogenesis and spermatogenesis by using a temperature-sensitive *Notch*

allele, *N^{ts1}*. In addition, we find that the molecular lesion associated with *N^{ts1}* is a missense mutation in the extracellular EGF homologous domain of *Notch*, and that the subcellular distribution of the protein is affected at non-permissive temperatures.

Materials and methods

PCR and Nucleotide sequencing

Genomic DNA for PCR was isolated from *N^{ts1}* adult flies as described in Grimwade et al. (1985). PCR amplification of *Notch* sequences was performed with the GeneAmp DNA amplification kit (Perkin Elmer Cetus, Norwalk, CT) and synthetic *Notch* primers (see below). The PCR reactions were done with the following program: (95°C for 45 seconds, 55°C for 1 minute, 72°C for 2.5 minutes) for 10 cycles, (95°C for 45 seconds, 58°C for 1 minute, 72°C for 5 minutes) for 20 cycles. Mutant DNA was amplified using five pairs of *Notch* synthetic primers (primers: S2860-A4213; S4202-A4981; S4970-A6361; S6340-A7681; S7650-A8854). Coordinates are given according to the position of the 3' end of each oligonucleotide relative to the *Notch* sequence in Wharton et al. (1985). ("S" refers to sense strand of this sequence, "A" to the antisense strand.) The products of the PCR reactions were treated with the Klenow fragment and subcloned into the *Sma*I site of the pEMBL18(+) vector (Dente et al., 1983). Multiple independent subclones were recovered for each region. Single-stranded DNA from these subclones was primed either with synthetic primers derived from wild-type *Notch* sequence or with the M13 universal primer, and sequenced by the dideoxynucleotide chain termination procedure (Sanger et al., 1977; Toneyguzzo et al., 1988) using Sequenase (United States Biochemical).

Immunocytochemistry of *Drosophila ovaries and testes*

1 to 2 day-old adult flies were fed with wet yeast for 3 to 5 days. Ovaries were then dissected by pricking the posterior abdominal region with a needle and pushing them out into 1×PBS. Ovaries were transferred into either a 15 ml plastic tube or a 2.0 ml polypropylene microcentrifuge tube and dissociated into ovarioles by either pipetting or manually shaking the tubes before fixation. Testes were manually dissected from adult males.

The fixation and staining procedures for ovarioles and testes using the anti-*Notch* monoclonal antibody C17.9C6 were essentially those described previously for imaginal discs (Fehon et al., 1991), with the following changes. Fixation was done in 4% paraformaldehyde. For non-fluorescent preparations, HRP-conjugated goat anti-mouse IgG (Jackson Laboratories) was used at a 1:500 dilution in BSN (40 mM NaCl, 50 mM KCl, 10 mM MgSO₄, 6 mM CaCl₂, 10 mM tricine, 20 mM glucose, 50 mM sucrose, 0.2% BSA, 3% normal goat serum, 0.1% Saponin) for two hours. HRP reactions were performed in 0.5 mg/ml diaminobenzadine (DAB) and 0.015% hydrogen peroxide for 3-5 minutes. To stain nuclei fluorescently, propidium iodide (150 μM in H₂O; Molecular Probes, Inc., Eugene, OR) was added after the final wash at a 1:3000 dilution, shaken for 1 minute and then rinsed before mounting. Alternatively, tissues were incubated in 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) in BSN for 10 minutes, followed by rinsing. Ovarioles and testes were mounted as previously described (Johansen et al., 1989; Fehon et al., 1991).

Microscopy

Confocal images were collected using either a Bio-Rad MRC-500 or a MRC-600 system attached to a Zeiss Axiovert compound

microscope. In all cases, the programs BASE and SCALE were run on images as they were collected (software provided by Bio-Rad). Where noted in the text, optical sections were combined using the PROJECT program. For photo reproduction, image files were transferred to a Macintosh IIfx computer either using Apple File Exchange or MacLink (DataViz, Inc.), converted to PICT file format using IPLabSpectrum (Signal Analytics Corporation) or Adobe Photoshop (Adobe Systems, Inc.), and arranged and annotated using Aldus Persuasion (Aldus Corporation).

Non-confocal photomicrographs (Figs 5 and 6D, E and F) were made using a Leitz Orthoplan 2 microscope equipped with DIC optics and epifluorescence. Fig. 5 is a HRP preparation that was changed to grey scale using Adobe Photoshop (Adobe Systems, Inc.). The DAPI and HRP preparations in Fig. 6D-F are from color slides that were scanned, using a Microtek ScanMaker 1850 (Microtek Lab, Inc.), into Adobe Photoshop and changed to grey scale.

Results

The expression of Notch during oogenesis

In situ hybridization analyses have revealed the existence of *Notch* transcripts in the ovaries, while genetic studies have indicated a maternal requirement for *Notch* activity in embryogenesis (Markopoulou and Artavanis-Tsakonas, 1989). The present study extends the genetic findings by examining the expression pattern of the Notch protein in ovaries. A monoclonal antibody (C17.9C6) that recognizes the intracellular domain of Notch (Fehon et al., 1990) was used to stain ovaries dissected from wild-type Canton-S females. For confocal microscopy, the ovaries were treated with either FITC- or CY3-conjugated secondary antibody; propidium iodide was used to stain the nuclei of the cells (see also Materials and methods). Throughout the following discussion, the terminology used to describe the various anatomical features of the ovarioles is the one used by King (1970), Mahowald and Kambysellis (1980) and Spradling (1991).

Germarium and Stages 1-6

The pattern of Notch expression distinguishes the three regions that comprise the germarium (Fig. 1A-C). Very low level staining is seen in the cytoplasm of the germline cells in region I, a mitotically active area containing clusters of up to 16 interconnected cystocytes. Higher levels are detected in region II, where mesodermal cells detach from the tunica propria and migrate, eventually engulfing each cluster of 16 cystocytes and thus producing the follicular envelope. Region II is characterized by cell clusters arranged across the width of the germarium that have a "convex, lens-shaped appearance" (Mahowald and Strassheim, 1970). In this region, low level cytoplasmic staining shows Notch expression in the cyst cells, while cells with elongated nuclei (Fig. 1A-B) show relatively high levels of Notch. We presume that the latter are the lens-shaped follicle cells that surround the egg chambers (Mahowald and Strassheim, 1970). The most posterior sixteen-cell cyst in the germarium resides in region III, where Notch is expressed at the apical surface of the cells comprising the follicular envelope (Fig. 1C). High levels of the protein are also seen in the boundary between regions II

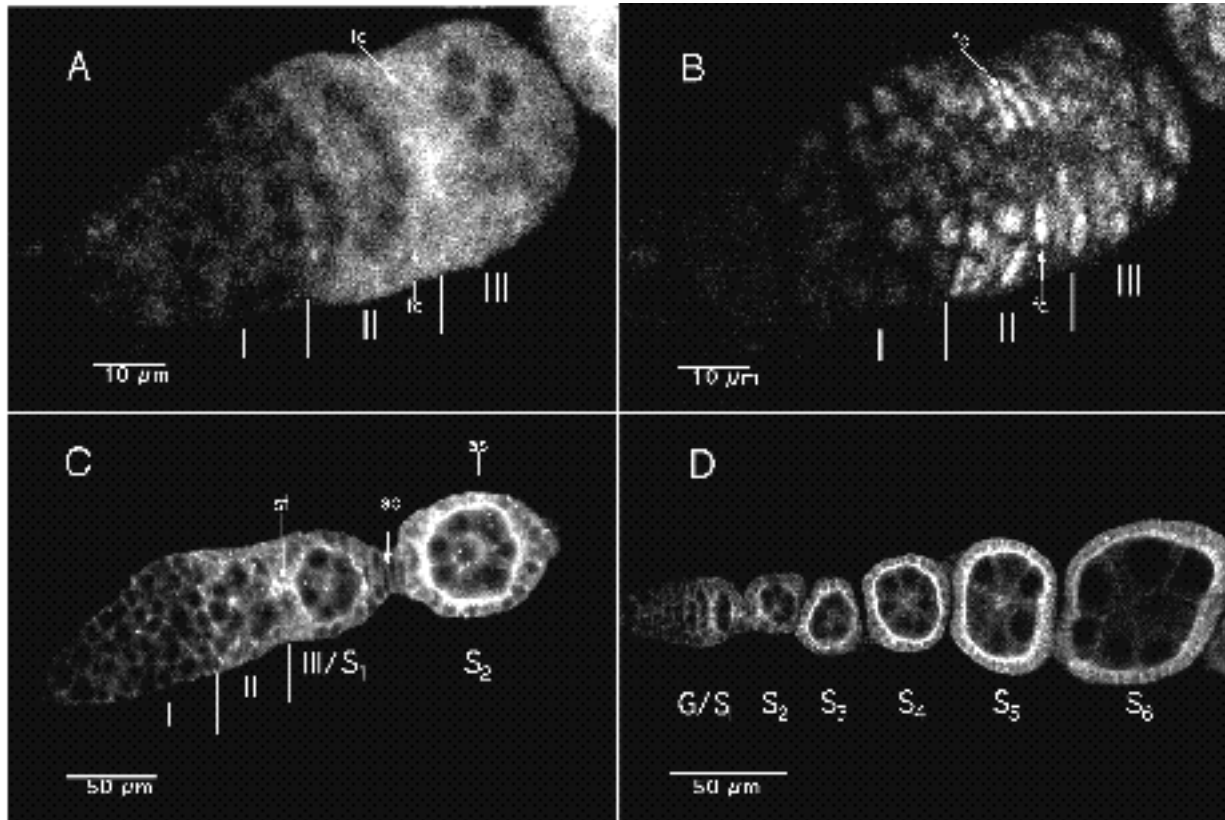


Fig. 1. Notch expression in the germarium and stages 1 through 6 of wild-type *Drosophila* egg chambers as shown by confocal microscopy, using the monoclonal antibody C17.9C6. Images A and B are a double-labelled optical section of a germarium expressing Notch (A) and propidium iodide (B). Note the increased Notch expression in Region II of A where mesodermal follicular cell invasion occurs (fc). The nuclei of these “convex, lens-shaped” cell clusters (Mahowald and Strassheim, 1970) are clearly seen in B. In (C), an optical section of a germarium and stage 2 egg chamber, high Notch expression is seen in the area between regions II and III where follicular stalk formation begins (sf). Some of the cells in this area will give rise to the stalk cells, while others will contribute to the follicular envelope. Note the stalk cells themselves (sc) do not express Notch. Notch is highly expressed at the apical surface (as) of the follicle cells from stages 1 through 6 (C and D). The nurse cells express Notch at a lower level throughout these stages.

and III, an area populated by follicular cells that will eventually form the follicular stalk connecting the vitellarium and the germarium (Fig. 1C; King, 1970). However, at later stages the stalk proper does not appear to express Notch as strongly as the other follicle cells (Fig. 1C, sc).

The follicle cells divide continuously up to stage 5 and cease mitoses during stage 6. Throughout this time, Notch is expressed abundantly and is highly polarized toward the apical end of the cell (Fig. 1D). Examined with immunofluorescence, Notch appears to be concentrated in vesicular structures within the cytoplasm located primarily near the apical surface and extending basally (Fig. 2A). After stage 6, when overall Notch expression decreases noticeably (Fig. 2A), these vesicular structures are much less numerous, and Notch protein appears to be primarily membrane associated. In contrast to the follicle cells, nurse cells express Notch in a non-polar manner and at much lower levels. Both the non-polar expression of Notch on nurse cell membranes and the reproducible low-level cytoplasmic staining are maintained throughout later stages.

Stages 7, 8, 9 and 10

At stage 7, the last previtellogenic stage, the egg chamber

begins to elongate as the follicle cells enlarge, begin polyploidization and become columnar (King, 1970). Even though Notch is expressed less intensely during this stage, its relative distribution remains comparable to the earlier stages in that the follicle cells continue to express higher levels than the nurse cells (Fig. 2B). Optical z-series done on this stage show that the distribution of Notch protein around the egg chamber is not totally uniform: the follicle cells apposed to the oocyte appear to express slightly more Notch protein than the others (data not shown). At stage 8, when yolk formation begins, Notch staining is at its lowest, but more expression is seen in the follicle cells abutting the oocyte (Fig. 2C).

Fig. 3A-C show Notch expression in stages 9 and 10. By the end of stage 9, most of the follicle cells that once covered the nurse cells have moved posteriorly over the oocyte (Fig. 3A). In general, Notch expression within the follicle cells has decreased, with the exception of the anterior-most follicle cells, which are still migrating posteriorly. At this stage, a group of follicle cells, the border cells (Fig. 3A), are in the midst of their migration from the anterior egg chamber, travelling between the nurse cells toward the anterior surface of the oocyte (Mahowald and Kambyzellis,

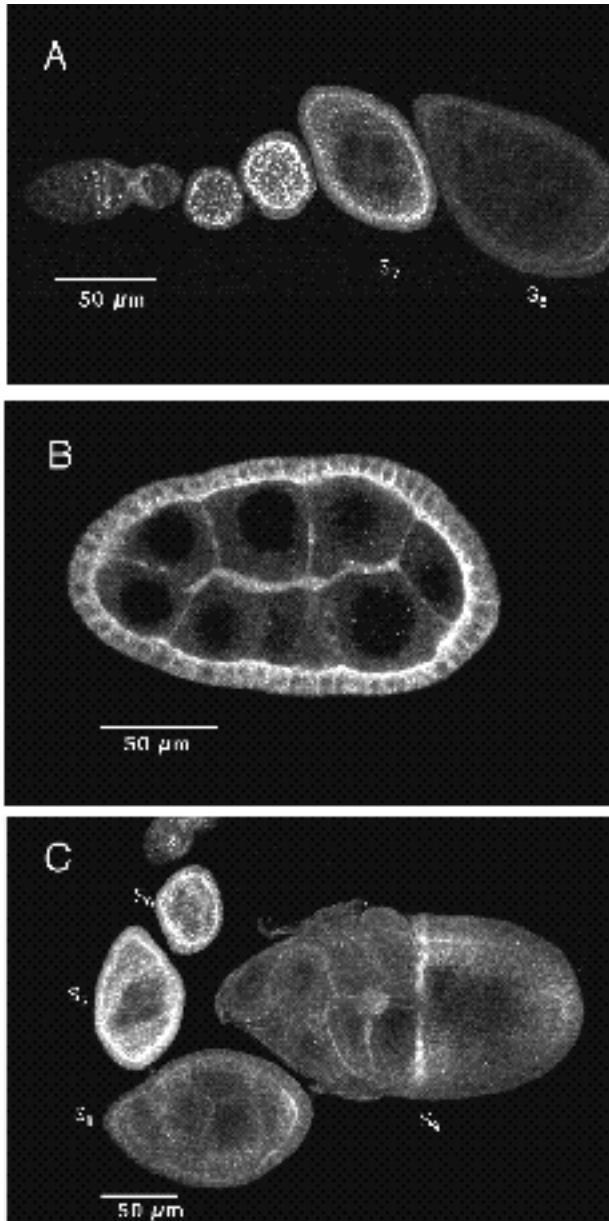


Fig. 2. Notch expression in early-to-mid stages of wild-type egg chambers as shown by confocal microscopy, using the monoclonal antibody C17.9C6. (A) is a linear projection of ten confocal optical sections (2 μm increments) of early-to-mid stage wild-type egg chambers. In early stages, Notch appears to be concentrated in vesicular structures within the cytoplasm and located primarily near the apical surface. After stage 6, these vesicular structures are much less numerous as Notch expression drops noticeably; Notch protein appears to be primarily membrane associated. (B) is an optical section of an early stage 7, wild-type egg chamber in which Notch continues to be expressed apically in the follicular envelope. Notch is also expressed at lower levels, both at the surface and within the cytoplasm of the nurse cells. The expression of Notch in the follicle cells drops noticeably during stage 7 and even more so by stage 8 (A and C). (C) is a linear projection of 10 confocal optical sections (2 μm increments) of stages 6, 7, 8 and what appears to be a stage 9 (or slightly later) egg chamber. At stage 8, the follicle cells show low expression, except for those abutting the oocyte. At stage 9, Notch is apically expressed in the follicle cells at the anterior end of the developing oocyte (C,S₉).

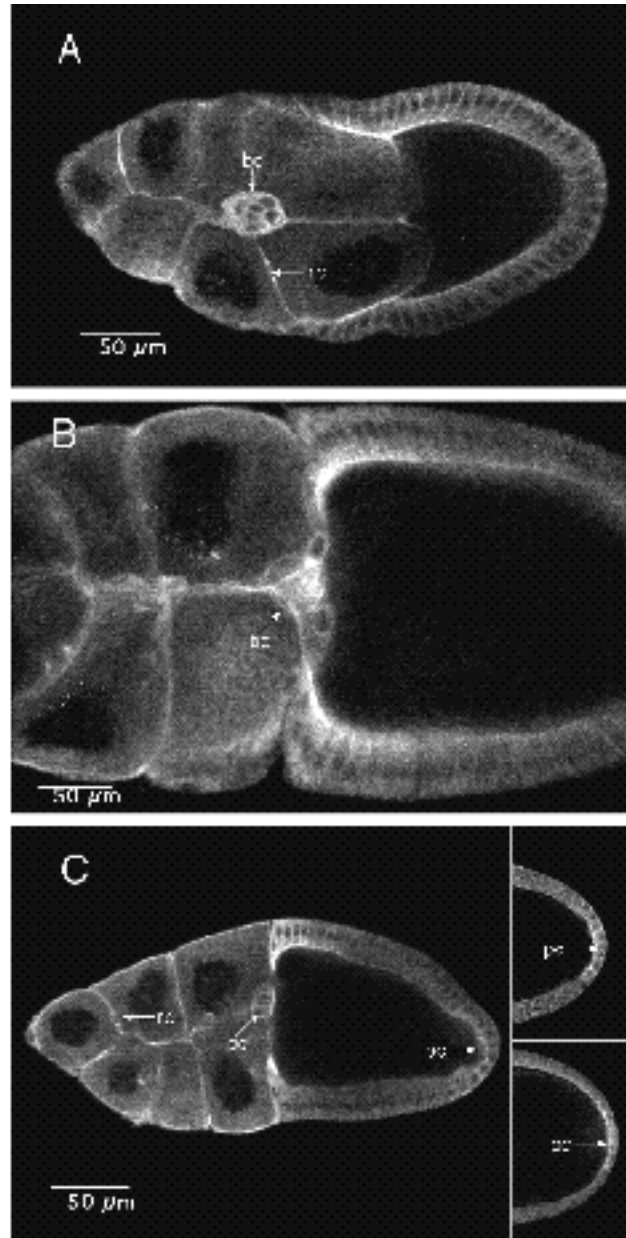


Fig. 3. Confocal optical sections showing Notch expression in stages 9 (A), 10A (B), and 10B (C) wild-type egg chambers. B is a linear projection of six sections (1.8 μm increments). The border cells (bc) show high levels of Notch expression throughout their migration (A, B, C); the nurse cells also express Notch at a lower level and clearly outline the ring canals (A, C, rc). Note Notch expression in the follicle cells at the anterior-dorsal and -ventral ends of the oocyte and in a few follicle cells at the posterior end of the oocyte (C, pc). Although Notch is strongly expressed at both the anterior-dorsal and anterior-ventral ends of the oocyte, it is consistently of greater intensity anterior-dorsally, where the oocyte nucleus lies (A, B, C).

1980; Spradling, 1991). Notch is highly expressed in the border cells. At stage 10A (Fig. 3B), these cells move dorsally where, at stage 10B, they continue to express Notch (Fig. 3C). At stage 10, all columnar follicle cells are in contact with the oocyte and the follicle cells at the anterior of

the oocyte start a centripetal migration (Mahowald and Kambyzellis, 1980; Spradling, 1991). At this time, the follicle cells have almost ceased to express Notch, with the notable exception of the centripetally migrating cells and a few cells at the very posterior end of the oocyte (Fig. 3C). The migrating cells at the anterior dorsal surface - those that are close to the oocyte nucleus (Mahowald and Kambyzellis, 1980; Spradling, 1991) - appear consistently more intensely stained (Fig. 3C). At these stages, the thin, squamous follicle cells that cover the nurse cells also express Notch (data not shown), while the oocyte continues to be devoid of Notch protein. In addition, Notch is found at the surface of the nurse cells, including the ring canals that connect them (e.g. Fig. 3A).

Stages 11, 12, 13 and 14

From stage 11 on, the follicular epithelium, which becomes thinner and starts secreting the endochorion, has essentially ceased to express Notch (Fig. 4A). However, Notch is expressed on the surface of the nurse cells and in the border cells. Notch is associated with the membranes of the nurse cells - including the ring canals (Fig. 4A) - which collapse over the nurse cell nuclei as the cytoplasm is lost during dumping (Fig. 4B) and eventually disappear as the nurse cells degenerate (Fig. 4C). The border cells, which are thought to be involved in the development of the micropyle - a conical chorion structure having a central hole serving as a sperm entry point - (King, 1970; Spradling, 1991), express Notch more strongly at their apical ends, forming a ring of expressing cells around the developing micropyle (Fig. 4C). While the micropyle itself and the developing dorsal appendages appear to stain for Notch (Fig. 4B-C), control experiments using non-specific antibodies also stain these structures (data not shown). We therefore cannot draw firm conclusions about Notch expression in these structures.

The molecular lesion of *N^{ts1}*

To elucidate the role of Notch in oogenesis, we examined the ovarian phenotypes of homozygous *N^{ts1}* females at non-permissive temperatures. Shellenbarger and Mohler (1978) have shown that the *N^{ts1}* allele at restrictive temperatures affects many developmental stages and developmental events. Given the pleiotropic effects of *N^{ts1}* and the complex structure of Notch, knowing the molecular lesion of the *N^{ts1}* mutation is essential in understanding the phenotypes associated with the *N^{ts1}* allele.

Intragenic meiotic recombination has shown that *N^{ts1}* is positioned between *split* and *N^{60g11}*, close to *Ax^{9B2}* (W.J. Welshons, personal communication). *split* is a missense mutation in the 14th EGF-like repeat of the protein (Hartley et al., 1987; Kelley et al., 1987); *N^{60g11}* maps N-terminally to the *notchoids*, which are mutations associated with molecular lesions near the C-terminal region of the protein (Xu et al., 1990); *Ax^{9B2}* is a missense mutation in the 23rd EGF-like repeat (Kelley et al., 1987).

To determine the molecular lesion associated with *N^{ts1}*, 6.23 kb spanning the sequences from the 16th EGF-like repeat to the end of the open reading frame was subdivided into five overlapping regions. Mutant DNA from each region was amplified by PCR reactions with ten different Notch synthetic primers (see also Materials and methods).

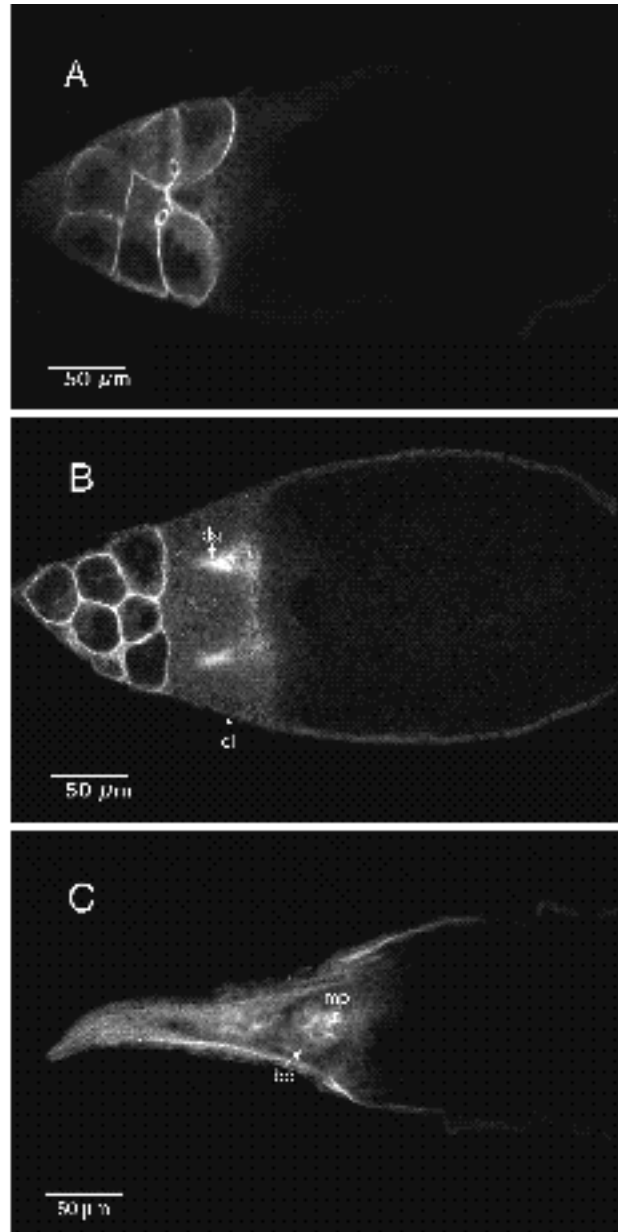


Fig. 4. Confocal optical sections of stages 11 (A), 12 (B) and 13 (C) wild-type *Drosophila* egg chambers. Notch expression is almost solely localized in the nurse cell boundaries, including the ring canals. By stage 12 (B), Notch continues to be present over the nurse cell cluster which has moved toward the anterior. The developing dorsal appendages (B,da) are autofluorescent as is the forming chorion layer (B,cl). At stage 13, Notch expression is lost along with the nurse cell remnants. Note that the ring-shaped border cells in stage 13 (C,bc) express Notch around the developing micropyle (mp)

Multiple independent subclones were recovered for each region and sequenced. Several changes from the published wild-type sequence were found (Wharton et al., 1985; Kidd et al., 1986; Hartley et al., 1987). Only one of these alterations results in a changed amino acid sequence: a G-to-A transition (nucleotide 4556, according to Wharton et al., 1985) resulting in a glycine-to-aspartic acid change at

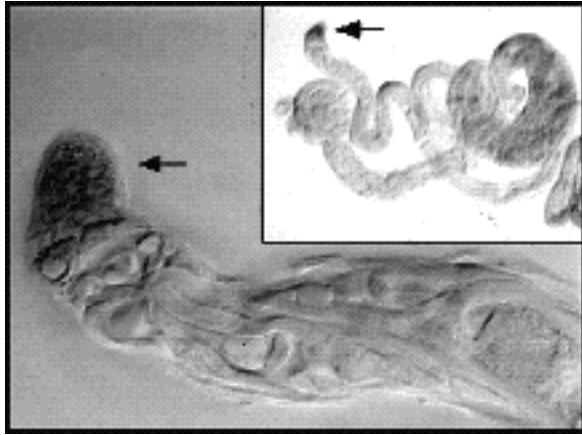


Fig. 5. DIC view of wild-type *Drosophila* testis (inset depicts entire testis), using the *Notch* monoclonal antibody C17.9C6 and HRP. Notch expression is seen at the tip of the testis (arrow), a region of gonial proliferation.

amino acid position 1272 within the 32nd EGF-like repeat (Wharton et al., 1985). We note that this glycine is highly conserved among EGF-like sequences. This position is compatible with the mapping data obtained by meiotic recombination. As is shown below, the *N^{ts1}* mutants produce protein at the non-permissive temperature. These findings are compatible with the notion that *N^{ts1}* is not a complete loss-of-function mutation.

The mutation N^{ts1} affects both female and male sterility

Eggs of normal morphology, capable of initiating embryogenesis following fertilization, can be produced from mothers bearing homozygous *Notch* mutant germline clones (Jimenez and Campos-Ortega, 1982; Wieschaus and Noell, 1986). Despite being zygotically heterozygous for *Notch*, such embryos fail to complete embryogenesis, indicating a maternal requirement of *Notch* for normal development. Consistent with these observations, both protein and transcripts are seen in the germline-derived cells. In addition, we observe high levels of Notch on the apical surface of the follicle cells, suggesting that *Notch* may be required in the somatic cells for normal oogenesis. In an attempt to address these issues, we examined the effects of the *N^{ts1}* mutation on female fertility and ovarian development.

Homozygous *N^{ts1}* females, which are fertile at permissive temperature (18°C), were mated to Canton-S wild-type males and kept at non-permissive temperature (29°C or at 32°C) for three days. Females treated in this manner failed to produce any fertile eggs. In contrast, *N^{ts1}* females carrying a copy of a transposon that contains all necessary *Notch* gene sequences (cos479, Ramos et al., 1989) were fertile under the same conditions (although such females laid less than 50% of the number of eggs laid by wild-type flies). The sterility of heat-treated *N^{ts1}* females is completely reversible when they are returned to the permissive temperature. We conclude from these results that wild-type *Notch* function is required for female fertility.

In the course of these experiments, we observed that the fertility of *N^{ts1}* males is dramatically reduced after exposure to non-permissive temperatures (29°C), while that of

the males carrying the transposon is relatively unaffected (data not shown). Consistent with an involvement of *Notch* in spermatogenesis, we find Notch expression in the region of gonial proliferation at the tip of the testis (Fig. 5; Lind-sley and Tokuyasu, 1980).

N^{ts1} mutant phenotypes in oogenesis

Both the expression pattern of wild-type Notch in ovaries and the finding that *N^{ts1}* animals are sterile at non-permissive temperatures indicate a requirement for *Notch* during oogenesis. We investigated further the function of *Notch* in oogenesis by examining the ovaries of homozygous *N^{ts1}* females after incubation at 29°C or 32°C for 24 or 72 hours. The ovaries were then stained for Notch using mAb C17.9C6. Approximately 20% of the ovarioles appeared abnormal after 24 hours of exposure at the non-permissive temperature, while, after 72 hours, the time that leads to complete sterility, almost all ovarioles appeared mutant. In contrast, *N^{ts1}* animals carrying Cos 479, the *Notch* transposon mentioned earlier, had wild-type ovaries.

Fig. 6 shows some typical mutant phenotypes. The phenotype that we observed most frequently is characterized by nurse cell-oocyte complexes that appear fused into large irregular shapes (Fig. 6A-E), which seem to result from the failure of the stage-1 egg chamber to separate from the germarium. As can be seen in Fig. 1C (sf), the follicle cells between regions II and III of the germarium, some of which will eventually give rise to the follicular stalk, express relatively high levels of Notch. If Notch expression is crucial for normal stalk development, and if normal egg chamber budding depends on a normal follicular stalk, then it is possible that the failure of budding in the *N^{ts1}* ovaries is the direct consequence of Notch malfunction.

DAPI staining shows that the irregularly shaped chambers contain germline nuclei of different ploidy (Fig. 6D-E). Consistent with the Notch expression seen in later stages, *N^{ts1}* ovaries are also associated with later mutant phenotypes. Phenotypes are especially obvious in the sizes of the oocytes and the formation of the dorsal appendages. Many of the eggs whose oocytes occupy only about 50% of an egg chamber start developing appendage structures. In addition, the dorsal appendages, which are secreted by two groups of follicle cells migrating anteriorly in stages 12-14, are malformed (Fig. 6F). It is not possible to establish the stage of these chambers. While the underlying cause for the observed phenotypes is unclear, we take our results to be more consistent with the notion that the phenotypes result from abnormal nurse cell cytoplasm transport to the oocyte rather than, for example, abnormal appendage formation.

Notch protein is clearly detected in follicle cells in *N^{ts1}* ovaries at the non-permissive temperature. However, the extreme apical polar localization of Notch seen in the wild type is much less obvious and, in some cases, has disappeared altogether (compare Fig. 6A to Fig. 1C-D and Fig. 2B). Additionally, as seen in Fig. 6A, the follicular envelope is sometimes non-contiguous; the propidium iodide labelling of this preparation showed that the gaps contained no nuclei, indicating absence of follicle cells (data not shown). Moreover, unlike the wild type, the level of expression between follicle cells is not uniform, that is,

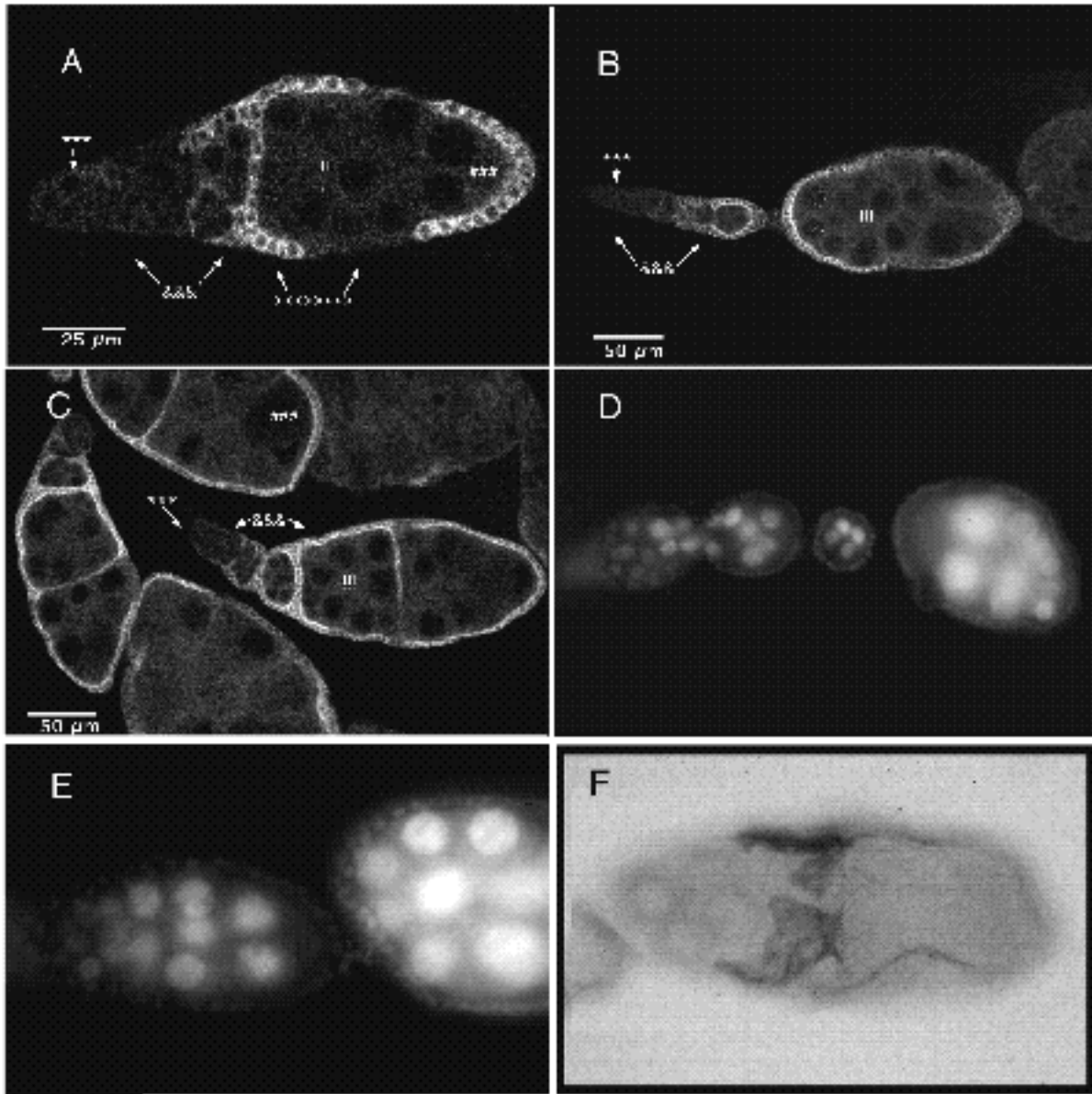


Fig. 6. (A-C) *Notch*-labelled confocal optical sections of *N^{ts1}* egg chambers heat-shocked for 72 hours at 32°C. These show characteristic heat-shocked phenotypes, for example: no obvious regional differentiation within the germarium and decreased or no *Notch* expression (***) ; unseparated germarium and presumptive later stages, resulting in misshapen, non-spherical egg chambers (&&&); increased, non-apical *Notch* expression in the follicle cells surrounding the egg chamber and missing follicle cells (><<><<>); loss of *Notch* expression on the surface of the nurse cells (!!!); distorted, seemingly missing or non-polar oocytes (###). (D, E) DAPI-stained *N^{ts1}* ovaries heat-shocked for 24 hours at 32°C. Note the differing ploidy of the nuclei within the same egg chamber. (F) HRP-stained *N^{ts1}* ovary showing dorsal appendages forming when the oocyte occupies not more than 50% of the ovarian chamber and having a distorted shape and larger-than-normal size. Although *Notch* antibody is used, wild-type dorsal appendages also show *Notch* expression in controls using only HRP.

some follicle cells express *Notch* at much higher levels than others (Fig. 6B-C). In the mutant, we also note that *Notch* is absent from nurse cells (Fig. 6A-C).

Discussion

Both mutant analyses as well as various expression studies revealed that the *Notch* locus is involved in a multitude of

events throughout development. The studies carried out thus far implicate *Notch* in regulative aspects of development and in the choice of cell fate (reviewed in Artavanis et al., 1991; Artavanis-Tsakonas and Simpson, 1991). In this paper, we have presented data regarding the expression of *Notch* in the ovaries and related it to the phenotypes associated with a conditional mutation at the *Notch* locus, *N^{ts1}*. *Notch* is expressed throughout oogenesis in somatic as well as germline-derived cells, displaying a pattern that is

dynamic at both cellular and subcellular levels. Furthermore, we find that oocyte development is blocked in *N^{ts1}* animals at non-permissive temperatures. Together, these data indicate that the *Notch* locus plays an important role in oogenesis.

Notch activity in the germline cells

Previous studies involving germline mosaics have shown that germline cells lacking *Notch* function form eggs that appear normal but give rise to inviable embryos (Jimenez and Campos-Ortega, 1982; Wieschaus and Noell, 1986). In situ hybridization studies have demonstrated *Notch* transcripts first in the nurse cells and then later on (stages 9 and 10) in the entire egg chamber. This suggests that *Notch* is transcribed in the nurse cells and, subsequently, the RNA is transported into the maturing oocyte (Markopoulou and Artavanis-Tsakonas, 1989). Although we do find *Notch* at the surface and in the cytoplasm of the nurse cells throughout oogenesis, we have not detected protein in the oocyte, contrary to what has been quoted as our finding by Ruohola et al. (1991). It therefore appears that the expression pattern of *Notch* in germline-derived cells reflects a dual role in oogenesis. On one hand, *Notch* RNA is transported from the nurse cells into the oocyte, where it is used during embryogenesis. On the other, *Notch* mRNA is translated in the nurse cells and presumably contributes to the development of the egg chamber and oocyte. Whether or not the mRNA that is translated in the nurse cells is different from that transported into the oocyte remains to be determined. It is worth noting, however, that preliminary evidence based on Northern analysis indicates that the early embryo has two species of *Notch* RNA - they differ by about two hundred nucleotides (C.C. Koczynski and M.A.T. Muskavitch, personal communication). One of these seems to be enriched in unfertilized eggs.

Immunofluorescent stainings of early embryos indicate that *Notch* protein expression cannot be detected before cellularization (Johansen et al., 1989; Fehon et al., 1991). Embryos hemizygous for a *Notch* deficiency, which lack any zygotic *Notch* gene product, are indistinguishable in intensity and pattern of staining from their wild-type siblings until after the onset of germ band extension (Fehon and Artavanis-Tsakonas, unpublished observations). Thus, maternal *Notch* mRNA is apparently translated into protein starting at the cellular blastoderm stage and continuing until germ band extension, at which time *Notch* embryos are readily distinguishable from their wild-type siblings.

Notch activity in somatic cells

The high level of *Notch* protein associated with the follicle cells, the disruption of egg chamber formation in the *N^{ts1}* mutant, and the demonstration by Jimenez and Campos-Ortega (1982) that *Notch* is not required in germline cells for the formation of the egg, all indicate that oogenesis requires *Notch* function in the somatic cells. It is worth emphasizing that the apparently homogeneous follicular envelope is composed of more than one cell type. Gene expression studies involving both antigen localization as well as enhancer traps have revealed the existence of subpopulations of follicle cells.

Notch expression in somatically derived cells is first

detected as they detach from the tunica propria to form the follicular envelope. All the follicle cells express *Notch*, especially the cells between region II and III of the germarium. Some cells in this particular area will eventually give rise to the stalk cells. Both phenotypes associated with early defects in the *N^{ts1}* mutation, fused egg chambers and the failure of egg chambers to separate from germarium, are consistent with a requirement for *Notch* function in these cells. These phenotypes are also compatible with the finding of Ruohola et al. (1991), which used a stalk-cell-specific marker to demonstrate that the *N^{ts1}* mutation blocks stalk cell formation.

In later stages, *Notch* is highly expressed in two migrating follicle cell groups: the border cells and the follicle cells at the anterior part of the developing oocyte, which migrate centripetally and eventually surround the oocyte. We did not observe a phenotype in the *N^{ts1}* mutant that could clearly be attributed to either border cell expression or the follicle cells synthesizing the dorsal appendages, but we did observe chambers arrested at a stage where the oocyte occupied approximately 50% of the egg chamber. It should be noted that we have examined extensively animals that have been treated at non-permissive temperatures for at least 24 hours. Shorter pulses have not been carried out systematically because of the low penetrance of *N^{ts1}* under those conditions. The high level of *Notch* expressed in the centripetally migrating follicle cells at this stage suggests that the reduction of *Notch* activity in the mutant may interfere with their migration. Consequently, oocyte development may be arrested prematurely and proper cytoplasmic discharge from the nurse cells to the oocyte could be blocked.

Finally, a small group of follicle cells abutting the posterior end of the oocyte express high levels of *Notch*. As revealed by enhancer trap lines, there are several genes that are specifically expressed in follicle cells at the two poles of the oocyte (Spradling 1991). Using a fasciclin III antibody, which normally recognizes only two pairs of cells at each pole (Spradling, 1991), Ruohola et al. (1991) demonstrated that *N^{ts1}* chambers have a larger number of fasciclin-positive cells at the posterior end. This observation, together with our own observation of increased *Notch* expression in these cells (Fig. 3C), is compatible with the notion that *Notch* function is required for the proper specification of these cells.

Notch has a polar subcellular distribution

One distinct aspect of *Notch* expression during development is that it is strongly polarized in certain, but not all, epithelia (Fehon et al., 1991). We do not yet know the functional significance of this difference, but it is clear that *Notch* is capable of a very dynamic subcellular expression pattern. This was particularly obvious in the follicle cells of the ovariole.

In the nurse and border cells, *Notch* does not have a polar distribution, while in the follicle cells surrounding the egg chamber, *Notch* is highly polarized. In addition, in early stages of oogenesis, *Notch* is seen in a punctate pattern close to or at the surface of the follicle cells, while later on *Notch* antigen is distributed more homogeneously and clearly polarized at the apical surface. Interestingly, the apical localization of *Notch* is disrupted in the *N^{ts1}* muta-

tion (Fig. 6). It is possible that this failure to polarize is at least partly responsible for loss of *Notch* function in the *N^{ts1}* allele.

Several tenable models could account for the failure of the *N^{ts1}* protein to display normal polarity toward the apical surface. It is possible that this mutation causes improper folding of the Notch protein during synthesis, perhaps interfering with transport from the ER, where the protein is synthesized, to the cell surface. However, the loss of polarity could represent a more direct interaction between the extracellular and intracellular domains of Notch. Alternatively, the failure of apical localization may reflect the inability of the mutant protein in the follicle cells to bind an appropriately polarized ligand. This hypothesis is particularly intriguing since, as we elaborate below, Notch is capable of interacting with other proteins.

Notch mediates cell fate choices during development

The biochemical function of Notch has not been fully elucidated, but there is mounting evidence at the genetic and molecular levels that it may function as a receptor. Using genetic mosaics, Heitzler and Simpson (1991) have shown that *Notch* appears to act as a receptor at least in choices involving sensory organs. We have recently demonstrated that Notch-expressing cells can interact with cells expressing either of the EGF homologous transmembrane proteins Delta or Serrate on their surface (Fehon et al., 1990; Rebay et al., 1991). Furthermore, we have shown that of the 36 EGF repeats of *Notch* only two are both necessary and sufficient to mediate these interactions (Rebay et al., 1991). Therefore Notch may act as a multifunctional receptor whose 36 EGF repeats form an array of ligand binding units, each of which may potentially interact with several different proteins during development (Rebay et al., 1991).

Phenotypic analyses have indicated that at least certain cell fate choices depend not only on *Notch*-mediated interactions but also on other, more specific, cell interaction mechanisms. For example, the very precise cellular architecture of the many cell types that build an ommatidium in the retina depends on a series of cell fate choices involving interactions between neighboring cells. While each consecutive fate decision depends on the activity of specific genes, it is also dependent on *Notch* activity (Cagan and Ready, 1989a, b). For example, R7 photoreceptor cell fate depends both on a very specific inductive interaction from the neighboring R8 cell (Krämer et al., 1991) and on *Notch* function (Cagan and Ready, 1989a).

Such considerations, in conjunction with the pleiotropic behaviour of Notch and the possibility that it may interact with several ligands, lead to the general hypothesis that fate choices between cell neighbors depend on at least two parallel, but not necessarily interdependent, mechanisms: (1) the actions of a developmentally broad Notch-mediated mechanism and (2) a more specific interaction mechanism(s), which is expected to vary between tissue types (Rebay et al., 1991; Artavanis and Simpson, 1991). The extreme model therefore would predict that most, if not all, cellular fate choices in development depend on Notch and that Notch expression in a group of cells implies that regulative events involving the choosing of cell fates are in progress.

Does Notch mediate interactions in oogenesis?

Notch expression is associated with groups of follicle cells (Figs. 1-3) that are known to give rise to specialized cells, namely the stalk cells and the cells at the posterior pole of the oocyte (Spradling, 1991). Indeed, as alluded to earlier, Ruohola et al. (1991) used markers specific for these cells to show that *N^{ts1}* ovaries have abnormal numbers of follicle cells: at non-permissive temperatures, *N^{ts1}* animals do not form stalk cells and appear to have more posterior cells than usual. Given the postulated general role of *Notch* in development and the observed expression pattern, it is reasonable to suppose that the differentiation of these follicle cells depends on *Notch*-mediated interactions between neighboring cells. However, the complexity of *Notch* expression in the ovariole and the non-specific and variable nature of the phenotypes of the *N^{ts1}* mutation at restrictive temperatures obliges us to be very cautious in interpreting the action of Notch in this tissue. In fact, one could argue that the extreme model mentioned above is too simplistic to explain entirely *Notch* action in the ovary.

During embryogenesis, larval or pupal development changes in *Notch* expression do not correlate well with cell movements (Fehon et al., 1991); yet, in the ovariole, high *Notch* expression is clearly associated with migrating cell populations. Whether or not it acts as a receptor, the adhesive properties of Notch (manifested by the aggregation of *Notch*- and *Delta*-expressing cells) may play a functional role in these cell populations. It will be particularly interesting to establish the relative distributions of Notch and Delta proteins in the migrating cell populations. Preliminary immunocytochemical evidence indicates that Delta is indeed expressed in the ovary (M.A.T. Muskavitch, personal communication), but a direct interaction with Notch during oogenesis has not been shown. However, at non-permissive temperatures, a *Delta* conditional mutation displays phenotypes that are very similar to those seen in *N^{ts1}* ovaries (Ruohola et al., 1991).

Although *Notch* expression in the follicular envelope may reflect *Notch*-mediated communication between the follicle cells, the conspicuously apical localization of the Notch protein raises the possibility that it may also be involved in the transmission of signals between the oocyte and the follicular envelope. In support of this, Ruohola et al. (1991) demonstrated that, in the *N^{ts1}* mutations, the spatial distribution of the anterior-posterior markers *oskar* and *bicoid* are altered: both antigens fail to localize at non-permissive temperatures. We have examined embryos laid by *N^{ts1}* females held at restrictive temperature for 24 hours and found that a very small proportion displayed polarity defects (data not shown). Both dorsal-ventral and anterior-posterior defects were observed, but the penetrance of that phenotype was very low (less than 1%) and thus its significance is unclear. Polarity phenotypes, especially anterior-posterior ones, may simply reflect the fact that *N^{ts1}* mutants have more posterior follicle cells rather than a direct disruption of *Notch*-mediated signals from those cells to the oocyte. However, the possibility that *Notch* mediates signals between somatically and germline-derived cells merits serious consideration.

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