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The *Drosophila toucan* (*toc*) gene is required in germline cells for the somatic cell patterning during oogenesis

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SUMMARY

We have characterized a new gene, called *toucan*, that is expressed and required in germline cells to promote proper differentiation of the somatic follicle cells. *toucan* mutant ovaries are defective in (i) the enclosure of newly formed germline cysts by the follicle cells, (ii) the formation of interfollicular stalks, (iii) the migration of the follicle cells over the oocyte and (iv) the formation of the eggshell. Over-expression of a *toucan* cDNA in the germline leads to the production of longer interfollicular stalks than wild-type ovaries, a phenotype that is the exact opposite of the *toucan* mutant phenotype. This observation shows that the formation of the interfollicular stalks depends not only on interactions among the somatic cells but also requires a germline signal. Moreover, dominant interactions have been observed between *toucan* and certain alleles of the *daughterless*, *Notch* and *Delta* genes, each of which is required in the somatic cells for the formation of egg chambers. *toucan* encodes for a large protein with a coiled-coil domain but has no other homology with known proteins. We propose that *toucan* participates in the production or localization of a germline-specific signal(s) that is required for the patterning of the follicular epithelium.

Key words: *Drosophila*, oogenesis, germline cell, follicle cell, *toucan*, cell patterning

INTRODUCTION

In *Drosophila* and most other animals, multiple interactions between germline and somatic cells are essential to trigger the development of the oocyte. In insects, the ovaries are composed of developing egg chambers arranged in tubular structures called ovarioles. The *Drosophila* ovary consists of 15-18 ovarioles. Each ovariole (Fig. 1A) contains a series of egg chambers at progressively more advanced stages of oogenesis (King, 1970; Spradling, 1993). Oogenesis begins with the division of an oogonial stem cell into another stem cell and a cystoblast at the anterior tip of each ovariole. The cystoblast divides four times to give rise to a cyst of 16 germline cells, which remain connected by intercellular bridges (ring canals). One of these 16 cells becomes the oocyte while the remaining 15 cells give rise to polyploid nurse cells. Proliferating somatic cells migrate from the periphery in region II of the germarium (Fig. 1A) to enclose the new germline cyst and to form the egg chamber (Spradling, 1993; Margolis and Spradling, 1995). The surrounding of a germline 16-cell cyst by a follicular epithelium involves controlled cell divisions and cell migrations, differential cell-cell adhesions and cell-shape changes. The coordination of all of these processes is triggered by multiple interactions between the germline cells and the somatic cells as well as among the somatic cells themselves. Genetic analyses have allowed the identification of genes involved in the development of the follicular epithelium. Two neurogenic genes, *brainiac* (*brn*) and *egghead* (*egh*), which are required in the germline, have been described as components of a signalling pathway for the regulation of germline-follicle cell adhesion (Goode et al., 1996b). Moreover, previous studies have shown that *brn* cooperates with the germline function of *gurken* (*grk*) and the somatic function of the *Drosophila* EGF Receptor gene (DER or *torpedo* (*top*)) to establish a continuous follicular epithelium around each cyst (Goode et al., 1992, 1996a). Four other genes, the proneural gene *daughterless* (*da*) and the neurogenic *Notch* (*N*), *Delta* (*Dl*) and *mastermind* (*mam*) genes, are also required in the somatic cells for the enclosure of germline cells (Cummings and Cronmiller, 1994; Xu et al., 1992; Bender et al., 1993). Dominant interactions among *da*, *N*, *Dl* and *mam* mutant alleles suggested that they belong to the same intercellular signalling pathway that triggers the fate and behavior of the somatic cells (Cummings and Cronmiller, 1994). Finally, the activity of the *hedgehog* (*hh*) gene has recently been described as an inductive signal affecting the control of follicle cell precursor divisions in region II of the germarium (Forbes et al., 1996).

Before leaving the germarium, 4-6 somatic cells, called stalk cells, (Fig. 1A) interleaf between the egg chambers to separate the new follicle from the previous one (King, 1970; Spradling, 1993). This event causes the complete individualization of the egg chambers, preventing direct contact between follicles. This separation needs the production of enough somatic cells and the acquisition of the stalk cell fate by some of these cells. *hedgehog* has been described to act in both processes whereas

N and *Dl* play a role only in the specification of multiple somatic cell sub-types (Forbes et al., 1996; Ruohola et al., 1991). To date, the germline cells have not been shown to be involved in stalk formation.

Signalling between germline and somatic cells occurs throughout oogenesis (Schüpbach, 1987). From stage 1 to stage 6 of oogenesis, the posteriorly positioned oocyte communicates with the polar follicle cells, which then adopt a posterior fate (Gonzalez-Reyes and St Johnston, 1994; Gonzalez-Reyes et al., 1995; Roth et al., 1995). This signalling leads to the repolarization of a microtubule network that directs the movement of the germinal vesicle towards the anterior margin of the oocyte during stage 8 (Fig. 1A) (Theurkauf et al., 1992). At this stage, the follicle cells adjacent to the oocyte nucleus adopt a dorsal fate in response to the *grk* signal from the oocyte. The establishment of the anterior-dorsal follicle cell fate is important during the later stages of oogenesis to trigger the correct formation of the eggshell and to permit the correct D/V polarization of the embryo (Schüpbach, 1987). Thus, the formation of the A/P and D/V developmental axes of the egg chamber and of the future embryo depends on multiple interactions between the oocyte and the surrounding follicle cells. These two processes are established by the Grk/DER intercellular signalling pathway.

The present study describes the role in the signalling between germline and somatic cells of a new female-sterile gene called *toucan* (*toc*). We show that *toucan* is involved in egg chamber development at several stages of oogenesis. We have strong evidence that *toucan* is required in the germline to trigger several morphogenetic events of the somatic cells. We propose that *toucan* participates in the production and/or distribution of one (or several) signal(s) from the germline cells to the somatic cells.

MATERIALS AND METHODS

Drosophila stocks

Fly culture and crosses were performed according to standard procedures. The *toucan*^{P} enhancer trap line was isolated as a recessive female-sterile mutation in a P-element-mediated mutagenesis (Sahut-Barnola et al., 1995), using P[*lacZ, ry*$^{+}$] as an enhancer detector (O'Kane and Gehring, 1987) and P[*ry*$^{+}$(D2-3)]99B as a transposase source (Laski et al., 1986; Robertson et al., 1988).

To remobilize the P element, the P[*ry*$^{+}$(D2-3)]99B jumpstarter strain was crossed into the *toucan*^{P} background to supply a source of transposase. Flies in which excision events had occurred were detected by scoring progeny for loss of the *rosy*$^{+}$ eye color marker carried by the P element. Three of these lines present a zygotic lethality (*toc*^{PR25} and *toc*^{PR50} are used in this study), seven display a reduced viability (less than 5% of the homozygotes eclose) (*toc*^{PR3}, *toc*^{PR12}, *toc*^{PR24} and *toc*^{PR26} are used in this study), ten have the same phenotype as *toc*^{P} and five have fully fertile females and complement the oogenesis defects seen with the other alleles.

The following fly strains were used: Canton S, *Df(2L)16X42* (23B; 23E1-2) (Bashaw and Baker, 1995), *Df(2L)JS31* (23A3-4; 23D) (Sekelsky, 1993), *cl da*^{2}*/CyO, y cl da*^{S22}*/CyO* (Cummings and Cronmiller, 1994), *brn*$^{fs.107}$*/FM3* (Goode et al., 1992), *93F/TM3* (Ruohola et al., 1991), the 8.2 line carrying two *P[w*$^{+}$*, ovo*^{D1}*]* transgenes (Mével-Ninio et al., 1994), *y*^{1} *N*^{ts1} *g*^{2} *f*^{1}*/C(1)DX y*^{1} *w*^{1} *f*^{1} and *Dl*^{9}*/In(3R)C e*^{1}. For complementation tests, the following lines were used: female-sterile mutations fs(2) *lto* RG3, *gourd*^{R133} and *gourd*^{QD67} (Schüpbach and Wieschaus, 1991), and lethal P-element insertions *l(2)01361, l(2)K00237, l(2)K08224* and *l(2)05527* (Bloomington Stock Center).

Egg chamber staining procedures

Ovaries were hand dissected in 0.7 M NaCl. For β-galactosidase activity detection, ovaries were fixed in 0.16% glutaraldehyde in phosphate-buffered saline (PBS) for 3 minutes. After washing in saline, ovaries were incubated at room temperature overnight in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 6.1 mM potassium ferrocyanide, 6.1 mM potassium ferricyanide and 0.2% X-Gal. Ovaries were rinsed in PBS, dissected and mounted in ethanol/glycerol (1/1). For DAPI staining of ovaries, tissues were fixed in 0.5% glutaraldehyde in PBS and rinsed twice in PBS. The ovaries were stained for 3 hours in a 1 μg/ml solution of DAPI in PBS and washed twice in PBS before mounting.

For egg shell examination, freshly laid eggs were mounted in Hoyer's medium (Van der Meer, 1977). All the microscopy was carried out on a Zeiss Axiophot equipped with differential interference contrast and epifluorescence optics.

Germline clones

The dominant female-sterile mutation *ovo*^{D1} was used as a tool to produce homozygous germline clones by mitotic recombination. Germline clones of the *toc*^{P} and *toc*^{PR3} alleles, were generated by γ-ray irradiation from a ^{137}Cs source using the 8.2 line with two *P[w*$^{+}$*, ovo*^{D1}*]* elements localized in 28A and 28B on the second chromosome (Mével-Ninio et al., 1994). Progeny from a cross between *w/w* ; *toc*^{P} (or *toc*^{PR3})*/CyO* virgin females and *w ovo*^{0}/Y ; *P[w*$^{+}$*, ovo*^{D1}*]*/+ males were irradiated with 1000 rads during the first larval instar to induce germline clones. 300 *w/w ovo*^{0} ; *toc*^{P}*/P[w*$^{+}$*, ovo*^{D1}*]* and 500 *w/w ovo*^{0} ; *toc*^{PR3}*/P[w*$^{+}$*, ovo*^{D1}*]* females were analyzed. These females were crossed to *w*/Y ; *toc*^{P} (or *toc*^{PR3})*/CyO*.

Nucleic acid procedures

The P-*lacZ* insertion of the *toucan*^{P} allele was used as a tag for cloning of sequences adjacent to the insertion by the inverse PCR method (Ochman et al., 1990). This fragment was used as a probe to screen a lambda genomic library (Tamkun et al., 1992) and an adult ovary cDNA library (J. L. Couderc and F. A. Laski, unpublished).

From two overlapping cDNAs, the complete *toucan* open reading frame followed by the 3′ untranslated region was reconstituted and cloned into p(COG) (Robinson and Cooley, 1997). P-element transformations were completed using standard procedures (Rubin and Spradling, 1982).

RNA was isolated from adult flies with the use of the sodium dodecyl sulfate (SDS)-phenol-chloroform procedure. Poly(A)$^{+}$ RNA were isolated on oligo(dT)-cellulose columns, size fractionated on formaldehyde-agarose gels (Sambrook et al., 1989) and transferred to Nytran (Schleicher and Schuell).

In situ hybridization

A biotin-labeled DNA probe, from a genomic DNA fragment adjacent to the *toucan*^{P} insertion site, was used for chromosome in situ hybridization (Ashburner, 1989). Whole-mount in situ hybridization to egg chambers was carried out according to Tautz and Pfeifle (1989) with the following modifications. Ovaries were dissected in PBS and fixed in heptane-saturated 4% paraformaldehyde, 0.1 M Hepes (pH 6.9), 2 mM $MgSO_4$ and 1 mM EGTA for 20 minutes. Ovaries were rinsed with PBT (PBS, 0.1% Tween 20) before proceeding with proteinase K treatment. Hybridization with digoxigenin-labeled RNA probes was performed at 55°C overnight and followed by washes in hybridization solution and a 1:1 mixture of hybridization solution and PBT at 55°C for 30 minutes each, and 2× 20 minutes in PBT at room temperature. Hybridized probe was detected using the Genius kit (Boehringer).

RESULTS

Characterization of a new locus involved in oogenesis

The *toucan* locus was identified as a recessive female-sterile mutation in an enhancer trap mutagenesis. In this line, a single P-*lacZ* enhancer trap is located at map position 23D (data not shown). The sterility can be reverted by P-element excision (see Methods) and the *toucan*^{P} mutation is able to complement the other genes that map in this region, indicating that the *toucan*^{P} mutation identifies a new gene (see Methods).

During oogenesis, the P-*lacZ* enhancer trap construct of *toucan*^{P} expresses β-galactosidase in a dynamic pattern in the germ cells. Expression initiates in germarial region IIA where cysts are forming, is maximal in 16-cell cysts in region IIB of the germarium, decreases when the egg chambers leave the germarium and has disappeared by the time the egg chambers reach stage 3 (Fig. 1B). The enhancer trap expression resumes in stage 8 egg chambers in one germ cell, the oocyte (Fig. 1C), where β-galactosidase accumulates specifically (Fig. 1D) until the end of oogenesis. This late pattern indicates that some promoters are active in the oocyte at stage 8 of oogenesis and probably before as has already been suggested (Grossniklaus et al., 1989). None of the eggs laid by *toc*^{P} homozygous females develop. About 30% of these eggs are shorter than wild type, have strongly reduced or absent dorsal appendages and do not have a flattened dorsal side (Fig. 1E). This indicates that *toucan* is required for proper oocyte development during late oogenesis. The expression pattern of the enhancer trap, however, suggests that the *toucan* locus is also required at earlier stages for the formation and maturation of egg chambers.

Females transheterozygous for the *toc*^{P} allele and a deficiency covering the 23D locus: *Df(2L)16X42* or *Df(2L)JS31* (see Methods), display ovaries with abnormal egg chambers. The same defects are also observed for 10 *toc* alleles (*toc*^{PR}) generated by imprecise excision of the P element at the *toucan* locus. Homozygous *toc*^{PR} females, when they exist, as well as transheterozygous females for all of the *toc*^{PR} alleles and the *toc*^{P} allele present several ovarian defects (see below), indicating that *toucan* is required during early oogenesis and that all of these mutations fall into a single complementation group. Since these *toc*^{PR} alleles behave in a manner similar to the two chromosomal deficiencies in combination with the *toc*^{P} allele, we refer to them as strong loss-of-function *toucan* alleles.

Strong *toucan* mutations affect the behavior of somatic cells at several steps of oogenesis

The ovarian morphology of young females (<5 days old) homozygous for strong *toucan* mutations has been examined. Whatever the alleles used, 80 to 90% of ovarioles contain one or more egg chambers in which many more than sixteen germline cells are enclosed in the same follicle (Fig. 2A-D). Some of these compound follicles present a partial interleafing of follicle cells between the germline cells (Fig. 2A). The germaria are often abnormally swollen due to an excess of germline cells. In addition, the morphological boundary between the germarium and the vitellarium is not precisely defined because some nascent egg chambers fail to bud off correctly from the germarium (Fig. 2B,C). The severity of the ovarian phenotype varies among different alleles. In some cases, the entire ovariole appears to be one giant egg chamber, which contains all of the cysts formed in the germarium (Fig. 2C). Two hypotheses can explain the formation of compound egg chambers: (1) a failure of the follicle cells to enclose individual germline cysts or (2) extra germline cystocyte divisions such as those that occur in *ovarian tumor* (*otu*) ovaries (Geyer et al., 1993). Three observations allow us to rule out this second hypothesis. First, DAPI staining shows that nurse cell nuclei inside a compound egg chamber vary in size (Fig. 2C). This difference reveals several levels of ploidy indicating that the nurse cells derive from cystoblasts with different birthdates. Second, in some compound egg chambers, two or more enlarged oocytes can be detected (Fig. 2D), whereas in *otu* mutant ovaries, no oocyte differentiates. Third, using rhodamine-phalloidin staining, we never observed more than four ring canals around the oocyte, which shows that the compound egg chambers are composed of several cysts of exactly 16 cells and argues against extra germline cystocyte divisions. Thus, in *toucan* mutant ovaries, the formation of compound egg chambers is due to the incorrect behavior of the somatic cells that should enclose the germ cell cysts.

The second defect concerns the separation of the egg chambers by interfollicular stalks. In *toucan* mutant ovaries, interfollicular stalks are never formed between two normal-sized (Fig. 2E) or compound egg chambers (Fig. 2A,B). Thus, the *toucan* gene is required to form a stalk of interfollicular cells between adjacent egg chambers. To define whether *toucan* is necessary for the differentiation of stalk cells or for their recruitment into stalks, we have used the 93F enhancer trap line, which expresses β-galactosidase specifically in the stalk cells (Ruohola et al., 1991). In half of the cases where two egg chambers are not properly separated by a stalk (out of at least one hundred), no expression of the stalk cell marker is detected (data not shown). In the other half, only one or two cells express the marker (Fig. 2F). These staining cells are never located between two adjacent follicles but are intergrated into the follicular epithelium surrounding the oldest germline cyst. Therefore, lack of wild-type *toucan* gene expression results in a decrease in the number of cells adopting a stalk cell fate. In addition, the cells that do express the stalk cells marker do not form stalks.

Another defect has been observed in stage 9 egg chambers. At this stage, in wild-type ovaries, most of the follicle cells move to cover the oocyte (Spradling, 1993). In strong *toucan* mutant alleles, an abnormal concentration of follicle cells around the nurse cells is detected in early stage 10A follicles (Fig. 2G,H). This effect is not due to a reduced size of the oocyte, which appears to be of normal size but rather, to a block or at least a delay of the migration of the follicle cells towards the oocyte. Thus, the *toucan* gene is also required for the correct migration of the follicle cells during later stages of oogenesis. It must be noted that the migration of the border cells is never disrupted in *toc*^{PR} ovaries, indicating that the migration of these cells is independent of the *toucan* function.

All of the ovarian defects observed in females homozygous for strong mutations in the *toucan* gene are also observed in transheterozygous combinations with the *toc*^{P} allele and are identical to those observed in transheterozygous *toc*^{P}/*Df(2L)JS31* or *toc*^{P}/*Df(2L)16x42* females (Fig. 2I and data not shown).

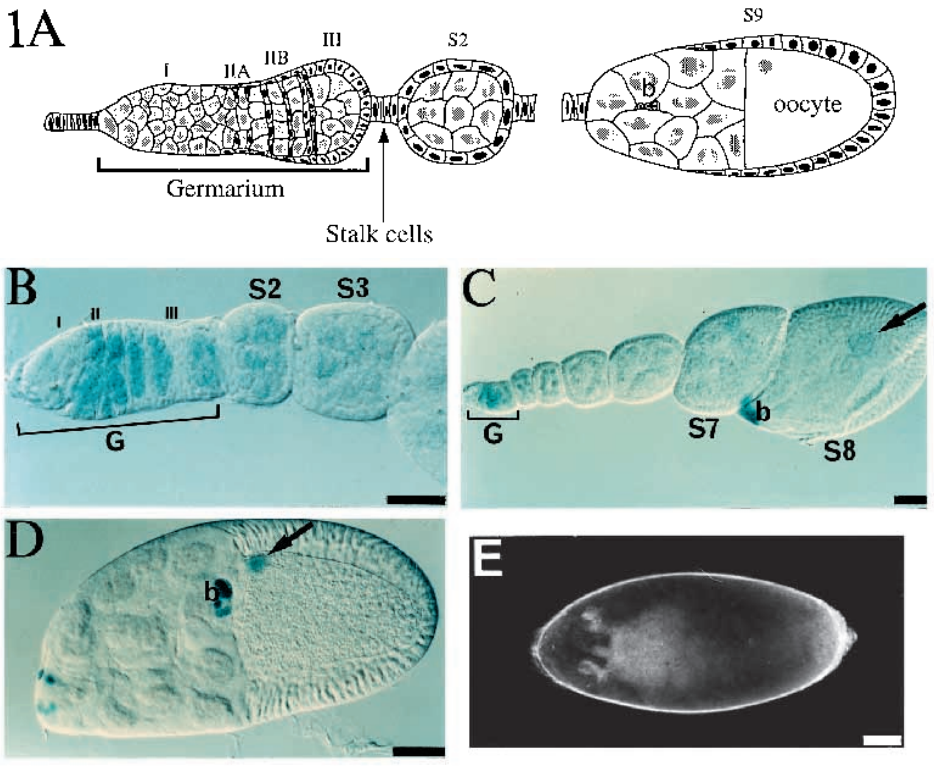

Fig. 1. Enhancer trap expression in the *toc*^{P} adult ovaries. (A) Schematic representation of an adult wild-type ovariole. The germline cell nuclei are in grey and the somatic cell nuclei are in black. The different regions of the germarium (G) are indicated by roman numbers. Stage 2 (S2) and stage 9 (S9) egg chambers are shown. Border cells (b). (B) Anterior part of a *toc*^{P} ovariole composed of a germarium and two early stage egg chambers. (C) Ovariole composed of the germarium and six egg chambers. The stage 8 (S8) follicle presents a labelled oocyte nucleus (arrow). The β-gal expression in the border cell nuclei is due to a vector position effect. (D) Stage 10A egg chamber presenting a strongly labelled oocyte nucleus (arrow). (E) Dorsal view of an egg from a *toc*^{P} mother with an extreme eggshell phenotype. Dark-field micrograph. Anterior is to the left as in the other figures. Scale bars represent 50 μm.

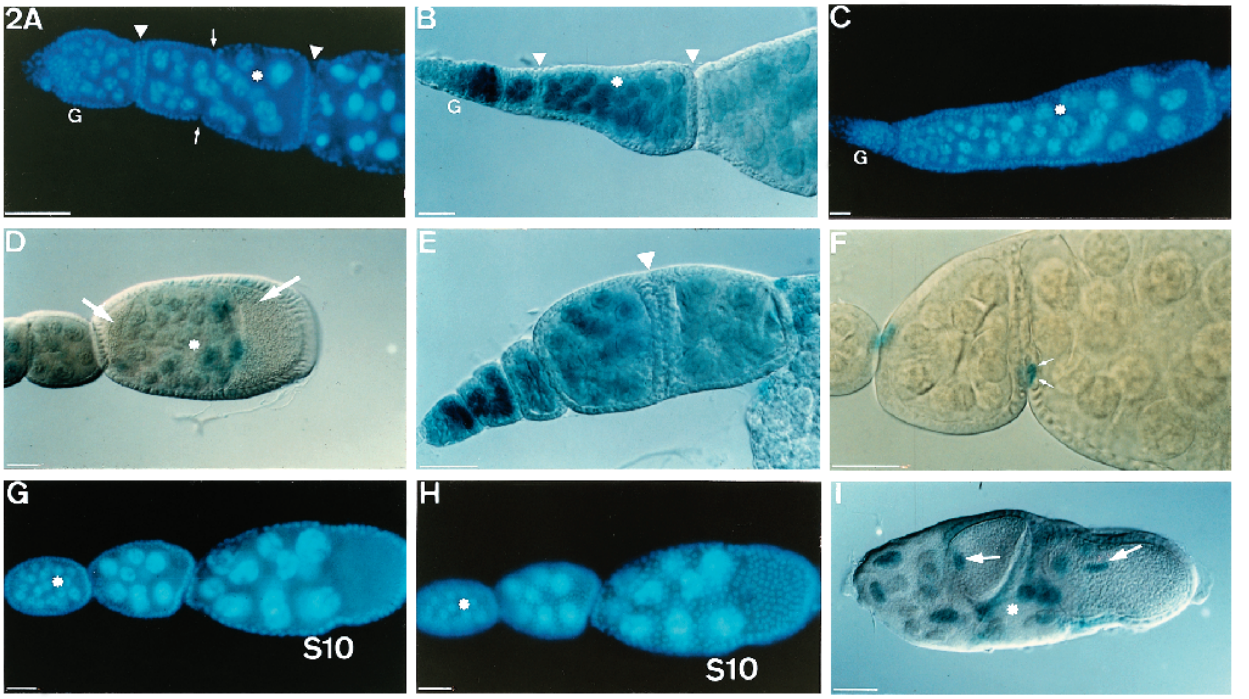

Fig. 2. Oogenesis defects in strong *toucan* alleles. (A,C,G,H) Ovarioles are stained with DAPI; (D,I) β-galactosidase comes from the P-*lacZ* insertion in the *toc*^{P} line, or (B,E) in the *toc*^{PR12} line. (F) β-galactosidase staining is due to the 93F enhancer trap line, which stains the stalk cells. Compound egg chambers are indicated with asterisks. The arrowheads point to the absence of stalks between adjacent egg chambers. G, Germarium. (A) A *toc*^{PR3}/*toc*^{PR3} ovariole has a swollen germarium and a compound egg chamber. The small arrows indicate the partial interleafing of follicle cells between two cysts. (B) A *toc*^{PR12}/*toc*^{PR12} ovariole with a compound egg chamber. (C) A *toc*^{PR3}/*toc*^{PR3} ovariole containing a germarium and only one large compound follicle. (D) A *toc*^{PR24}/*toc*^{P} compound egg chamber. The arrows indicate the two oocytes at opposite ends of the follicle. (E) A *toc*^{PR12}/*toc*^{PR12} ovariole presenting two correctly formed egg chambers which are not separated by an interfollicular stalk. (F) Two adjacent *toc*^{PR3}/*toc*^{PR3} follicles. The arrows indicate the two cells expressing the stalk cell marker. (G) A *toc*^{PR26}/*toc*^{PR26} mutant ovariole. In the stage 10 (S10) egg chamber, the follicle cells have not migrated. (H) The same ovariole as in (G) with the focus on the follicle cells. (I) A compound *toc*^{P}/*Df(2L)JS31* egg chamber. The arrows indicate the two oocyte nuclei. Scale bars represent 50 μm in all panels except in panel F (100 μm).

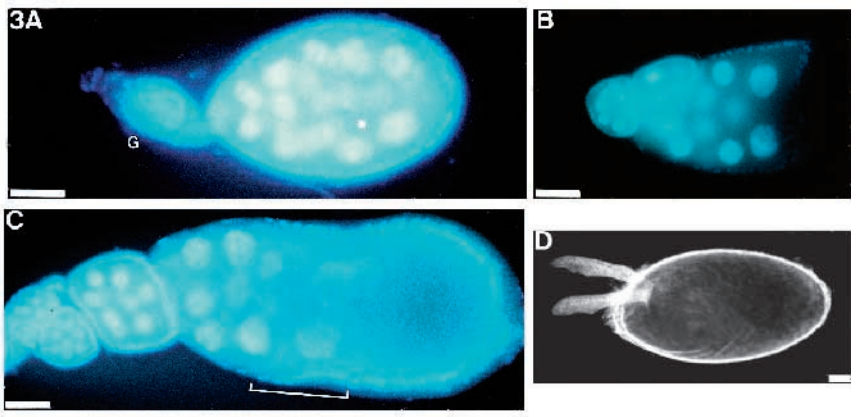

Fig. 3. Ovary and eggshell defects obtained in toc^{PR3} (A-C) or toc^{P} (D) germline clones. Ovaries are stained with DAPI. (A) Ovariole composed of a germarium followed by a single compound egg chamber. (B) Stalkless follicles abnormally aligned throughout the ovariole. (C) Ovariole with a stage 10A egg chamber: the bracket indicates the follicle cells which still cover the nurse cells. (D) Dark-field micrograph of the chorion of an egg from a toc^{P} germline clone. The dorsal side is up. Scale bars represent 50 μm.

The *toucan* gene is required in the germline cells

The lack of wild-type *toucan* function leads to abnormalities in the migration and differentiation of the somatic cells at several steps of oogenesis. Some of these processes are known to be regulated by interactions between somatic and germline cells. To determine whether the *toucan* ovarian phenotype is due to a germline or a somatic requirement, mutant germline clones for the strong allele toc^{PR3A} were generated (see Methods). In about 1% of females, a mosaic clone was observed. In these clones, the somatic cells are wild type whereas the germline cells are homozygous for the toc^{PR3A} mutation. For each ovary, 1-5 ovarioles contain developing and mature egg chambers, confirming that the ovo^{D} mutation is not present in the germline cells of these ovarioles. Very few mosaic eggs are laid and they never hatch. Fig. 3A shows an ovariole composed of a germarium and a compound egg chamber containing two cysts. 15% of the mosaic egg chambers present this phenotype. At least ten ovarioles

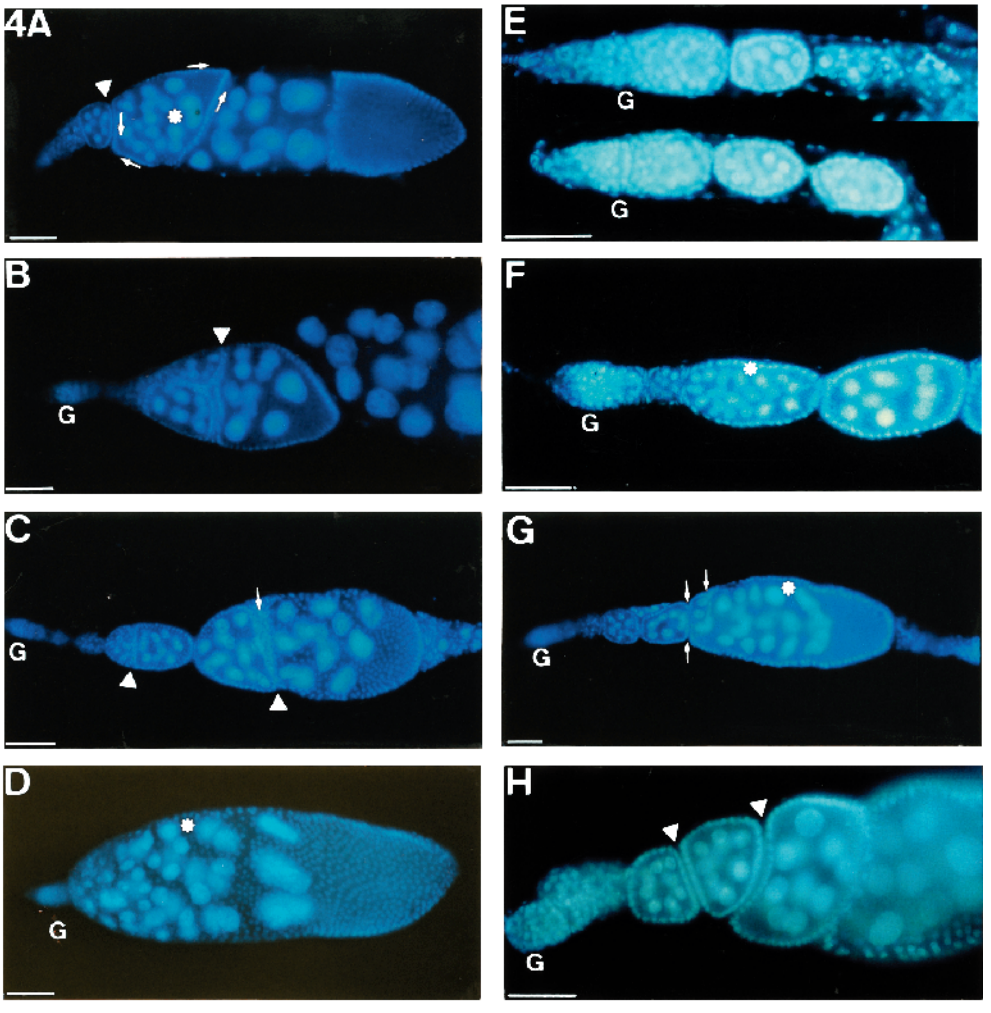

Fig. 4. Ovary defects due to dominant interactions between *da*, *N*, *Dl* and *toc* mutations. Ovaries are stained with DAPI. Compound egg chambers are indicated with asterisks. Arrowheads point to the absence of the stalks between adjacent egg chambers. G, Germarium. (A) A toc^{PR3}/da^{S22} ovariole with a compound follicle. The follicle cells move towards the two oocytes located at opposite ends of the follicle (small arrows). (B) Abnormally aligned egg chambers in toc^{PR12}/da^{2} ovariole. (C,D) toc^{PR25}/da^{2} ovarioles. (C) The ovariole displays stalkless follicles and an interleafing of unorganized follicle cells (small arrow) between adjacent egg chambers. (D) An ovariole containing only one very large compound egg chamber. (E) Two $toc^{PR12}/+$; $+/N^{ts1}$ ovarioles consisting of a swollen germarium and one or two early egg chambers. (F,G) $toc^{PR50}/+$; $+/Dl^{9}$ ovarioles with compound egg chambers. The small arrows in G show the partial interleafing of follicle cells between two germline cysts. (H) A $toc^{PR25}/+$; $+/brn^{fs.107}$ ovariole. Some egg chambers are not separated by a stalk. Scale bars represent 100 μm in A-D, G and 50 μm in E, F and H.

produced by differents clones are composed of normal egg chambers that are not separated by interfollicular stalks (Fig. 3B). Most of the time, these egg chambers are not correctly aligned within the ovariole. In stage 10 egg chambers, the migration of follicle cells over the oocyte is not completed, suggesting that the migration is delayed or blocked (Fig. 3C). The *toucan* gene is, therefore, required in germline cells for the migration of the somatic cells during the formation and maturation of egg chambers and for the formation of interfollicular stalks.

toucan mutant germline clones have been also produced with the toc^{P} allele. Fertilized eggs laid by these females never develop and clearly show abnormal morphological structures (Fig. 3D). In the three mosaic clones obtained, all of the laid eggs (more than 30 eggs were examined) are round and smaller than wild-type eggs and the respiratory appendages are reduced. The disruption of *toucan* in germline cells only is therefore sufficient to prevent the somatic tissue from building a correct eggshell and to prevent the development of the future embryo.

toucan interacts with *daughterless*, *Notch* and *Delta* in the formation of the egg chambers

Mutant ovaries for particular alleles of *da*, *Dl*, *N* and *brn* present the same range of defects as those observed with *toucan*, including compound egg chambers and stalkless follicles (Cummings and Cronmiller, 1994; Bender et al., 1993; Xu et al., 1992; Goode et al., 1992). Dominant interactions have been observed in double heterozygous combinations between *da*, *N* and *Dl* alleles. To test the interaction between *toucan* and these genes, ovaries from double heterozygous females were examined. No defect is observed in any single heterozygotes for these genes.

A synergistic dominant interaction is observed between toc^{PR} mutations in combination with da^{2} and da^{S22} mutations (respectively, a null and an extreme hypomorphic allele). 80 to 90% of the ovarioles display giant egg chambers and stalkless follicles (for details, see Fig. 4A-D). The abnormalities observed in these ovaries are very similar to the *toucan* ovarian phenotype (compare Figs 2C and 4D). The severity of the phenotype increases with the strength of *toc* and *da* alleles used ($toc^{PR3}/da^{S22} < toc^{PR12}/da^{2} < toc^{PR25}/da^{2}$). Thus, the levels of activity of both genes are crucial at common steps of egg chamber formation. Oogenesis is also severely disrupted in N^{ts1}/+ ; toc^{PR12}/+ females after 3 days at the restrictive temperature (32°C). All of the ovarioles present a germarium that is swollen by an abnormal accumulation of germline cysts (Fig. 4E). Only a few egg chambers bud off, suggesting that the process of egg chamber formation is more strongly affected in this combination than in homozygous mutant ovaries of each gene separately. A dominant, but not synergistic, interaction is observed in ovaries from doubly heterozygous for toc^{PR50} and Dl^{9}, where compound follicles are observed at lower frequency (Fig. 4F,G). In toc^{PR25}/+ ; $brn^{fs.107}$/+ females, the ovaries look wild type. We have detected very rare stalkless follicles but no compound egg chambers (Fig. 4H). Together, these dominant interactions indicate that *toucan* is acting in the same signalling pathways for the formation of the egg chamber as *da*, *N* and *Dl*.

toucan mRNA is expressed in the germline cells in a dynamic pattern throughout egg chamber development

Two transcription units were identified in the region adjacent to the P insertion in the *toucan* locus (Fig. 5A). The first group of cDNAs corresponds to the transcript of the *mother against dpp* (*mad*) gene (Sekelsky et al., 1995), which is located 3 kb upstream from the *toucan* P-*lacZ* element insertion site. This gene is expressed during oogenesis in the nurse cells from stage 9 onwards and its expression is not affected in homozygous $toucan^{P}$ flies (data not shown). The second group of overlapping cDNAs collectively form an 8.1 kb cDNA which hybridizes to genomic fragments located from just upstream of the insertion to 70 kb downstream. This cDNA encodes a

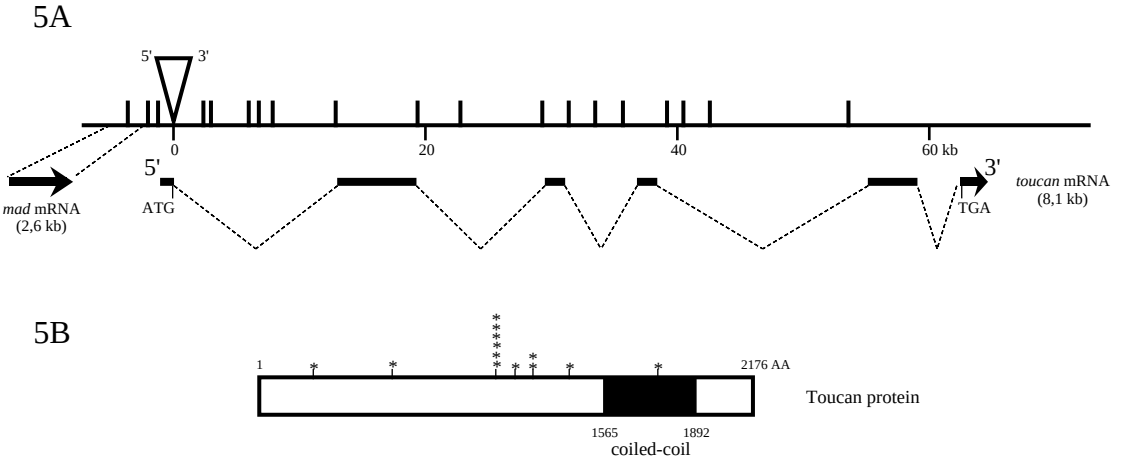

Fig. 5. Molecular characterization of the *toucan* gene. (A) The top line shows the genomic map of the *toucan* gene. The vertical bars indicate the *Eco*RI restriction sites. The P(*lacZ, ry*$^{+}$) element (large triangle) is inserted between the first two nucleotides of the first intron. Below, the *mad* and *toucan* transcription units are shown. The relative size of the *toucan* exons and their positions relative to the genomic map are indicated. The*toucan* cDNA sequence can be obtained via EMBL accession number: Y14157. (B) Schematic representation of a potential Toucan protein. The predicted coiled-coil region (P>0.85) is diagramed by a black box. The [KR] [ST] P putative phosphorylation motifs are indicated by asterisks.

protein of 2176 amino acids with a predicted molecular mass of 235 kDa (Fig. 5B). No overall homology between Toucan and any protein in the databases was found, using FASTA and BLAST programs, indicating that it is a novel protein. Preliminary sequence analyses indicate that the Toucan polypeptide contains a coiled-coil domain of 300 amino acids in its carboxy-terminal region (Fig. 5B), as predicted by the Lupas algorithm (Lupas et al., 1991). This domain contains 39 hydrophobic heptad repeats, forming a helical domain with a hydrophobic face and a hydrophilic face. Interaction along the hydrophobic faces of two Toucan proteins may form a coiled-coil rod. Thirteen [KR] [ST] P motifs are present mostly in the center of the protein (residues 1034-1365) (Fig. 5B). This motif has been described in neurofilament proteins as a phosphorylation site for a cdc2-like kinase (Lew and Wang, 1995).

Using the 5′ part of the cDNA as a riboprobe, two transcripts are detected by Northern blot in wild-type flies; a major 8 kb transcript and a minor 7 kb transcript (Fig. 6) were seen. In heterozygous or homozygous toc^P mutant flies, the level of both transcripts is reduced and additional transcripts of 4.5 and 2.0 kb appear. In homozygotes, the 7 kb transcript is no longer detectable. The truncated transcripts are detected only if the riboprobe includes sequences upstream of the insertion site, suggesting that these shorter transcripts end within the insertion. These results strongly suggest that the 7 and 8 kb transcripts correspond to the transcripts of the *toucan* gene and that the insertion of the P(*lacZ, ry+*) element in the toc^P line disrupts expression of both transcripts.

In wild-type ovaries, *toucan* mRNA is first detected in the germarium as soon as the 16-cell cysts are formed (Fig. 7A). This pattern is maintained in stage 1 and 2 egg chambers. During mid-oogenesis (stage 3 to stage 8), the *toucan* mRNA is restricted to the most posterior part of oocyte (Fig. 7B,C). Expression then becomes undetectable in the oocyte but starts to be expressed in the nurse cells at stage 9 (Fig. 7D). This expression increases strongly during stages 10A and 10B (Fig. 7E) and the *toucan* mRNA again accumulates at the posterior end of the oocyte. All of the *toucan* mRNA in the nurse cells enters the oocyte at the end of oogenesis leading to a uniform distribution in the early embryo (Fig. 7F). In conclusion, the expression of the *toucan* gene is detected only in the germline cells with a spatiotemporal pattern consistent with its germline requirement throughout oogenesis.

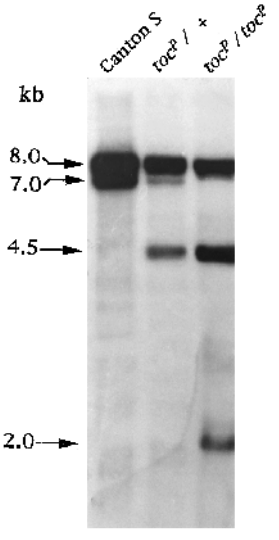

Fig. 6. The *toucan* gene encodes two transcripts. Poly(A)$^+$ RNA from wild-type (lane1), toc^P/+ (lane 2) and toc^P/toc^P mutant (lane 3) flies were hybridized with an antisense *toucan* RNA probe overlapping the first exon.

Overexpression of the *toucan* gene in germline cysts generates long interfollicular stalks

The *toucan* cDNA was specifically expressed in germline cells using the *otu* promoter (Robinson and Cooley, 1997). Overexpression of *toucan* in the germline in wild-type females leads to the formation of normal egg chambers containing a single 16-cell cyst (Fig. 7A). A large proportion of the laid eggs develop into wild-type embryos. The egg chambers are separated from each other, however, by stalks of 10-15 cells compared to 4-6 in wild-type ovaries. Thus, *toucan* overexpression in germline cells is sufficient to give rise to the formation of long stalks. Moreover, these extra interfollicular cells are either correctly organized into a stalk (Fig. 7A,C) or form a cluster around one of the central interfollicular cells (Fig. 7B). This proves that a germline signal is required to trigger the formation of a correct stalk, and that the process of egg chamber separation does not solely depend on the interactions among somatic cells that have been previously described (Ruohola et al., 1991; Larkin et al., 1996).

DISCUSSION

A germline signal is required for interfollicular stalk formation

toucan mutant ovaries and *toucan* mutant germline clones produce stalkless follicles, whereas the overexpression of a *toucan* cDNA, from a germline-specific promoter, leads to opposite defects: i.e. the production of giant stalks. This indicates that a germline signal is required for the formation of interfollicular stalks and that this signal is dependent on the level of Toucan activity. Based on preliminary sequence analyses, *toucan* does not seem to encode a secreted or a transmembrane protein. Thus, toucan probably does not correspond to the signal itself, but it could be involved in its production or distribution.

Previous studies have shown that the formation of interfollicular stalks depends on the regulation of somatic cell precursor divisions (Forbes et al., 1996) and on interactions among the somatic cells themselves through *daughterless*, *Notch* and *Delta* activities (Ruohola-Baker et al., 1991; Larkin et al.,1996). Due to its early expression in the germarium, *toucan* could be influencing in any of these processes. The absence of stalks in ovaries from transheterozygous for *toc* and either *da*, *N* or *Dl* indicates that all of these genes participate in the same signalling pathway and that interactions between the germline and the somatic cells are also crucial for this process. Based on the results of constitutively active Notch expression, it has been proposed that stalk cell fate depends on a binary decision among the somatic cells (the instructive model) (Larkin et al., 1996). However, in the long stalks produced by germline Toucan overexpression, all of the interfollicular cells express the 93F stalk cell marker (data not shown). Altogether, our results are consistent with the prohibitive model (Larkin et al., 1996) in which the somatic cells are maintained in an uncommitted state by the Notch signalling pathway and are induced to differentiate by external signals, i.e. a germline signal. Moreover, the formation of normal stalks

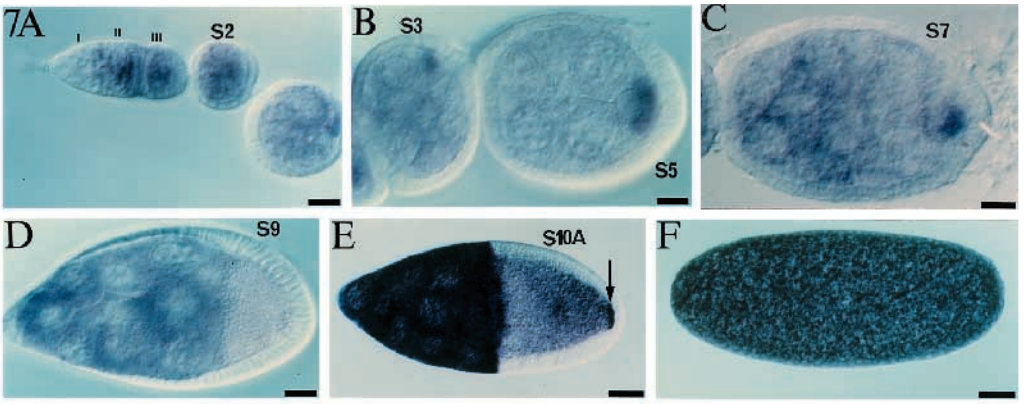

Fig. 7. The *toucan* mRNA is expressed in the germline cells of wild-type ovaries. In situ hybridization to whole-mount egg chambers and embryos was performed using a digoxigenin-labeled antisense *toucan* RNA probe. (A) Anterior part of an ovariole presenting a germarium and a stage 2 (S2) egg chamber. The different parts of the germarium are indicated with roman numbers. (B,C) Specific accumulation of the *toucan* transcripts at the posterior margin of the oocyte in early egg chambers (B) and in a stage 7 egg chamber (C). (D) A stage 9 egg chamber. The cytoplasm of the nurse cells is labelled. (E) In a stage 10A egg chamber, *toucan* mRNA is heavily expressed in nurse cells and accumulates at the posterior end of the oocyte (arrow). (F) Embryo at the syncytial blastoderm stage presenting a uniform distribution of the *toucan* transcript. Scale bars represent 50 μm except in panel D (25 μm).

requires Hedgehog activity in somatic cells to control the proliferation of the somatic cell precursors (Forbes et al., 1996). It is reasonable to propose that *toucan* regulates the production and/or distribution of one germline signal necessary to coordinate the rate of somatic cell precursor divisions with the rate of 16-cell cyst production.

toucan interacts with the neurogenic genes for enclosure of the cyst

The patterning of the follicular epithelium around the germline cyst requires the activities of the neurogenic genes *Notch* (*N*) and *Delta* (*Dl*), and the proneural *daughterless (da)* gene in the somatic tissue (Cummings and Cronmiller, 1994; Xu et al., 1992; Bender et al., 1993). Mutant ovaries homozygous for the *da*, *N* or *Dl* genes display abnormal enclosure of follicles as do double heterozygous combinations between these three genes, indicating that they all belong to the same signalling pathway for this process. The same defects in egg chamber formation have been observed in *toucan* mutant ovaries and in double heterozygous ovaries for *toucan* and *da*, *N* or *Dl*. This indicates that *toucan* encodes a previously undescribed component of the *da*, *N* and *Dl* signalling system and that this system, which triggers regulatory events that promote follicle cell migration and the acquisition of differential cell adhesion properties, is dependent of the germline. When the process of egg chamber formation begins (region II of the germarium), *N*, *Dl* and *da* are expressed in the prefollicular cells, whereas *toucan* mRNA is present in the germline cysts. *toucan* may be involved in the production or the distribution of a germline signal that regulate N, DL and Da activities. The control of cell-surface molecule expression such as Notch and Delta must be crucial for the establishment of differential cell surface properties. N and Dl have extracellular domains that have been shown to be involved in cell sorting processes (Fehon et al., 1990) and which may help to build the follicular epithelium around the cyst. The transcription factor Da may regulate the expression of adhesion or cell-cell recognition molecules necessary for this process.

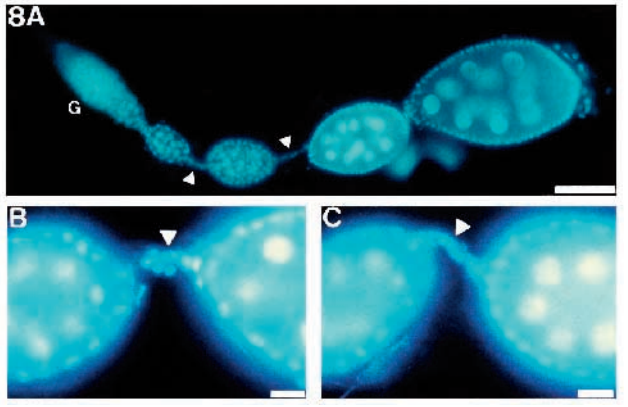

Fig. 8. Germline-specific overexpression of a *toucan* cDNA in wild-type female. Ovaries are stained with DAPI. The arrowheads point to giant stalk structures between adjacent egg chambers. G, Germarium. (A) Ovariole with two long interfollicular stalks. (B,C) Magnification of giant interfollicular structures. The cells are not correctly organized into a stalk in B. Scale bars represent 50 μm in A and 10 μm in B and C.

Recent results have shown that two other neurogenic genes, *brainiac* and *egghead*, are critical germline components that modify the adherence properties of the follicular epithelium around the cyst (Goode et al., 1996b). Although no dominant interaction has been observed between *toucan* and *brainiac* mutations, we cannot exclude the possibility that *toucan* is required for this process. So far, however, we have never observed defects in the adhesion of the follicle cells to the cyst that result in gaps in the follicular epithelium in *toucan* mutants.

A germline signal for the migration of the follicle cells over the oocyte

Analysis of the *toucan* expression pattern shows that this gene is also transcribed in the germline during mid-oogenesis. The *toucan* transcript accumulates specifically in the oocyte from stage 3 to stage 8. This expression is probably responsible for the defect in follicle cell migration over the oocyte during stages 8-9 in *toucan* mutant ovaries. This process may depend on the production of a new high affinity ligand on the oocyte membrane or a new receptor on the surface of the follicle cells (Spradling, 1993). Consistent with the germline requirement and the expression pattern of the *toucan* gene, we postulate that *toucan* is required for the production of this ligand, which

controls the gathering of the follicle cells around the oocyte. Goode et al. (1996b) have shown that, in *brn* and *egh* mutant ovaries, the follicle cells migrate too fast to cover the oocyte. This behavior is the exact opposite of that observed in *toucan* mutant ovaries. One attractive model would be that *toucan* negatively regulates the activities of the Brn and Egh transmembrane proteins.

Is *toucan* involved in the establishment of the anteroposterior (A/P) and the dorsoventral (D/V) axes?

We have shown that *toucan* plays a key role in several signalling systems between the germline and the somatic cells. *toucan* may also be required for the two morphogenetic processes triggered by the grk/DER pathway: establishment or maintenance of the A/P and the D/V polarity of the egg chamber. First, the spatiotemporal expression of the *toucan* gene is compatible with a requirement for *toucan* in these signalling systems. Second, preliminary studies have shown that ovaries from strong *toucan* mutations present mislocalized oocyte nuclei in some stage 10 egg chambers (data not shown). This phenotype is similar to those described in *grk*, *top* and *cni* mutant egg chambers and reflects an abnormal A/P polarity of the oocyte cytoskeleton. Third, $toucan^{P}$ homozygous females lay eggs with dorsal defects. This eggshell phenotype is weaker than that of *grk* or *top* (Schüpbach, 1987), suggesting that the dorsal signalling is not completely disrupted in *toucan* mutants but that some dorsal follicle cells do not adopt a correct fate. Embryos from $toucan^{P}$ homozygous females die before cellularization, preventing analysis of embryonic cuticles. Altogether, these observations suggest that *toucan* could be closely linked to the Grk-Egfr signalling pathway. It will be interesting to determine whether *toucan* is required for the production or the distribution of the Grk protein.

In conclusion, the *toucan* gene plays a key role in signalling between the germline cells and the somatic cells to build a mature egg chamber. Its main function seems to be to allow the correct production and/or localization of germline signal(s) directed towards the somatic cells. Analysis of the subcellular localization of the Toucan protein will allow us to define its precise role in such signalling pathways.

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