Int. J. Dev. Biol. 43: 207-217 (1999)

Original Article

Requirement of *Drosophila l(2)gl* function for survival of the germline cells and organization of the follicle cells in a columnar epithelium during oogenesis

CÉCILIA DE LORENZO, DENNIS STRAND and BERNARD M. MECHLER*

Department of Developmental Genetics, Deutsches Krebsforschungszentrum, Heidelberg, Germany

ABSTRACT The *lethal(2)giant larvae* gene, or *l(2)gl*, encodes a widely expressed cytoskeletal protein which acts in numerous biological processes during embryogenesis and oogenesis, including cell proliferation, and morphogenetic movements. Having identified the nucleotide change occurring in the $l(2)gl^{ts3}$ sequence, we produced by site-directed mutagenesis the identical change leading to the substitution of a serine by a phenylalanine at position 311 of $p127^{l(2)gl}$ and introduced the modified $l(2)gl^{F311}$ gene into $l(2)gl^{-}$ flies. The transgene can fully rescue the development of *l(2)gl* flies raised at 22°C but causes drastic effects on their development at 29°C confirming the temperature sensitivity of the phenylalanine substitution at position 311. Fertility of females, albeit not of males, was strongly affected. Temperature-shift experiments and microscopic examination of ovaries showed that the mutation blocked egg chamber development at the onset of vitellogenesis (stages 8-9) with growth arrest of the oocyte, incomplete follicle cell migration over the oocyte associated with abnormal organization of the follicular epithelium, and apoptosis of the germline cells, as measured by TUNEL assays. By comparison to wildtype, we found that $p127^{F311}$ is already reduced in amount at 22°C and delocalized from the cytoskeletal matrix, albeit without affecting the apical localization of myosin II, a major partner of p127. At 29°C, the level of $p127^{F311}$ is even more reduced and the distribution of myosin-II becomes markedly altered at the apices of the follicle cells. These data indicate that during oogenesis p127 plays a critical function at the onset of vitellogenesis and regulates growth of the oocyte, follicle cell migration over the oocyte and their organization in a palisadic epithelium, as well as viability of the germline cells.

KEY WORDS: *Drosophila, l(2)gl, myosin-II, cytoskeleton, oogenesis, apoptosis*

Introduction

In recent years interest in *Drosophila* oogenesis has grown considerably because development of new genetic strategies and use of refined techniques in molecular and cell biology have provided ways to analyze the mechanisms underlying the morphogenesis of complex tissues. In these respects, the ovary of *Drosophila* made of 16 ovarioles, each representing an independent egg assembly line, constitutes a particularly suitable model system to investigate the sequence of events which lead a germline stem-cell with a number of associated somatic cells to develop into an egg chamber and in turn this egg chamber to form a mature egg (King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993).

During this complex process, essentially three types of cells act in concert, namely the oocyte, the germline-derived nurse cells, and the somatically derived follicle cell populations. The origin and differentiation of the three types of cells and their integration into a functional egg chamber require cell proliferation, cell growth, cell shape changes, alterations in cell contacts and cell migrations. The pathway which leads to these changes can be divided into two sets of events. The first is a cascade of regulatory processes involving cell-cell signals and changes in gene transcription. The second set of events includes activation of effector molecules which generate changes in cell shape. Effector molecules are often cell adhesion or cytoskeletal proteins and tend to act in multiple and/or redundant functions. If many upstream regulators of oogenesis have been described, fewer effectors have been identified and they remain

Abbreviations used in this paper: l(2)gl, lethal (2) giant larvae; nmMHC, nonmuscle myosin II heavy chain.

***Address for reprints:** Department of Developmental Genetics, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. FAX: (49) 6221-424552. e-mail: dev.genetics@dkfz-heidelberg.de

0214-6282/99/$15.00
© UBC Press
Printed in Spain
www.lg.ehu.es/ijdb

poorly characterized. Therefore, the analysis of the connection between regulators and effectors is of cardinal importance for understanding how the activation of a specific regulator may spatially and temporally trigger defined coordinate changes in the shape and position of cells during oogenesis. Many of the movements are thought to be driven by motor proteins, such as the actin-based motor protein nonmuscle myosin II, and recent analyses have shown that a hypomorphic mutation in *spaghetti squash*, or *sqh*, which encodes the nonmuscle myosin II regulatory light chain, can disrupt several distinct steps during oogenesis indicating that myosin II function is required in numerous morphological events during egg chamber development (Edwards and Kiehart, 1996; Jordan and Karess, 1997).

Although *Drosophila* nonmuscle myosin II likely binds several proteins in the cells, only three of them have been so far characterized: filamentous (F-)actin, the above mentioned myosin II regulatory light chain, and the p127 protein encoded by the *lethal(2)giant larvae* tumor suppressor gene, or *l(2)gl* (Strand *et al.*, 1994a). Our recent studies have shown that p127 can bind to a discrete domain of the myosin tail near its carboxyl end. Through such binding p127 can prevent the *in vitro* formation of myosin II filaments, indicating that the binding of p127 may maintain myosin II in a non-contractile state and genetic analysis has shown that indeed p127 functions as a negative regulator of myosin II activity (G. Merdes, D. Strand, Z. Su, D. Kiehart and B.M. Mechler, personal communication). Moreover, immuno-histochemical analysis of morphogenetic movements during *Drosophila* embryogenesis has revealed that myosin structures involved in surface contraction are constantly depleted in p127 further supporting the concept that p127 is associated with a non-contractile form of myosin II.

Inactivation of *l(2)gl* leads to malignant transformation of the neuroblasts in the larval brain hemispheres and tumors of the imaginal discs (Gateff and Schneiderman, 1969) and produces abnormalities in other tissues (Hadorn, 1961; Mechler, 1991). As previously shown, the *l(2)gl* gene product, or p127 (Jacob *et al.*, 1987), acts as a cytoskeletal component in both the peripheral matrix underlying the plasma membrane and the cytoplasmic cytoskeleton in a number of tissues throughout development (Strand *et al.*, 1994b). Furthermore, p127 forms high molecular mass complexes made essentially of homo-oligomers (Jakobs *et al.,* 1996) with which at least ten additional proteins are associated (Strand *et al.*, 1994a). Among the proteins interacting with p127, in addition to nonmuscle myosin II heavy chain (Strand *et al.*, 1994a), we identified a yet unknown serine-kinase whose activation leads to a specific phosphorylation of p127 and results in the dissociation of myosin II from p127 without affecting p127 oligomerization (Kalmes *et al.*, 1996). The ability of p127 to bind myosin II is not only limited to *Drosophila* but was shown to occur also between the human homologs of both proteins (Strand *et al.*, 1995) further supporting the notion that mechanisms similar to those uncovered in *Drosophila* may also govern the intracellular organization of human cells.

Pioneering work of Hadorn and his collaborators showed that, among the pleiotropic pattern of defects resulting from the inactivation of *l(2)gl*, male and female gonads were differentially affected with the male germ cells degenerating during early larval development and the female germ cells remaining unaffected during the whole larval development (Hadorn and Gloor, 1942; Gloor, 1943; Hadorn, 1961). When *l(2)gl* ovaries are transplanted into genetically wild-type larvae (Hadorn, 1937), the implanted ovaries develop synchronously with the metamorphosing host and at the time of hatching reach the same stage as wild-type ovaries. However, the development of the implanted *l(2)gl* ovaries is arrested with egg chambers displaying degenerated nurse cells and aberrant organization of the follicle cell layer. These data were interpreted as showing a difference in the specificity of the lethal phase with an earlier "lethal crisis" in germ cells than in somatic cells, whose differentiation was even thought not to be affected by the mutation (Gloor and Hadorn, 1942; Hadorn and Gloor, 1942; Gloor, 1943; Hadorn, 1961). Further studies involving pole cell transplantation and genetic mosaics reveal, however, that *l(2)gl* function is required in both germline and somatic cells for producing mature eggs (Szabad *et al.*, 1991) and immunohistochemical examination shows that during oogenesis p127 is indeed expressed in both germline and follicle cells (Strand *et al.*, 1994b).

Since amorphic *l(2)gl* mutations can prevent egg development in mosaic animals, we have investigated the *l(2)gl* function by taking advantage of the availability of a temperature-sensitive allele of *l(2)gl*. For this purpose we used the $l(2)gl^{ts3}$ allele which was previously induced by W. Hanratty (Manfruelli *et al.*, 1996; Baek and Hanratty, 1997). In order to eliminate effects of second site mutations and to confirm that the temperature sensitivity of this allele results from an amino acid substitution, we determined first the nucleotide sequence of the whole coding domain of the $l(2)gl^{ts3}$ allele and identified the site of the molecular lesion. We then created by site-directed mutagenesis an equivalent change in a wild-type *l(2)gl* gene and by using *P*-element mediated transformation and genetic crosses we introduced the modified *l(2)gl* gene into *l(2)gl*-deficient flies which were then subjected to phenotypic analysis. Our data show that exposure to a restrictive temperature results in the specific arrest in egg chamber development at mid-oogenesis with apoptosis of the germline cells and incomplete migration and abnormal organization of the follicle cells over the oocyte. These findings reveal that the *l(2)gl* gene contributes to different cell-specific processes involving growth of the oocyte, organization of the follicle cells over the oocyte and survival of the germline cells.

Results

Temperature induced sterility of $l(2)gl^{F311}$ females

Nucleotide sequence analysis of the $l(2)gl^{ts3}$ gene revealed a single nucleotide change corresponding to C to T transition at nucleotide position 7762, as defined by Jacob *et al.* (1987) (EMBL Accession Number M17022). This change resulted in the substitution of a serine residue by a phenylalanine residue at amino acid position 311. Following construction of a *P-CaSpeR* transposon (Pirrotta, 1986) containing the synthetically modified gene carrying a T at position 7762, designated as $P\text{-}[l(2)gl^{F311}]$, we introduced by *P*-element mediated transformation this transposon into the genome of *y w* flies. Transgenic flies carrying the modified gene were recovered, and the $P\text{-}[l(2)gl^{F311}]$ gene was tested for its ability to rescue the development of $l(2)gl^{4}$ animals which carry a terminal deletion of the left arm of chromosome 2 uncovering the *l(2)gl* gene (Mechler *et al.*, 1985). Three different lines containing an independently inserted $P\text{-}[l(2)gl^{F311}]$ transgene were found to fully rescue the development of $l(2)gl^{4}$ flies when raised at the permissive

temperature of 22°C. However, drastic effects on the development of these flies were noticed when they were reared at a restrictive temperature of 29°C. In particular, we found that the fertility of *P-[l(2)gl^{F311}]* or *l(2)gl^{F311}* females was strongly reduced when maintained at 29°C. By contrast, the fertility of the males was not affected. These results demonstrated that the temperature sensitivity is uniquely caused by the change of a serine into a phenylalanine at amino acid position 311 in the p127 protein.

In order to determine which stage of oogenesis is affected by the restrictive temperature we investigated in more detail the effects of temperature shifts on the production of eggs laid by *l(2)gl^{F311}* females. When newly hatched females raised at 22°C were immediately shifted to 29°C, we found that they were unable to produce eggs. However, when *l(2)gl^{F311}* females were first maintained for two to three days at 22°C and then shifted to 29°C, these females displayed a nearly normal rate of egg laying during the first day at 29°C. Then the rate of egg laying gradually decreased to reach after 3 days at 29°C about 5% of the control rate. This result indicates that the critical phase affected by the *l(2)gl^{F311}* mutation takes place during a period extending between early to mid-oogenesis and that late oogenesis is relatively unaffected. In particular, we observed that the eggs laid by *l(2)gl^{F311}* females at 29°C displayed a normal morphology and could be fertilized although their development was blocked when the eggs were maintained at this temperature. By contrast, when eggs laid for two hours by *l(2)gl^{F311}* females maintained at 29°C were then incubated at 22°C, their development was found to be normal indicating that the alteration occurring at 29°C in the p127^{F311} protein is reversible.

The l(2)gl^{F311} mutation blocks egg chamber development at the onset of vitellogenesis

To further define the morphological defects which occur during oogenesis we examined the ovaries of *l(2)gl^{F311}* females which were shifted to 29°C immediately after hatching and reared for two additional days at 29°C (Fig. 1B), and compared their structure to those of wild-type ovaries which were treated identically (Fig. 1A). By comparison to wild-type ovaries, the ovaries of *l(2)gl^{F311}* females exhibited a severe reduction in their overall size which can be attributed to a lack in late stage egg chambers. A similar observation was made with ovaries of females carrying the original *l(2)gl^{ts3}* mutation, although the ovaries displayed an even more pronounced reduction in the size of the larger egg chambers (Fig. 1C). By contrast, the morphology of ovaries from *l(2)gl^{F311}* females reared at 22°C was indistinguishable from that of wild-type ovaries. Further examination of individual ovarioles allowed us to more precisely determine the stage of egg chamber development which is affected by the *l(2)gl^{F311}* mutation.

A *Drosophila* ovary consists of a cluster of 15-17 ovarioles, each representing an independent egg assembly line. The tapered anterior tip of each ovariole forms the germarium which is made of germline and somatic stem cells whose progeny are subsequently organized into egg chambers. Each egg chamber is formed of 16 cystocytes of which one will differentiate as the oocyte and the other 15 as nurse cells. The egg chambers, which become surrounded by a layer of somatic cells, leave the germarium and develop progressively as they move posteriorly within the ovariole. Their development has been divided into fourteen stages beginning with the formation of the egg chamber in the posterior region

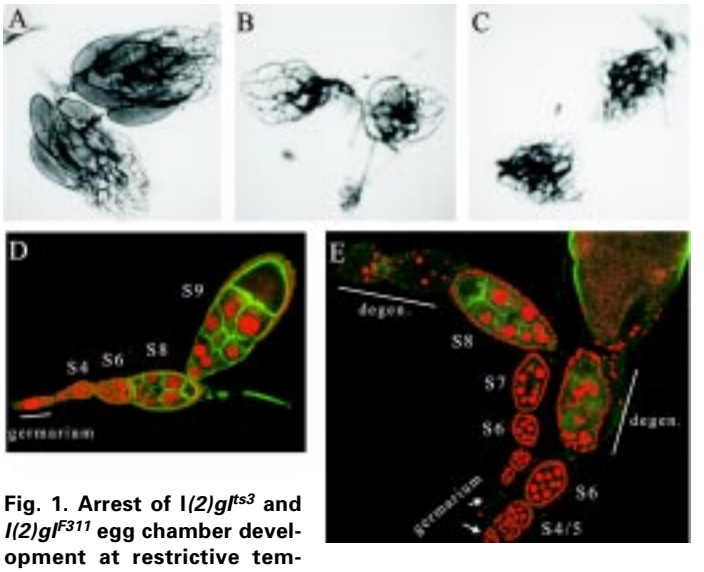

Fig. 1. Arrest of l*(2)gl^{ts3}* and *l(2)gl^{F311}* egg chamber development at restrictive temperature. *Dissected ovaries of* **(A)** *wild-type,* **(B)** *l(2)gl^{ts3}, and* **(C)** *l(2)gl^{F311} females reared for 2 days at 29°C. In l(2)gl^{ts3} and l(2)gl^{F311} the ovaries display an extreme reduction in size. Egg chambers of* **(D)** *wild-type and (E) l(2)gl^{F311} ovarioles stained with FITC-conjugated phalloidin (green) to reveal actin-rich structures and propidium iodine (red) for DNA with degenerating (degen) egg chambers.*

of the germarium followed by the previtellogenic stages 2-7 (King, 1979; Spradling, 1993). During these stages, the egg chambers increase gradually in size, albeit remaining roughly spherical. They are uniformly covered by a monolayer of approximately 1000 follicle cells and linked to the adjacent egg chambers by short chains of interfollicular stalk cells. At stage 7 the egg chambers become elongated and, from stage 8, the yolk proteins which are essentially synthesized in the fat bodies and released in the hemolymph accumulate by an endocytic process in the oocyte. Between stages 9 and 13 the follicle cells undergo a characteristic set of migrations that are significant for the final organization of the egg. In particular, between stages 9 and 10, most of the follicle cells migrate posteriorly, surrounding the oocyte within the egg chamber and leaving only about 80 thin epithelial cells over the nurse cells. At stage 9, a small group of about 10 cells, called the border cells, migrate from the anterior end of the egg chamber between the nurse cells and come to lie at the anterior border of the oocyte. These cells secrete the micropylar cone and the canal through which the fertilizing sperm enters. The covering layer of follicle cells first secretes the vitelline membrane during stages 9 to 11 which are concluded by the discharge of the content of the nurse cells into the oocyte at stage 11. During the final stages 12 to 14, the follicle cells secrete the successive layers of the chorion and the two long tapering appendages of the anterior dorsal surface and a chorionic plaque at the posterior end of the egg whereas the nurse cells and their nuclei regress completely. Finally, the resulting mature egg is released into the oviduct.

Examination of 5 day old *l(2)gl^{F311}* females which were reared for three days at 29°C revealed a marked depletion in egg chambers of advanced stages, i.e. beyond stage 9. A few ovarioles were found to contain a nearly mature egg but in these cases the egg was found to follow a degenerating egg chamber (Fig. 1E). In all the other ovarioles, the most developed egg chambers

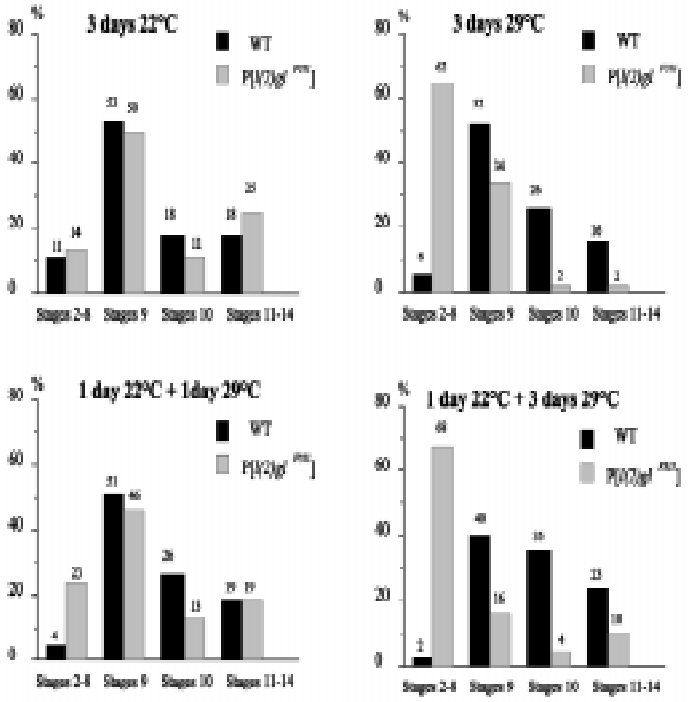

Fig. 2. Development of egg chambers in wild-type and *l(2)gl^{F311}* ovarioles after different temperature shift regimens. *The stage of ovariole development in wild-type (black) and l(2)gl^{F311} (gray) females reared at 22°C or 29°C for the periods of time indicated in each panel was analyzed in at least 100 ovarioles. The most advanced stage of egg chamber development in each ovariole was determined by confocal microscopy as depicted in Figure 1D and E, and their relative abundance are indicated as percentages above the bars. Restrictive temperature blocks egg chambers development in l(2)gl^{F311} females at stages 8-9.*

display degenerating characteristics while egg chambers in early stages of development (stages 1 to 8) appeared to be normal (Fig. 1D,E). In particular, the defective egg chambers exhibited a pronounced size reduction with abnormally arranged nurse cells and a disorganized cytoskeleton. These observations suggest that the critical phase affected by the *l(2)gl^{F311}* mutation occurs at stage 9 of oogenesis. To sustain this inference, we performed a series of shift experiments with either newly hatched females or one day-old females and scored the stages of the most-advanced egg chambers present in over one hundred individual ovarioles. As shown in Figure 2, this analysis revealed that the distribution of egg chamber stages is similar in both wild-type and *l(2)gl^{F311}* ovaries when the females were maintained at 22°C. By contrast, when females were shifted to 29°C immediately after hatching and reared at this temperature for three days we observed a dramatic change in the distribution of the most advanced egg chamber stages in *l(2)gl^{F311}* ovarioles. The *l(2)gl^{F311}* ovaries were found to predominantly accumulate stage-2 to -9 egg chambers and were essentially lacking egg chambers of more developed stages (Fig. 2B). When we examined the ovaries of newly hatched females which had been first kept one day at 22°C and then shifted for two days at 29°C, we found almost no difference in the developmental stages reached by egg chambers in ovaries of both wild-type and *l(2)gl^{F311}* females. We only noticed a slight decrease in the number of egg chambers at stage 10 and a corresponding increase in the number of young egg chambers. However, when similar females had been kept for three days at 29°C, we noticed a drastic reduction in the number of advanced stage egg chambers in *l(2)gl^{F311}* ovaries and a reciprocal increase in the number of young egg chambers. Then similar proportions of egg chamber stages were found in the ovarioles of *l(2)gl^{F311}* females maintained for either five, seven or eleven days at 29°C (data not shown). These results further indicate that the critical phase affected by the *l(2)gl^{F311}* mutation takes place at the transition between previtellogenesis and vitellogenesis at a time when egg chambers undergo pronounced structural changes with yolk deposition and follicle cell migration.

l(2)gl^{F311} blocks growth of the oocyte, formation of an orderly organized columnar epithelium following migration of the follicle cells over the oocyte and induces apoptosis in the germline cells

In order to monitor the morphogenetic processes which are affected during oogenesis by the *l(2)gl^{F311}* mutation, we examined by confocal microscopy egg chambers of four day-old wild-type and *l(2)gl^{F311}* females kept for three days at 29°C. First, we investigated the structure of the egg chambers by staining with FITC-conjugated phalloidin the actin-based cytoskeletal matrix underlying the plasma membrane and the specialized structures interconnecting the germ cells, called the ring canals, and by labeling the DNA with propidium iodide. This analysis revealed a similar organization in the germarium and previtellogenic egg chambers of both wild-type and mutant ovarioles. In particular, the stage-2 to -8 egg chambers of *l(2)gl^{F311}* ovarioles are surrounded by a single layer of follicle cells and their germ cells are connected by normally looking ring canals. Furthermore, the size of the *l(2)gl^{F311}* nurse cells nuclei is as large as in wild-type.

Structural abnormalities become visible with stage-9 egg chambers when, in the case of wild-type, the oocyte becomes larger than the nurse cells and the follicle cells begin to migrate over the oocyte forming first a gradient of increasingly elongated cells (Fig. 3A,B) which ultimately results at stage 10A in a demarcated monolayer of columnar cells at the posterior of the egg chamber and a layer of squamous cells at the anterior (Fig. 3C,D). Simultaneously a group of about 6-10 follicle cells, the border cells, located at the anterior end of the egg chamber migrate between the nurse cells and become apposed to the anterior border of the oocyte at the time when the other follicle cells complete their posterior migration over the oocyte. Examination of *l(2)gl^{F311}* ovarioles showed that the most advanced egg chambers can reach stage 9 with a large proportion of the follicle cells being located over the oocyte (Fig. 3E-H). By comparison to a similarly developed wild-type egg chamber (Fig. 3B), the oocyte size of a *l(2)gl^{F311}* stage-9 egg chamber is smaller (Fig. 3H) indicating that yolk uptake may be deficient. In all stage-8/9 egg chambers which have been examined, the layer of follicle cells over the oocyte forms an antero-posterior gradient of increasingly elongated cells whose shape is irregular and whose nuclei, instead of forming a regular row, tend to be irregularly spaced (Fig. 3E-H). No sharp transition between columnar cells and squamous cells could be detected. These observations indicate that the follicle cells were first able to migrate normally over the oocyte, but unable to achieve their migration. Over the posterior end of a degenerating *l(2)gl^{F311}* egg chamber as shown in Figure 4C and D, the columnar

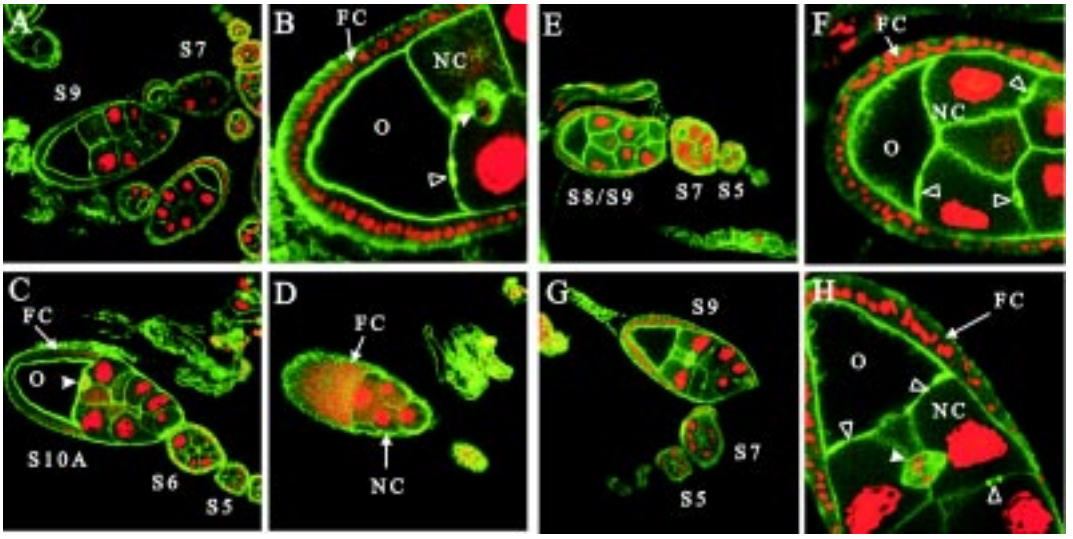

Fig. 3. Wild-type and *l(2)gl*^{F311} egg chambers during follicle cell migration maintained for 3 days at 29°C. *Confocal images of (A-D) wild-type egg and (E-H) l(2)gl*^{F311} *chambers stained for actin (green) with FITC-conjugated phalloidin and DNA (red) with propidium iodide.* **(A** *and* **B)** *Ovariole of a stage-9 egg chamber (S9) in which the follicle cells (FC) are in the process of migration over the oocyte (O) resulting in an anterior-posterior gradient of cell thickness. (B) A higher magnification of the stage-9 egg chamber shown in (A) reveals the regular spacing of nuclei in the columnar epithelial cells over the oocyte and the migrating border cells (closed arrowheads) passing between the nurse cells (NC).* **(C** *and* **D)** *In a more advanced stage-10A egg chamber, the migration of the follicle cells over the oocyte and the border cells has been completed for both types of cells with few follicle cells remaining over the nurse cells. Note the relatively dense distribution of the nuclei in the columnar epithelial layer of follicle cells over the oocyte which fills about 40% of the egg chamber. C is an optical section through the center of the egg-chamber whereas D is a more superficial section of the same egg chamber. Position of ring canals in nurse cells is indicated by open arrowheads.* **(E-H)** *Incomplete migration of both follicle and border cells in l(2)gl*^{F311} *stage-9 egg chambers of females held three days at restrictive temperature. The follicle cells have only partially migrated over the oocyte forming a layer of irregularly aligned nuclei. In addition, the oocyte is partially crumpled and relatively small in size. (F) Enlarged equatorial optical section of the egg-chamber shown in E reveals the distorted alignment of the nuclei over the oocyte.* **(G** *and* **H)** *Optical sections through a stage-9 egg chamber corresponding to the most-developed non-degenerating egg-chamber that can be observed in l(2)gl*^{F311} *ovaries maintained at 29°C. Migration of both follicle and border cells over the oocyte is incomplete. Although the oocyte displays a regular shape, its size is reduced by comparison with that of an oocyte in a wild-type stage-9 egg chamber. (H) Higher magnification of G.*

cells can become extremely elongated. We also noticed that, in the most advanced stage-9 egg chambers, as shown in Figure 4B, the border cells have apparently reached their final destination as indicated by their lateral alignment, however, they remained located within the layer of nurse cells bordering the oocyte and were never found apposed to the anterior border of the oocyte, although the distance of their migration within the egg chamber may correspond to the length needed to reach the anterior border of a normally growing oocyte. This finding indicates that the migration of border cells is not affected in *l(2)gl*^{F311} egg chambers.

One of the most obvious aspects of the *l(2)gl*^{F311} ovaries held at 29°C deals with the degeneration of the most distally located egg chambers in more than half of the ovarioles. In these egg chambers the nuclei are very condensed and fragmented and the phalloidin-stained cytoskeletal matrix which normally decorates the cortical domain of the nurse cells and the oocyte becomes disrupted in the most advanced degenerating egg chambers. A similar disruption is observed in the follicle layer in which a residual phalloidin-stained material accumulates at the apex of the most anteriorly located cells. Furthermore, the reduction in volume of the egg chamber is accompanied by a thickening and disorganization of the follicle layer characterized by overlapping cells (Fig. 4C,D). The relative smaller volume of the most severely disorganized egg chambers and the strongly condensed and fragmented structure of the polyploid nurse cell nuclei indicate a rapid degeneration of the germ cells which is highly reminiscent of cell death resulting from apoptosis.

To confirm that apoptosis occurs in the degenerating egg chambers, we performed a TUNEL assay on ovaries taken from *l(2)gl*^{F311} females which were held for three days at 29°C. Figure 4H-I shows a degenerating egg chamber stained with propidium iodide and labeled with fluorescein-dUTP by using terminal transferase. In this egg chamber all pycnotic nurse cell nuclei were intensively stained with propidium iodide and reacted positively in the TUNEL assay whereas in earlier stage egg chambers the normally looking nuclei were diffusely stained with propidium iodide and remained unlabeled by terminal transferase. These results clearly demonstrated that egg chambers containing pycnotic nuclei underwent apoptosis. Usually, when an egg chamber was positively reacting in the TUNEL assay, all nurse cell nuclei were labeled whereas no follicle cell nuclei or only a small proportion of them were positively reacting. This observation suggests that the apparent synchronization of programmed cell death in the nurse cells may result from the presence of functional ring canals between these cells which may mediate an apoptotic signal. No such signal may take place between the follicle cells which are devoid of visible cytoplasmic connection. Using other indicators of cell death by apoptosis, such as acridine orange which detects altered plasma membrane permeability and 123-rhodamine which reveals increased mitochondrial activity, we found that egg chambers in advanced stages of degeneration showed uptake of both dyes (data not shown). However, in our hands, pycnosis of nuclei was the most reliable and direct criterion for detecting the earliest sign of apoptosis (Fig. 4E-G). In an early apoptotic stage-9 egg chamber shown in Figure 4E-G, the nurse cell nuclei are fragmented and pycnotic but are still in their original position in the egg chamber. As revealed by phalloidin staining, the actin cytoskeleton retains its organization and the ring canals are still present between

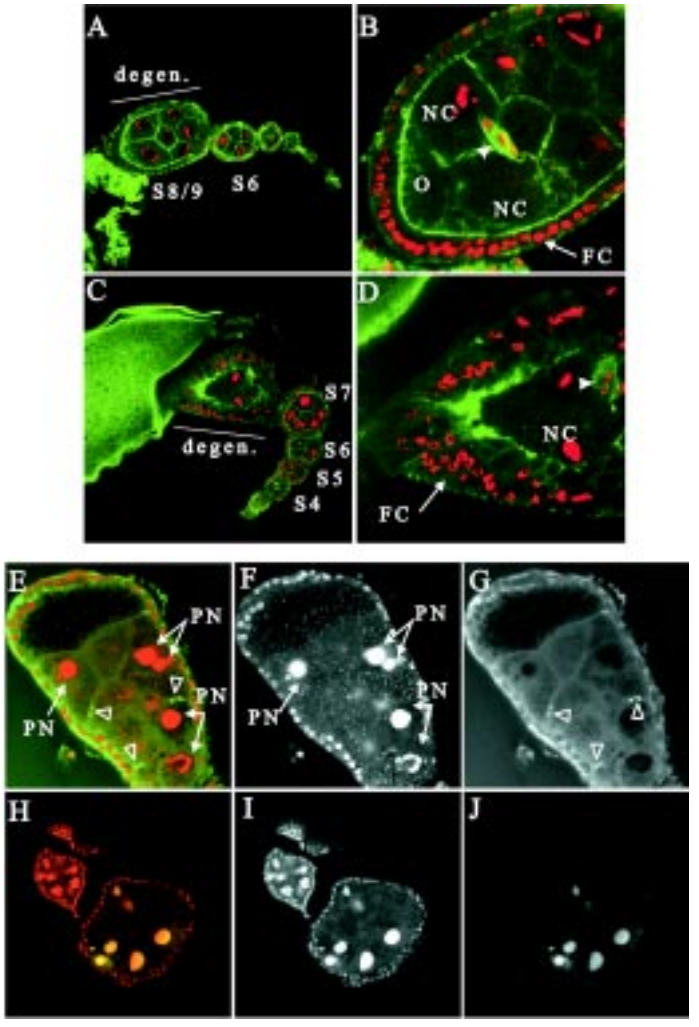

Fig. 4. Advanced epithelial abnormalities coincide with the presence of apoptotic nurse cells in degenerating *l(2)gl^{F311}* egg chambers. *(A-G) Confocal images of egg chambers from ovaries of l(2)gl^{F311} females maintained 3 days at 29°C and stained for actin (green) with FITC-conjugated phalloidin and DNA (red) with propidium iodide.* **(A)** *Stage-8 egg chamber with earlier signs of degeneration as revealed by pycnosis of the nuclei in the nurse cells and a thickening of the epithelial layer at the anterior pole of the egg chamber without a concomitant emaciation of the follicle cells over the nurse cells.* **(B)** *Stage-9 egg chamber with an oocyte (O) of extremely reduced size. The migration of follicle (FC) and border cells (BC) is incomplete with the border cells forming a flattened disc between pycnotic nurse cells (closed arrowheads).* **(C)** *Ovariole with a degenerating egg chamber displaying an almost complete dissolution of the oocyte and nurse cells resulting in thickening and contortion of the follicle cells at the anterior of the egg chamber. Note the presence of apparently intact border cells in the centrally located region of the egg chamber.* **(D)** *Higher magnification of C.* **(E-G)** *Confocal images of a stage-8-9 egg chamber with all nurse cell nuclei becoming simultaneously pycnotic, albeit remaining predominantly at their original position in the egg chamber. Migration of both follicle and border cells has not yet been initiated but the onset of the apoptotic process of the oocyte and nurse cells leads to a dissolution of the content of these cells resulting in an abnormal organization of the follicle cells. (F) DNA channel of E and (G) actin channel of* **(E)**. **(H)** *Confocal image of an ovariole stained for DNA with propidium iodide (red) and labeled with fluorescein conjugated dUTP in the presence of deoxyterminal transferase (yellow). The TUNEL assay shows that pycnotic nuclei of the nurse cells in the most developed egg chamber are positively reacting.* **(I)** *DNA channel of H and* **(J)** *TUNEL assay channel of H.*

the nurse cells (Fig. 4G). In more advanced stages (Fig. 4B,D), there is no more trace of organized cytoskeleton structure and the ring canals have disappeared.

All together the data resulting from the structural analysis of the degenerating egg chambers demonstrate that the *l(2)gl^{F311}* mutation causes distinct effects during mid-oogenesis. First, the growth of the oocyte is impaired, second, the ultimate phase of migration of the follicle cells over the oocyte and their organization in a tightly apposed columnar epithelium are altered and, third, apoptosis of the germline cells is induced and leads to a rapid and complete collapse of the egg chamber.

Instability of p127^{F311} is increased at restrictive temperature

In order to examine the effects of the phenylalanyl substitution at position 311 on the stability of p127 we determined by Western blot analysis the level of p127 expression in wild-type and *l(2)gl^{F311}* ovaries at permissive and restrictive temperatures. Western blot analysis of proteins extracted from ovaries of four day-old females kept for three days at either 22°C or 29°C revealed that by comparison to wild-type ovaries the amount of p127^{F311} is markedly reduced at permissive temperature (Fig. 5A). This reduction was even more pronounced at restrictive temperature, indicating that the phenylalanyl substitution directly affects the stability of the p127 protein.

This finding prompted us to determine whether the p127^{F311} instability would be reflected in its association with nonmuscle myosin II heavy chain. We investigated the amount of nonmuscle myosin II heavy chain present in p127 immuno-complexes, extracted from wild-type and mutant ovaries maintained at permissive or restrictive temperatures. As shown in Figure 5B, we found that the level of myosin II associated with p127 remained constant in ovaries of both wild-type or *l(2)gl^{F311}* mutant females whether they were maintained at 22°C or 29°C. This result indicates that the level of interaction between p127 and myosin II is apparently not affected by the phenylalanine substitution at position 311 and in view of the reduced level of the mutant protein present at 29°C suggests even that the binding of myosin II may contribute to the stabilization of the mutant p127^{F311} protein.

Delocalization of the p127^{F311} from the plasma membrane

During early oogenesis, p127 is intensively expressed in all nurse cells, oocytes and follicle cells (Strand *et al.*, 1994). We examined first wild-type ovaries maintained at 29°C and found, as shown in Figure 6 A-C, that p127 displays a normal dual intracellular location with a uniform distribution in the cytoplasm and a marked concentration along the plasma membrane. In the nurse cells and the oocyte, p127 decorates the plasma membrane separating these cells, whereas in follicle cells p127 is strongly localized along the lateral sides of the cells and virtually absent from the basal and apical membranes of these cells. By contrast, in *l(2)gl^{F311}* ovaries from females held at either permissive (Fig. 6B',D-F) or restrictive temperatures (Fig. 6B'',G-I), the p127^{F311} protein was found to be essentially absent from the cortical layer associated with the plasma membrane and diffusely distributed in the cytoplasm of both germline and follicle cells. Furthermore, under identical laser scanning parameters we detected a reduced staining of p127 in *l(2)gl^{F311}* previtellogenic egg chambers maintained at 29°C reflecting a lower abundance of the mutated protein at 29°C.

Disruption of nonmuscle myosin-II organization in l(2)gl^{F311} follicle cells takes place at restrictive temperature

In wild-type previtellogenic egg chambers (stages 6 to 9), nonmuscle myosin II is essentially concentrated at the apices and at the basal (outer) ends of the follicle cells (Edwards and Kiehart, 1996). Examination of *l(2)gl^{F311}* egg chambers maintained at 22°C showed that myosin-II is concentrated on both apical and basal domains of the follicle cells (Fig. 6D,E). At restrictive temperature the organization of myosin II in the follicle cells is markedly disrupted with formation of brightly stained clumps and aggregates of various sizes located along the apical and basal domains (Fig. 6G,H). Moreover, by comparison to p127^{F311}, nonmuscle myosin II appears to remain relatively stable and is still detectable in collapsing egg chambers in which nearly no trace of p127 could be detected (Fig. 6I). These data show that the phenylalanyl substitution at position 311 of p127 affects its stability and induces a delocalization of the mutant protein from the cytoskeletal matrix. This delocalization can be already observed at permissive temperature. However, when the temperature is raised, the mutation leads to a greater reduction in the amount of the mutant protein and produces a visible disruption of the organization of nonmuscle myosin II resulting ultimately in a developmental arrest of oogenesis.

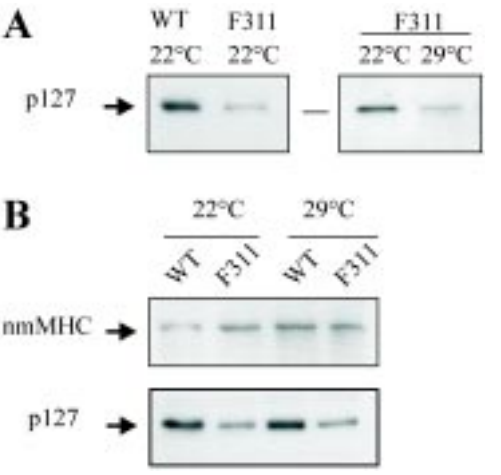

Fig. 5. The amount of p127^{F311} protein in ovaries is reduced at permissive (22°C) and even further reduced at restrictive (29°C) temperatures suggesting instability. (A) *Western blot analyses of total ovarian proteins detected with anti-p127 antibodies. By comparison to wild-type, the amount of p127 protein present in l(2)gl^{F311} ovaries maintained at 22°C is already reduced and even further reduced when the females were reared at 29°C. Loading of equivalent amounts of proteins was controlled by Coomassie Blue staining of SDS-PAGE gels loaded with identical amount of ovarian protein extracts.* **(B)** *Western blot analysis of ovarian proteins immunoprecipited with anti-p127 and detected with (upper panel) anti-myosin II antibodies and reprobed with (lower panel) anti-p127 antibodies showing that the relative amount of myosin II bound to p127 remains constant in all experimental conditions.*

Discussion

In this report we show that *Drosophila* oogenesis can be specifically blocked by the substitution of a serine by a phenylalanine at position 311 of the p127 protein, which corresponds to the mutation detected in the coding sequence of the chemically induced *l(2)gl^{ts3}* allele previously isolated by W. P. Hanratty (Manfruelli *et al.*, 1996; Baek and Hanratty, 1997). By introducing a nucleotide change in an otherwise wild-type *l(2)gl* sequence, we demonstrate through transgenesis that this amino acid substitution confers the expected temperature sensitivity, alters the stability of the p127 protein, and produces specific developmental defects during oogenesis.

Our phenotypic analysis reveals that the cytoskeleton associated p127 protein can be involved into complementary processes exerting simultaneously structural as well as signaling functions. The alteration in the palisadic organization of the follicles cells over the oocyte indicates that p127 can contribute to the structure of the cell by presumably stabilizing the plasma membrane. Involvement in a signaling pathway is supported by the finding that all nurse cells become apoptotic in stage-9 egg chambers. These two defects affecting both germline and somatic tissues constitute the major phenotypic characteristics occurring during mid-oogenesis at the onset of vitellogenesis when major structural rearrangements of the egg chamber take place. Our results extend the pioneering work made by Ernst Hadorn and Hans Gloor in the 1940s (Gloor and Hadorn, 1942; Hadorn and Gloor, 1942; Gloor, 1943; reviewed in Hadorn, 1961) showing that *l(2)gl* controls late ovarian development, as well as those made by Szabad *et al.* (1991). These latter authors demonstrated by genetic mosaics and transplantation of *l(2)gl*-deficient pole cells that both tissues are required for egg production, but no detailed description was reported on the morphological alterations occurring in these tissues and no further investigations were conducted for explaining the cause of the female sterility.

During *l(2)gl^{F311}* oogenesis the processes impaired by a shift at 29°C involve the growth of the oocyte, the final step in the migration of the follicle cells over the oocyte and their organization in a regular palisadic epithelium, as well as the survival of the nurse cells and oocyte. These events occur normally at the transition phase between previtellogenesis and vitellogenesis. No morphological abnormalities could be detected in *l(2)gl^{F311}* ovaries during their previtellogenic development. This is in contrast to previous observations made on *l(2)gl^{ts3}* ovaries maintained at restrictive temperature (Manfruelli *et al.*, 1996) showing a fusion between the germarium and the youngest egg chambers, a marked accumulation of follicle cells at both extremities of the egg chambers, and a lack of interfollicular stalk between the egg chambers. These differences indicate that, either, second site modifiers enhance the *l(2)gl^{ts3}* phenotype, or different experimental conditions than ours were used during their analysis. We favor the first hypothesis since we have noticed that heteroallelic *l(2)gl^{ts3}/l(2)gl^{F311}* ovaries display an *l(2)gl^{F311}* phenotype.

Biochemically, we can show that the *l(2)gl^{F311}* mutation alters the stability of the p127 protein and its intracellular localization. Although these two features can already be noticed at permissive temperature, no developmental abnormality could be detected at 22°C. Phenotypic effects are only noticed when *l(2)gl^{F311}* females are reared at 29°C. At this temperature the relative amount of the p127^{F311} protein is further reduced and corresponds to about one tenth of the level of p127 in wildtype ovaries. However, as revealed by the analysis of the *l(2)gl^{+t24}* transgenic flies in which a C-

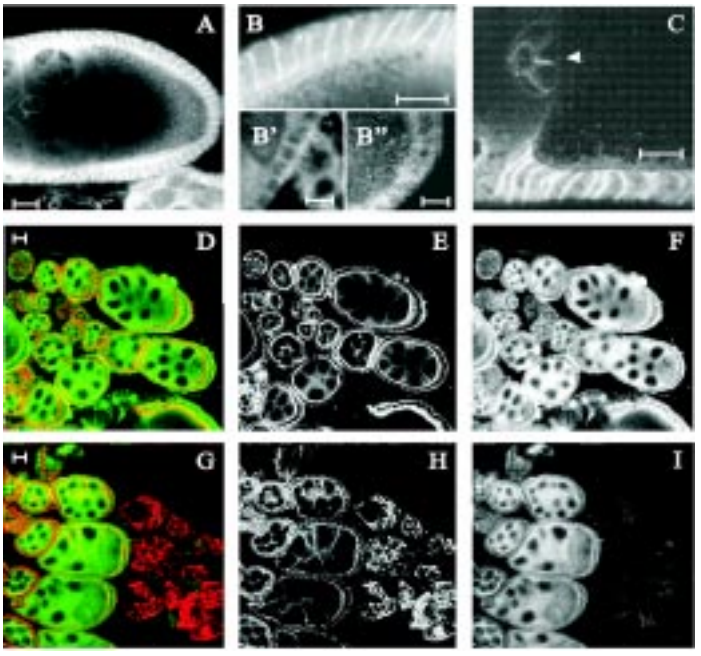

Fig. 6. Delocalization of p127^{F311} from the cytoskeletal matrix underlying the plasma membrane and temperature-dependent disruption of myosin II organization at the apex of follicle cells lining *l(2)gl^{F311}* egg chambers. (A,B *and* **C)** *Confocal images of wild-type stage-10A egg chambers maintained at 29°C and stained for p127, stage-9 egg chambers from ovaries of l(2)gl^{F311} females maintained for 3 days at* **(B')** *22°C or* **(B'')** *29°C. (D-E) Confocal images of ovarioles from l(2)gl^{F311} females maintained for 3 days at (D-F) 22°C or (G-I) 29°C and stained for (E and H) myosin II (red) and (F and I) p127 (green). In the wild-type stage-10A egg chamber, the follicle cells form a columnar epithelium over the oocyte in which p127 staining strongly decorates the lateral plasma membranes and is diffusely distributed in the cytoplasm of the follicle cells. In l(2)gl^{F311} stage-9 egg chambers, the distribution of p127 is more diffuse already (B') at permissive temperature and (B'') the membrane staining is completely abolished at restrictive temperature. As shown in* **(C)**, *border cells (arrowhead) are faintly stained with p127 by comparison to the follicle cells. The diffused cytoplasmic distribution in nurse cells and oocyte is more pronounced in less advanced egg chambers. (B) is an enlargement of A. In l(2)gl^{F311} egg chambers reared at 22°C or 29°C, p127 is diffusely distributed in the cytoplasm and virtually delocalized from the cytoskeletal matrix at 29°C. By contrast, a difference can be noticed in the pattern of distribution of myosin II between both temperatures. At 22°C myosin II displays a uniform distribution at the basal and apical domains of the follicle cells and a discontinuous distribution when the ovaries were held at 29°C. Notice that immunostaining for p127 is barely detectable in degenerating egg chambers which contain, however, detectable amount of myosin II. Bar, 20 μm.*

terminally truncated p127 protein is expressed at less than 10% of the normal level (Strand *et al.*, 1991), a strong reduction in the expression of p127^{t24} protein is insufficient to cause sterility. No alteration in the *l(2)gl^{+t24}* oogenesis can be noticed with the exception that the eggs are flaccid, albeit viable (data not shown). These data suggest that the lower level of p127^{F311} detected at 29°C is not sufficient to explain the disorganization of the egg chamber. Consequently, we have to infer that the temperature shift induces structural changes in p127^{F311}, which in turn may critically affect its interaction with other proteins.

One of the known proteins interacting with p127 is nonmuscle myosin II heavy chain (Strand *et al.*, 1994a) and our analysis showed that indeed the myosin layer surrounding the egg chamber at the apices of *l(2)gl^{F311}* follicle cells is disrupted at 29°C. A simplified explanation would be that the association between p127 and myosin II would be altered at 29°C resulting in the dissociation of p127 from myosin and in the disorganization of the myosin cytoskeleton. However, semi-quantitative Western analysis showed that irrespective of the temperature the amount of myosin II bound to p127 remained constant indicating that a reduced binding of p127 for myosin II is inadequate to explain the disruption of the myosin layer at 29°C. We have thus to infer that the *l(2)gl^{F311}* mutation may influence the association of p127 with a third protein contributing to the organization of the myosin cytoskeleton. At 29°C, the interaction between this protein and the p127-myosin II complex is disrupted and leads to the disaggregation of the myosin-based cytoskeleton. The existence of this protein is further supported by the results of deletion mapping experiments which revealed that amino acid residue 311 is presumably located outside of the myosin binding (G. Merdes and B. M. Mechler, personal communication). Furthermore, targeted modifications of evolutionary conserved amino acids in the vicinity of the residue 311 were found to exert no effect on the *in vitro* binding of myosin whereas modifications of other more distally located residues were able to either enhance or decrease the affinity of p127 towards myosin II (data not shown). These results indicate that residue 311 is outside of the myosin II binding domain and may thus correspond to the binding site of a new component involved in the organization of the myosin cytoskeleton and required during stages 9/10 of oogenesis.

The disruption of the cortical layer of myosin II at the apices of *l(2)gl^{F311}* follicle cells suggests that interaction between the follicle cells may be disrupted in a similar way as dorsal closure is affected in *zipper* embryos (Young *et al.*, 1993). In particular, the residual amount of myosin II associated with the apices of the follicle cells could be sufficient for initiating the migration of these cells over the oocyte by reducing the apical surface in contact with the oocyte but insufficient for the terminal phase of migration. In this context we would argue that *l(2)gl* is required for completing the migration of the follicle cells and establishing a regularly structured epithelium. As seen at 29°C, the *l(2)gl^{F311}* follicle cells lose their cuboidal shape, become elongated and display an apparent normal apico-basal polarity but are unable to complete their conversion into a regular columnar epithelium. The distortion of their shape and their irregular alignment indicate that p127 should contribute to one of the ultimate steps leading to the organization of a regular palisadic epithelium by presumably acting on the cytoskeletal components consolidating the lateral membrane.

The onset of vitellogenesis constitutes one of the important control points of oogenesis. At eclosion, the oldest egg chambers reach stage 7 and more than 24 h are needed to produce a mature egg. In freshly eclosed females, juvenile hormone is required for the oocyte to initiate vitellogenin uptake (Postlethwait and Weiser, 1973; Raikhel and Dhadialla, 1992). Moreover, if protein supply is not adequately provided to the female, the yolk is produced in low amounts and the majority of the egg chambers progress only up to stage 8. Further development is arrested until a sufficient amount of protein is again made available. Under such limiting conditions, vitellogenesis proceeds at a reduced rate in a limited number of egg chambers. Prolonged oogenesis is particularly observed in the case of mutants characterized by a reduced rate of protein synthesis. Mutations in the *bobbed* and *mini* genes encoding rRNA

(Mohan, 1971) and 5S RNA (Procunier and Tartof, 1975), respectively, as well as mutations in different *Minutes* genes (Walker, 1985), encoding ribosomal proteins, delay oogenesis with accumulation of previtellogenic egg chambers. The ovarioles display, however, no signs of degeneration. In the case of homozygously mutated females in the *string of pearls* gene, which codes for the ribosomal protein S2 (Cramton and Laski, 1994), the egg chambers develop normally up to stage 5 (King, 1970) and become arrested at this point without evidence of apoptosis in the most advanced egg chambers. Similarly, inability of the oocyte to incorporate yolk proteins resulting from mutations in the vitellogenin receptor encoded by the *yolkless* (*yl*) gene (Schonbaum *et al.*, 1995) blocks the growth of the oocyte without preventing oogenesis to proceed up to stages 13 and 14 when the egg chambers collapse (Waring *et al.*, 1983; DiMario and Mahowald, 1987). The *yl* genetic defect exerts no obvious morphological abnormalities within the nurse cells or the follicle cells of stage-10 egg chambers and has been shown by pole cell transplantation (Waring *et al.*, 1983) and mitotic recombination (Perrimon *et al.*, 1986) to be strictly germline dependent. Thus, although growth of *l(2)gl*^{F311} oocytes is markedly reduced at 29°C, neither a decreased rate of protein synthesis nor a failure in vitellogenin uptake are sufficient to explain the pleiotropic effects observed in *l(2)gl*^{F311} egg chambers when they reach stage 8/9.

Since the growth of *l(2)gl*^{F311} oocytes was consistently underdeveloped, the question then arises whether the incomplete migration of the follicle cells could not be simply caused by the limited space available at the surface of the oocyte. However, we observed that the *l(2)gl*^{F311} border cells migrating between the cluster of nurse cells move to a distance which corresponds to the length needed to reach the anterior limit of a normally growing oocyte, suggesting that the process of follicle cell migration is not directly affected. Apparent normal migration of border cells has also been noticed in mutations characterized by a defective posterior migration of the follicle cells producing an open-ended chorion phenotype which results essentially from a lack of centripetal migration of the follicle cells at stage 10 (Schüpbach and Wieschaus, 1991). Interestingly, in these egg chambers, like in *l(2)gl*^{F311} egg chambers, the border cells migrate to the position where the anterior end of the oocyte should be located if it would have grown normally.

A series of mutations causing developmental arrest at mid-oogenesis at the onset of vitellogenesis with degeneration of the egg chambers have been reported (King, 1970, 1979; King and Mohler, 1975; Mahowald and Kambysellis, 1980; Cooley *et al.*, 1988; Schüpbach and Wieschaus, 1991; Spradling, 1993; Verheyen and Cooley, 1994). None of these mutations displays phenotypic characteristics identical to those seen in *l(2)gl*^{F311} at 29°C. Among the collection of female sterile mutations produced by Schüpbach and Wieschaus (1991), we have examined in more detail the ovaries of *midway (mdy)* females whose oogenesis is arrested at stage 8/9 and found that the ovariole organization differs markedly from the structure of *l(2)gl*^{F311} ovarioles. In particular the *mdy* ovarioles are made of tightly stacked and elongated egg chambers without any interfollicular stalk. Moreover, in stage 8/9, the follicle cells surround entirely the egg chamber and in degenerating egg chambers the follicle cells accumulate at their posterior end, albeit without becoming columnar in shape.

Mutations at the loci *tiny (ty)* and *diminutive (dm)* cause degeneration of the egg chambers at mid-oogenesis with abnormal differentiation of the follicle cells located over the oocyte (King and Burnett, 1957; King and Vanoucek, 1960), but in the case of *ty* the follicular layer is often double layered over the oocyte and in the case of *dm* the follicle cells lining the oocyte remain cuboidal and degenerate before their transition to columnar epithelium. The inability of *dm* follicle cells to elongate indicates that *dm* should exert a temporally distinct function than *l(2)gl*. Of particular interest is the recent molecular isolation of the *dm* gene which encodes the *Drosophila* Myc protein (Gallant *et al.*, 1997; Schreiber-Agus *et al.*, 1997). The *Drosophila* Myc protein can interact with vertebrate Max and can also cooperate with activated H-RAS to malignantly transform primary mammalian cells (Schreiber-Agus *et al.*, 1997). Similar to *l(2)gl* in *Drosophila*, the mammalian *myc* proto-oncogene has been linked to multiple aspects of eukaryotic cell function, including the regulation of cell growth, differentiation and death (Henriksson and Lüscher, 1996).

One of the striking characteristics of the *l(2)gl*^{F311} mutation is the rapid death of the germ line cells which, at 29°C, occurs at the transition between stages 8 and 9 during a period of time which maximally extends up to 6 h (Spradling, 1993). The question arises whether apoptosis of the germ line cells is a direct effect of the *l(2)gl*^{F311} mutation or the sum of a series of defects occurring at this time of the egg development. To answer such a question it is necessary to dispose of probes for investigating the induction of activators of apoptosis. For this purpose we have determined whether we could detect an enhanced expression of the known activators *reaper* (*rpr*) and *head involution defective* (*hid*) in ovaries of females maintained at 29°C by comparison to those of females reared at 22°C. However, either by *in situ* hybridization or by using semi quantitative RT-PCR for detecting expression of these genes, we were unable to find any difference between the mutant and wild-type ovaries (data not shown), suggesting that these genes may not be required for regulating apoptosis during oogenesis. These results are supported by recent experiments showing that during late oogenesis normal apoptosis of the nurse cells is independent from the expression of the clusters of positive regulators of apoptosis, *rpr*, *hid* and *grim* located within the deficiency *Df(3)H99* (Foley and Cooley, 1998) but requires the presence of a yet unknown positive effector. Characterization of a nurse cell specific effector of apoptosis will provide ways to understand how the *l(2)gl*^{F311} mutation might trigger this effector. Furthermore, during oogenesis, impairment of *spaghetti squash* (*sqh*) function, which regulates the activity of nonmuscle myosin II, leads to abnormal egg chamber formation characterized by a reduced number of nurse cells which often are multinucleated indicating a failure in cytokinesis (Jordan and Karess, 1997). However, despite these defects the *sqh* egg chambers can attain the size and shape expected for stages 11-12 without displaying any sign of cell death indicating that impairment of nonmuscle myosin II function is not sufficient for inducing apoptosis of the nurse cells. Likewise, mutations affecting the neurogenic genes *egghead*, *brainiac* or *Notch* (Goode *et al.*, 1996) or the tumor suppressor gene *discs large 1* (Goode and Perrimon, 1997) which cause marked defects in epithelial morphogenesis apparently produce no premature cell death of the nurse cells indicating that abnormal epithelial morphogenesis is alone not sufficient for inducing apoptosis.

In conclusion, our studies revealed the pleiotropic effects produced during oogenesis by the substitution of a single amino acid in p127 and showed that this protein contributes to the organization of the follicle cells in a columnar epithelium and to the survival of the germline cells. In the future, studies of targeted mutations in p127

should allow a more precise understanding of the multiple functions exerted by this protein and ultimately will permit one to place the *l(2)gl* network at a nexus where signals are integrated in the execution of the various programs governed by this gene.

Materials and Methods

Genetics and molecular biology

DNA extracted from homozygous *l(2)gl*^{ts3} flies (Manfruelli *et al*., 1996; Baek and Hanratty, 1997) provided by Dr. Michel Sémériva (Marseille, France), were digested with HindIII or Spe1 and inserted into the *pBluescript SK*$^{+}$ phagemid vector (Stratagene Cloning Systems, La Jolla, CA) digested with the corresponding restriction enzymes. 5.10^{6} phagemid-carrying XL-1 Blue colonies were screened by using a purified ^{32}P-labeled EcoRI fragment containing the entire *l(2)gl* coding sequence in the cDNA clone Ec173 (Jacob *et al*., 1987). Five overlapping clones covering the entire coding domain of the *l(2)gl* gene were sequenced by standard methods and the sequence was compared to the *l(2)gl* gene sequence as defined by Jacob *et al.* (1987).

A transformation construct with a targeted replacement of a cytosine by an thymidine at position 7762 of the *l(2)gl* sequence was made by first cloning into the *pAlter-1* plasmid (Promega Corp., Madison, WI) a 5.7 kb Kpn1 *l(2)gl* genomic fragment beginning at position 7482 and extending into the polylinker of the *pGEM4* plasmid vector. This fragment was then modified by using the Altered Sites II *in vitro* Mutagenesis Systems (Promega Corp.) and the oligonucleotide sequence CAATT-GTGTGT**T**CGTTCACGC (with the substituted nucleotide indicated in bold letter) following the instructions provided by the manufacturer. A 2 kb Sph1-SacII DNA fragment (pos. 7727-9727) containing the substituted nucleotide was then used to replace the corresponding fragment in a 13.1 kb EcoR1 DNA fragment containing the whole *l(2)gl*$^{+}$ genomic sequence (Jacob *et al*., 1987) and cloned into *pCaSpeR* vector (Pirrotta, 1986). *P*-element transformations were completed by standard protocols (Rubin and Spradling, 1982; Jacob *et al*., 1987; Opper *et al.,* 1987; Török *et al*., 1993).

Immunohistochemistry

For all stainings, ovaries were dissected in Ringer's solution and fixed for 10 min in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4), rinsed in PBS, and blocked for 2 h at room temperature or overnight at 4°C with 25% goat serum in PBS plus 0.1% Tween-20. The blocking solution was removed. For double staining with p127 and myosin II sera, ovaries were first incubated overnight at 4°C with rabbit anti-p127 antibodies and the mouse anti-myosin II monoclonal antibody provided by D. Kiehart (Durham, NC) at a concentration of 5 μg/ml and 2 μg/ml, respectively, in PBS with 3% goat serum and 3% bovine serum albumin. After three successive washes in PBS for 1 h or longer, the ovaries were stained for 1 h with Cy3-labeled goat anti-rabbit immunoglobulins and Cy5-labeled goat anti-mouse immunoglobulins (Dianova, Hamburg, Germany). After 1 h washing in PBS, the ovaries were mounted in elvanol and inspected under a confocal laser scanning Zeiss microscope (Carl Zeiss Jena GmbH, Jena, Germany). For double stainings of DNA and filamentous actin, the ovaries were first treated for 2 h with RNaseA (400 μg/ml in PBS), washed with PBS and stained for 2 h at room temperature with 1 μg/ml FITC-conjugated phalloidin (Sigma, St. Louis, MO) and 5 μg/ml propidium iodine, washed overnight at 4°C in PBS, mounted in elvanol and examined under a confocal laser scanning Zeiss microscope.

For detecting apoptosis we used the TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay adapted from Gavrieli *et al.* (1992). Dissected ovaries were fixed overnight at 4°C in 4% paraformaldehyde in PBS, washed three times with PBS, incubated with 20 μg/ml proteinase K in 10 mM Tris-HCl, pH 8.0, for 15 min at room temperature, and washed in double distilled water four times for 2 min each. The ovaries were shortly preincubated in TdT buffer (30 mM trisma base, 140 mM sodium cacodylate, pH 7.2 and 1 mM cobalt chloride) and incubated for 2 h at 37°C in 100 μl TdT buffer containing 30 units terminal deoxynucleotidyl transferase and 30 nmol fluorescein-12-dUTP (Boehringer, Mannheim, Germany). The tissues were washed three times with H_2O, treated for 30 min at room temperature with RNaseA (400 μg/ml), washed three times with H_2O, stained for 30 min at room temperature with 5 μg/ml propidium iodine, washed again three times with H_2O and finally mounted in elvanol for examination under a confocal laser scanning Zeiss microscope.

Acknowledgments

The authors thank all the colleagues in the lab for support and helpful discussions and M. Sémériva for providing the original l(2)gl^{ts3} *stock. C. De Lorenzo was supported by a fellowship awarded by the Commission of the European Union (ERBCHBGCT 930245). This work was supported by grants from the Bundesministerium für Forschung und Technologie (Klinischer-Biochemischer Forschungsverbund), the Commission of the European Union (Contract BMH1-94-1572) and a contribution of the Swiss Cancer League.*

References

BAEK, K-H. and HANRATTY, W. (1997). The *lethal(2)giant larva (l(2)gl)*, a recessive oncogene, is required during embryonic and post-embryonic development in *Drosophila*. *Cancer Lett. 111*: 233-238.

COOLEY, L., KELLEY, R. and SPRADLING, A. (1988). Insertional mutagenesis of the *Drosophila* genome with single P elements. *Science 239*: 1121-1128.

CRAMTON, S.E. and LASKI, F.A. (1994). *strings of pearls* encodes *Drosophila* ribosomal protein S2, has *Minute*-like characteristics and is required during oogenesis. *Genetics 137*: 1039-1048.

DIMARIO, P.J. and MAHOWALD, A.P. (1987). *Female sterile (1) yolkless*: a recessive female sterile mutation in *Drosophila melanogaster* with depressed numbers of coated pits and coated vesicles within the developing oocytes. *J. Cell Biol. 105*: 199-206.

EDWARDS, K.A. and KIEHART, D.P. (1996). *Drosophila* nonmuscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. *Development 122*: 1499-1511.

FOLEY, K. and COOLEY, L. (1998). Apoptosis in late stage *Drosophila* nurse cells does not require genes within the *H99* deficiency. *Development 125:* 1075-1082.

GALLANT, P., SHIIO, Y., CHENG, P.F., PARKHURST, S.M. and EISENMAN, R.N. (1997). Myc and Max homologs in *Drosophila*. *Science 274*: 1523-1527.

GATEFF, E. and SCHNEIDERMAN, H.A. (1969). Neoplasms in mutant and cultured wild-type tissues of *Drosophila*. *Natl. Cancer Inst. Monogr. 31*: 365-397.

GAVRIELI, Y., SHERMAN, Y. and BEN-SASSON, S.A. (1992). Identification of programmed cell death *in-situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol. 119*: 493-501.

GLOOR, H. (1943). Entwickungsphysiologische Untersuchung an der Gonaden einer letalrasse (*lgl*) von *Drosophila melanogaster*. *Rev. Suisse Zool. 50*: 339-394.

GLOOR, H. and HADORN, E. (1942). Vergleich der Schädigungen im Ovar von *Drosophila melanogaster* bewirkt durch einen Letalfaktor und ein Sterilitätsgen. *Arch. Jul. Klaus-Stiftg. VererbForsch 17:* 438-440.

GOODE, S. and PERRIMON, N. (1997). Inhibition of patterned cell shape change and cell invasion by *discs large* during *Drosophila* development. *Genes Dev. 11:* 2532-2544.

GOODE, S., MELNICK, M., CHOU, T.-B., and PERRIMON, N. (1996). The neurogenic genes *egghead* and *brainiac* define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis. *Development 122*: 3863-3879.

HADORN, E. (1937). Transplantation of gonads from lethal to normal larvae in *Drosophila melanogaster*. *Proc. Soc. Exp. Biol. Med. 36*: 632-634.

HADORN, E. (1961). *Developmental Genetics and Lethal Factors*. Methuen and Co. Ltd., London, UK.

HADORN, E. and GLOOR, H. (1942). Die Auswirkung eines Letalfaktors (*lgl*) bei *Drosophila melanogaster* aus Wachstum und Differenzierung der Gonaden. *Rev. Suisse Zool. 49*: 228-236.

HENRIKSSON, M. and LÜSCHER, B. (1996). Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res. 68*: 109-182.

JACOB, L., OPPER, M., METZROTH, B., PHANNAVONG, B. and MECHLER, B.M. (1987). Structure of the *l(2)gl* gene of *Drosophila* and delimitation of its tumor supressor domain. *Cell 50*: 215-225.

JAKOBS, R., DE LORENZO, C., SPIESS, E., STRAND, D. and MECHLER, B.M. (1996). Homo-oligomerization domains in the *lethal(2)giant larvae* tumor suppressor protein, p127 of *Drosophila. J. Mol. Biol. 264*: 484-496.

JORDAN, P. and KARESS, R. (1997). Myosin light chain-activating phosphorylation sites are required for oogenesis in *Drosophila. J. Cell Biol. 139*: 1805-1819.

KALMES, A., MERDES, G., NEUMANN, B., STRAND, D. and MECHLER, B.M. (1996). A serine-kinase associated with the p127-*l(2)gl* tumor suppressor of *Drosophila* may regulate the binding of p127 to nonmuscle myosin II heavy chain and the attachment of p127 to the plasma membrane. *J. Cell Sci. 109*: 1359-1368.

KING, R.C. (1970). *Ovarian Development in Drosophila melanogaster,* Academic Press, New York, London and San Francisco.

KING, R.C. (1979). *Oogenesis in Drosophila.* Academic Press, New York, London and San Francisco.

KING, R.C. and BURNETT, R.G. (1957). Oogenesis in adult *Drosophila melanogaster*. V. Mutations which affect nurse cell nuclei. *Growth 21*: 263-280.

KING, R.C. and MOHLER, J.D. (1975).The genetic analysis of oogenesis in *Drosophila melanogaster*. In *Handbook of Genetics* (ed. R.C. King), Vol. 3, Plenum New York, Plenum. pp. 757-792.

KING, R.C. and VANOUCEK, E.G. (1960). Oogenesis in adult *Drosophila melanogaster*. X. Studies on the behavior of the follicle cells. *Growth 24*: 333-338.

MAHOWALD, A.P. and KAMBYSELLIS, M.P. (1980). Oogenesis. In *The Genetics and Biology of Drosophila* (Eds. M. Ashburner and T. R. F. Wright), Vol. 2D, Academic Press, New York. pp. 141-224.

MANFRUELLI, P., ARQUIER, N., HANRATTY, W.P. and SÉMÉRIVA M. (1996). The tumor suppressor gene, *lethal(2)giant larvae (l(2)gl)*, is required for cell shape change of epithelial cells during *Drosophila* development. *Development 122*: 2283-2294.

MECHLER, B.M. (1991). The fruitfly *Drosophila* and the fish *Xiphophorus* as model systems for cancer studies. *Cancer Surv. 9*: 505-527.

MECHLER, B.M., MCGINNIS, W. and GEHRING, W.J. (1985). Molecular cloning of *lethal(2)giant larvae*, a recessive oncogene of *Drosophila melanogaster*. *EMBO. J. 4*: 1551-1557.

MOHAN, J. (1971). Influence of the RNA content on oogenesis in the *bobbed* mutants of *Drosophila melanogaster. J. Embryol. Exp. Morphol. 25*: 237-246.

OPPER, M., SCHULER, G. and MECHLER, B.M. (1987). Hereditary suppression of *lethal(2)giant larvae* malignant tumor development in *Drosophila* by gene transfer. *Oncogene 1*: 91-96.

PERRIMON, N., MOHLER, L., ENGSTRÖM, L. and MAHOWALD, A.P. (1986). X-linked female-sterile loci in *Drosophila melanogaster. Genetics 113*: 695-712.

PIRROTTA, V. (1986). Cloning *Drosophila* genes. In *Drosophila, a practical approach* (Ed. D.R. Roberts) IRL Press, Oxford, UK, pp. 83-110.

POSTLETHWAIT, J.H. and WEISER, K. (1973). Vitellogenesis induced by juvenil hormone in the female sterile mutant *apterous*^{4} in *Drosophila melanogaster*. *Nature New Biol. 244*: 284-285.

PROCUNIER, J.D. and TARTOF, D.D. (1975). Genetic analysis of the 5S RNA genes in *Drosophila melanogaster. Genetics 81*: 515-523.

RAIKHEL, A.S. and DHADIALLA, T.S. (1992). Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol. 37*: 217-251.

RUBIN, G.M. and SPRADLING, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science 218*: 348-353.

SCHONBAUM, C.P., LEE, S. and MAHOWALD, A.P. (1995). The *Drosophila yolkless* gene encodes a vitellogenin receptor belonging to the low density lipoprotein receptor superfamily. *Proc. Natl. Acad. Sci. USA. 92*: 1485-1489.

SCHREIBER-AGUS, N., STEIN, D., CHEN, K., GOLTZ, J.S., STEVENS, L. and DEPINHO, R.A. (1997). *Drosophila* Myc is oncogenic in mammalian cells and plays a role in the *diminutive* phenotype. *Proc. Natl. Acad. Sci. USA 94*: 1235-1240.

SCHÜPBACH, T. and WIESCHAUS, E. (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics 129*: 1119-1136.

SPRADLING, A.C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster* (Eds. M. Bate and A. Martinez-Aria). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 1-70.

STRAND, D., JAKOBS, R., MERDES, G., NEUMANN, B., KALMES, A., HEID, H.W., HUSMANN, I. and MECHLER, B.M. (1994a). The *Drosophila lethal(2)giant larvae* tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. *J. Cell Biol. 127*: 1361-1373.

STRAND, D., RASKA, I. and MECHLER, B.M. (1994b). The *Drosophila lethal(2)giant larvae* tumor suppressor protein is component of the cytoskeleton. *J. Cell Biol. 127*: 1345-1360.

STRAND, D., TÖRÖK, I., KALMES, A., SCHMIDT, M., MERZ R. and MECHLER, B.M. (1991). Transcriptional and translational regulation of the expression of the *l(2)gl* tumor suppressor of *Drosophila melanogaster*. *Adv. Enzyme Regul. 31:* 339-350.

STRAND, D., UNGER, S., CORVI, R., HARTENSTEIN, K., SCHENKEL, H., KALMES, A., MERDES, G., NEUMANN, B., KRIEG-SCHNEIDER, F., COY, J.F., POUSTKA, A., SCHWAB, M. and MECHLER, B.M. (1995). A human homologue of the *Drosophila* tumour supressor gene *l(2)gl* maps to 17p11.2-12 and codes for a cytoskeletal protein that associates with nonmuscle myosin II heavy chain. *Oncogene 11*: 291-301.

SZABAD, J., JURSNICH, V.A. and BRYANT, P.J. (1991). Requirement for cell-proliferation control genes in *Drosophila* oogenesis. *Genetics 127*: 525-533.

TÖRÖK, I., HARTENSTEIN, K., KALMES, A., SCHMITT, R., STRAND, D. and MECHLER, B.M. (1993). The *l(2)gl* homologue of *Drosophila pseudoobscura* suppresses tumorigenicity in transgenic *Drosophila melanogaster*. *Oncogene 8*: 1537-1549.

VERHEYEN, E. and COOLEY, L. (1994). Looking at oogenesis. In *Methods in Cell Biology. Drosophila melanogaster: Practical Uses in Cell and Molecular Biology* (eds. L.S.B. Goldstein and E.A. Fyrberg). Vol. 44. Academic Press, San Diego, Ca. pp. 545-564.

WALKER, V.K. (1985). Yolk polypeptide gene expression in *Minute Drosophila* females. *Biochem. Genet. 23*: 363-378.

WARING, G.L., DIORIO, J.P. and HENNEN, S. (1983). Isolation of germ line-dependent female-sterile mutation that affects yolk specific sequestration and chorion formation in *Drosophila. Dev. Biol. 100*: 452-463.

YOUNG, P.E., RICHMAN, A.M., KETCHUM, A.S. and KIEHART, D.P. (1993). Morphogenesis in *Drosophila* requires nonmuscle myosin heavy chain function. *Genes Dev. 7*: 29-41.

Received: August 1998
Accepted for publication: November 1998