Nup154, a New *Drosophila* Gene Essential for Male and Female Gametogenesis Is Related to the *Nup155* Vertebrate Nucleoporin Gene

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Abstract. The *Nup154* gene of *Drosophila* encodes a protein showing similarity with known nucleoporins: rat Nup155 and yeast Nup170 and Nup157. Hypomorphic mutant alleles of *Nup154* affected female and male fertility, allowing investigation of the gene function in various steps of oogenesis and spermatogenesis. *Nup154* was required in testes for cyst formation, control of spermatocyte proliferation and meiotic progression. In ovaries, *Nup154* was essential for egg chamber development and oocyte growth. In both the male and female germ line, as well as in several other cell types, the Nup154 protein was detected at the nuclear membrane, but was also present inside the nucleus. Intranuclear localization has not previously been described for rat Nup155 or yeast Nup170 and Nup157. In mutant egg chambers the Nup154 protein accumulated in the cytoplasm, while it was only barely detected at the nuclear envelopes. FG repeats containing nucleoporins detected with mAb414 antibody were also mislocalized to a certain extent in *Nup154* mutant alleles. This suggests that Nup154 could be required for localizing other nucleoporins within the nuclear pore complex, as previously demonstrated for the yeast Nup170. On the other hand, no evident defects in lamin localization were observed, indicating that *Nup155* mutations did not affect the overall integrity of the nuclear envelope. However, ultrastructural analyses revealed that in mutant cells the morphology of the nuclear envelope was altered near the nuclear pore complexes. Finally, the multiplicity of phenotypes observed in *Nup154* mutant alleles suggests that this gene plays a crucial role in cell physiology.

Key words: nucleoporin genes • *Drosophila melanogaster* • egg chamber development • male gametogenesis • meiosis

The early stages of adult *Drosophila melanogaster* gametogenesis have common features in the two sexes (for reviews see Fuller, 1993; Spradling, 1993). Germ line stem cells are located at the anterior tip of both ovaries and testes. Each germ line stem cell divides asymmetrically producing a stem cell and a sister cell that becomes the mitotic "founder" of a 16-cell syncytial cyst. Somatic cell differentiation is different in the two sexes. In testes, stem cell–type division of two somatic progenitor cells generate two cyst cells that surround each newly formed primary spermatogonial cell. Signaling from germ cells is required to avoid further proliferation of these somatic cells (Gonczy and DiNardo, 1996). On the contrary, in ovaries, somatic follicle cells proceed through several division cycles and encapsulate each 16-cell cyst, as it passes through region 2b of the germarium (Margolis and Spradling, 1995).

Thereafter, germ line differentiation pathways diverge in the two sexes. In male cysts, primary spermatocytes switch from cell division to a phase of cell growth and gene expression and finally enter meiotic divisions, followed by differentiation in 64 spermatids. During male meiosis, chromosomes undergo the extensive remodelling characteristic of this process (Fuller, 1993). In females, one of the germ line–derived 16 cells becomes the oocyte, while the other sister cells become nurse cells. By stage 3 of egg chamber development, oocyte chromosomes condense into a karyosome and the nucleus remains in meiotic prophase until the end of oogenesis (Mahowald and Kam-

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bysellis, 1980). Nurse cell chromosomes are initially paired and undergo several rounds of endoreplication (Hammond and Laird, 1985). At stage 4 of development, the organization in polytene chromosome bands is lost and five blocks of chromatin become apparent (Spradling, 1993; Keyes and Spradling, 1997). At later stages, nurse cell–condensed chromatin progressively decondenses (King, 1970), facilitating the high transcription levels of components required for oocyte growth (Tirronen et al., 1993; Keyes and Spradling, 1997).

In this paper we present the isolation and characterization of mutations in a *Drosophila* gene encoding a predicted nucleoporin homologous to rat Nup155 and yeast Nup170 and Nup157 nucleoporins (Radu et al., 1993; Aitchison et al., 1995*b*). Original mutant alleles initially called *tulipano* (*tlp*), were recovered in a genetic screen for female sterile mutations. According to nucleoporin nomenclature, which indicates the various proteins on the basis of their molecular weight, we have named the gene *Nup154*. Very few *Drosophila* nuclear pore–associated proteins and genes have been identified so far (Frasch et al., 1988; Jongens et al., 1994; Berrios et al., 1995; Zimowska et al., 1997), although over the past few years many genes encoding yeast and metazoan nucleoporins have been isolated and sequenced (Davis, 1995). Moreover, mutant collections have been useful to analyze nuclear pore complex (NPC)[1] structure in yeast (Doye and Hurt, 1995), but genetic data exploring nucleoporin functions in higher eukaryotes have been up to now reported for only CAN/Nup214 protein in mouse (van Deursen et al., 1996). Finally, the role of nuclear membrane components in organizing nuclear functions is largely unknown.

We report the cloning and characterization of the *Nup154* gene, analyze its protein product localization and involvement in various steps of male and female gametogenesis, and discuss the importance and interest of the gene function in relation to the structural defects shown by mutant alleles. The availability of the first *Drosophila* mutants in a gene showing homology with known nucleoporins provides a useful genetic tool for the future isolation of mutations in other genes coding for NPC components in this organism. Their study will in turn help elucidate the physiological and developmental role of nucleoporins in metazoa.

Materials and Methods

Strains

Drosophila stocks were raised at 25°C on a standard sucrose-corn-meal-yeast medium. The *l(2)01501* strain was provided by Dr. U. Schaefer (Max-Planck-Institut für biophysikalische Chemie, Gottingen, Germany).

Molecular Analyses

Standard techniques were used for RNA and DNA manipulations, genomic and cDNA library screening (Chomczynski and Sacchi, 1987; Sambrook et al., 1989). Subclones were generated by insertion of DNA restriction fragments, or PCR-amplified products, into appropriate site(s) of the pGem3 vector.

A genomic library constructed by partial Sau3A digestion of Oregon R genomic DNA and ligation into the BamHI site of the EMBL3 vector (Frischauf et al., 1983) was provided by Dr. V. Pirrotta (Department of Zoology, University of Geneva, Geneva, Switzerland). An embryonic cDNA library in λgt10 (Poole et al., 1985) was provided by Dr. T. Kornberg (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). Nucleotide sequencing was performed using synthetic oligonucleotide primers and the Thermo Sequenase–radiolabeled terminator cycle sequencing kit (Amersham International, Little Chalfont, UK) according to manufacturer's instructions.

In Situ Hybridizations

In situ hybridizations with digoxigenin-labeled (Boehringer Mannheim GmbH, Mannheim, Germany) probes on whole-mount ovaries were performed as described (Tautz and Pfeifle, 1989), with minor modifications.

Eggs Collection and Cuticle Preparations

Eggs were collected at 24°C on small agar plates. After two 20-min precollections, eggs were collected at different intervals to obtain meiotic stages and first mitotic divisions. Eggs were dechorionated in 50% bleach, washed in distilled water, and then devitellinized as described (Warn and Warn, 1986), except for a final fixation step with acetone at −20°C for 5 min.

Cuticle preparation was performed as described (Schupbach and Wieschaus, 1986). Eggs were mounted in Hoyers/lactic acid 1:1 and cleared at 65°C.

Generation of anti-Nup154 Antibodies and Western Blot Experiments

To generate anti-Nup154 antibodies, a *Nup154* cDNA fragment coding for amino acids 253–645 of the predicted Nup154 protein was cloned in the bacterial expression plasmid pQE-31, fused in frame with its 6× his tag coding region (QIAExpress; QIAGEN Inc., Valencia, CA). The recombinant protein was expressed in the *Escherichia coli* bacterial strain M15(pREP4) and affinity purified over a NiNTA column according to manufacturer's instructions. Polyclonal antibodies directed against the fusion protein were produced in rabbit and affinity purified using the antigen immobilized on nitrocellulose filters. For Western blot analyses the proteins (35 μg/lane) were separated on 10% SDS–polyacrylamide gels (Laemmli, 1970) using a mini-PROTEAN II gel apparatus (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred onto nitrocellulose filters in transfer buffer (25 mM Tris [Sigma Chemical Co., Poole, UK], 192 mM Glycine [Sigma Chemical Co.], and 20% methanol) at a constant voltage (100 V) for 1 h at 4°C using a miniature trans-blot module (Bio-Rad Laboratories). Membranes were blocked for 16 h in blocking buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 5% nonfat dry milk, 0.05% Triton X-100). Anti-Nup154 antibodies were used at 1:100 dilution in blocking buffer for 2 h at room temperature. After washing (three times for 10 min in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Triton X-100) immunoblots were incubated with HRP-conjugated goat anti–rabbit antibodies (Bio-Rad Laboratories) (1:16,000 in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Triton X-100, 1 h at room temperature), washed as above, and then incubated in ECL+Plus (Amersham International) detection reagents according to vendor instructions.

Immunohistochemistry

Fixation and antibody staining of hand-dissected ovaries were carried out as previously described (Bopp et al., 1993). Primary anti-Nup154 antibodies were used at 1:20 dilution and detected using FITC-conjugated sheep anti–rabbit IgG secondary antibodies (Boehringer Mannheim GmbH) at 1:100 dilution. mAb414 (Berkeley Antibody Co., Richmond, CA) was used at 1:10,000 dilution and detected using rhodamine-conjugated goat anti–mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:200 dilution. DNA staining was performed by 5-min incubation in PBS containing 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole), followed by extensive washes in PBS. Stained egg chambers were mounted in Aquamount and analyzed with conventional epifluorescence or with a Zeiss laser confocal microscope attached to a Zeiss Axiofot microscope (Carl Zeiss, Oberkochen, Germany). The images were processed in Photoshop 3.05 (Adobe Systems Inc., Mountain View, CA).

Embryo staining with Hoechst 33258 and antibodies against β-tubulin was carried out as previously described (Riparbelli and Callaini, 1996).

1. *Abbreviation used in this paper*: NPC, nuclear pore complex.

Testes from larvae, pupae, and adult flies were dissected in PBS, placed in a small drop (0.5 μl) of 5% glycerol in PBS on a glass slide and covered with a coverslip. The slides were frozen on a copper bar precooled in liquid nitrogen or directly in liquid nitrogen, and after removal of the coverslip with a razor blade, were immersed in methanol for 10 min at −20°C and in acetone for 5 min at −20°C. γ-Tubulin and cyclin B were detected using 1:100 dilutions of Rbcs1 or Rb271 antibodies (provided by W.G.F. Whitfield, University of Dundee, Dundee, UK), respectively. Incubations with both antibodies were carried out overnight at 4°C. In double-staining experiments, β-tubulin was detected by subsequent incubation of the samples with anti–β-tubulin mAb (Boehringer Mannheim Corp., Indianapolis, IN), used at 1:200 dilution, for 1 h at room temperature. Nuclear envelope was identified with an mAb raised against human fibroblast vimentin (Biocine, Siena, Italy) that was previously shown to recognize nuclear membrane in early *Drosophila* embryos (Riparbelli and Callaini, 1992). The appropriate secondary antibodies, either goat anti–mouse or goat anti–rabbit IgG (Cappel Laboratories, Malvern, PA) coupled with FITC or TRITC, were used at 1:600 dilution and incubated for 1 h at room temperature. DNA was stained by 3–4 min incubation in PBS containing 1 μg/ml Hoechst 33258. Samples were mounted on glass microscope slides in 90% glycerol containing 2.5% *n*-propyl gallate (Giloh and Sedat, 1982). Fluorescence observations were made on a Leitz Aristoplan microscope equipped with FITC, TRITC, and UV filters.

Electron Microscopy

Testes from pupae and adult flies were fixed in trialdehyde solution (Kalt and Tandler, 1971) for 2 h at room temperature or overnight at 4°C. After rinsing in 0.1 M cacodylate buffer, pH 7.2, samples were postfixed in 1% osmium tetroxide for 2–3 h, bulk stained in 1% uranyl acetate in distilled water, dehydrated in a graded series of ethanol, treated with propylene oxide, embedded in Epon-Araldite mixture, and then polymerized at 60°C for 48 h. Random and serial sections were cut using an LKB Nova ultramicrotome and a diamond knife. Sections were collected on copper grids, stained with uranyl acetate and lead citrate, and then observed and photographed with a Philips CM10 electron microscope (Philips Electron Optics, Mahwah, NJ).

The cDNA sequence data reported in this paper are available from GenBank/EMBL/DDBJ under accession number Y17111.

Results

Isolation of the tulipano Mutation

In a large screen for *P* element-induced mutations in region 32 of the second chromosome, several female-sterile and semi-sterile lines were isolated. Complementation tests indicated that two of them identified the same gene and were allelic to *l(2)01501*, a lethal *P* insertion in 32D1 band (Karpen and Spradling, 1992). We called the mutation *tulipano* (*tlp*) and the alleles we isolated *tlp1* and *tlp2*, because of the peculiar truncated aspect of the mutant eggs. We later renamed the gene *Nup154* and maintained the original names to indicate the mutation and the alleles isolated. Remobilization of the *P* element from *l(2)01501* lead to the isolation of viable and fertile lines, providing evidence that the lethal mutation and the failure to complement *tlp1* and *tlp2* was indeed due to presence of the transposon. The two alleles fully complemented other female-sterile mutations mapping in the region, including *hold up*, *abnormal oocyte*, and *wavoid like* (Sandler, 1977).

In homozygous condition, both *tlp1* and *tlp2* not only exhibited female semi-sterility but also induced complete male sterility.

Cloning of the Nup154 Gene

Genomic regions flanking the *P* element insertion site were obtained by plasmid rescue from both *tlp1* and *tlp2*. A small DNA fragment immediately adjacent to the transposon was used to screen a wild-type genomic library. A fragment, spanning the *P* insertion site was, in turn, used to isolate cDNAs of different lengths from a 0–3 h embryonic cDNA library. In Fig. 1 the sequence of a cDNA, reconstructed from overlaps of three partial cDNA clones, is reported, together with its putative translation product. The predicted protein is composed of 1,365 amino acid residues, having a calculated mol wt of 154 kD. It contains 32 cysteine residues, 12 potential *N*-linked glycosylation sites and several potential phosphorylation sites for protein kinase C, casein, and tyrosine kinases. Computer searches identified no hydrophobic stretches at the NH_2 terminus (Nielsen et al., 1997), suggesting that the protein has no signal peptides. Two small regions enriched in proline are present in the middle of the protein. Computer Blast analysis (Altschul et al., 1990; Gish and States, 1993) of the putative translation product revealed a striking similarity all along the rat Nup155 nucleoporin (32.4% iden-

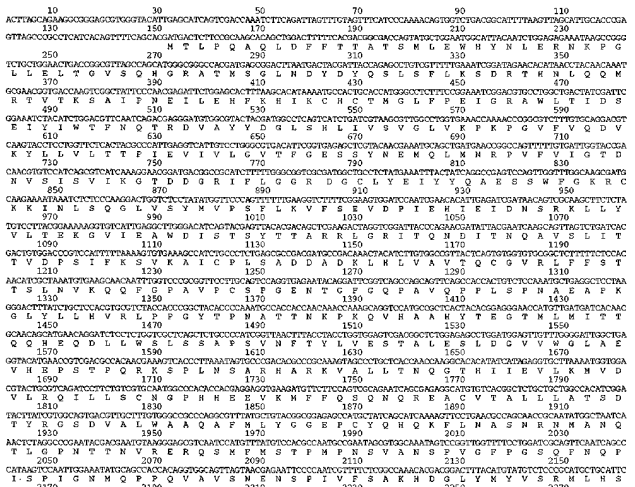

Figure 1. Nucleotide and deduced amino acid sequences of *Nup154* cDNA. The sequence was reconstructed from overlaps of three partial cDNAs. Translation of the open reading frame is shown from the ATG at position 152. The cDNA is 4,368 nt long, including 119 nt of 3′ untranslated sequence. These sequence data are available from GenBank/EMBL/DDBJ under accession number Y17111.

tity in 1,358 residues), (Radu et al., 1993). Yeast Nup170, and Nup157, reported as structurally related to rat Nup155 (Aitchison et al., 1995*b*), also show similarities with the *Drosophila* protein, mostly in two blocks, in the NH_2-terminal (32.5% identity in a 191-residue region) and the COOH-terminal (21.5% identity in a 395-residue region) halves, while the central region, as well as the first and last 150–amino acid regions, are not conserved. The *Drosophila* Nup154 protein, like rat Nup155 and yeast Nup170 and Nup157 nucleoporins, did not have the XFXFG, FG, or GLFG repeats present in the most numerous nucleoporin sub-family (Davis, 1995; Fabre et al., 1995).

Nucleo-cytoplasmic Transport Activity in tlp Alleles

Nucleoporins are known to be involved in both protein and mRNA transport across nuclear membranes (Gerace, 1992). We therefore investigated if polyA$^+$ RNA accumulated in nurse cell nuclei of *tlp1* ovaries by in situ hybridization, using as probe a population of antisense DNA molecules produced by reverse transcription of polyA$^+$ RNA extracted from wild-type ovaries. The results (Fig. 2) indicated that the global level of RNA transport in the mutant (Fig. 2 *b*) is comparable with that of wild type (Fig. 2 *a*), at least in the tested tissue and within the resolution limits of the technique. Because of the presence of pseudotumorous egg chambers in *tlp* mutants (see below and Fig. 6 *b*), we chose as a marker for protein nuclear import in the ovary the Sex lethal (Sxl) protein, which undergoes a relocalization process from cytoplasm to nucleus in wild-type germaria (Bopp et al., 1993). In *tlp1* the nuclear import of Sxl occurs normally (data not shown). From these data we obviously cannot exclude that trafficking of other proteins, as well as of specific RNA molecules could be impaired in *tlp* mutants.

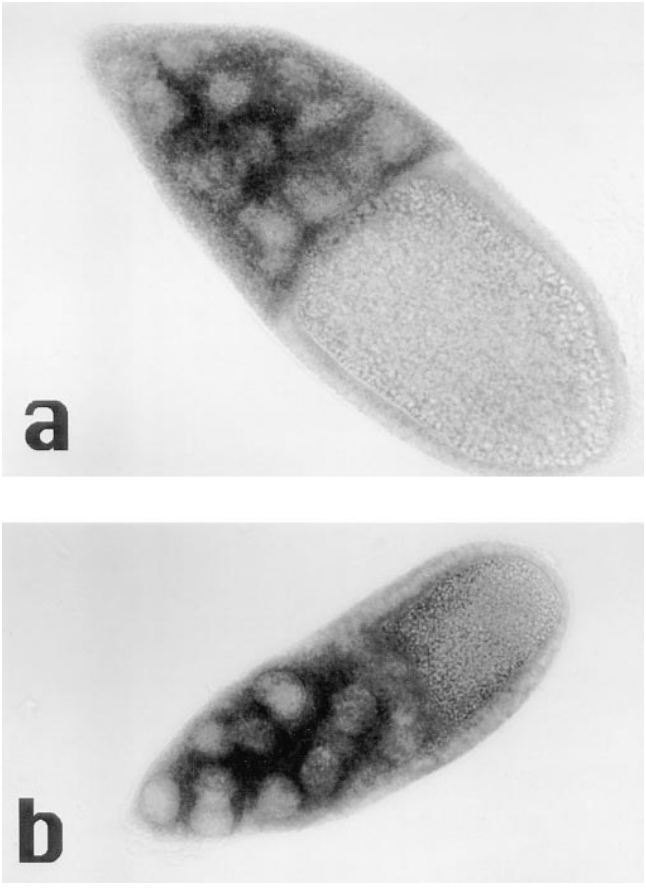

Figure 2. Nucleo-cytoplasmic mRNA transport in *tlp1* homozygous egg chambers. PolyA$^+$ RNA extracted from wild-type ovaries was reverse transcribed to produce digoxigenin-labeled antisense molecules that have been used in in situ hybridization experiments on wild-type (*a*) and *tlp1* (*b*) egg chambers. The level of global RNA transport in the mutant is comparable with that observed in wild type.

Nup154 Protein Immunolocalization

Polyclonal antibodies directed against a recombinant fragment of the Nup154 protein were obtained and tested in Western blot analyses. A protein of the expected molecular weight was detected in both 0–6 h embryo (Fig. 3 *a*, lanes *1* and *2*, and *b*, lane *1*) and ovarian (Fig. 3 *b*, lane *2*) extracts. To investigate Nup154 localization in developing egg chambers, immunofluorescence experiments were performed. In the wild type, the protein was present in the germarium and throughout egg chamber development, both in germ line and soma, around the nuclear periphery (Fig. 3 *c*). In stage 10 egg chambers it was also present in a network inside the nuclei (Fig. 3 *e*), where its distribution seemed to overlap with that of chromatin (Fig. 3 *f*). This was even more clear in the oocyte nucleus, where chromosomes were condensed in a central karyosome (Fig 3 *d*) and anti-Nup154 antibodies identified a very bright central dot (Fig. 3 *c*). The intranuclear localization of Nup154 protein was also observed in spermatocytes and other cell types (data not shown).

Since very few data are available on nucleoporin localization in *Drosophila* and intranuclear localization has not been reported for rat Nup155 and yeast Nup170 and Nup157, we then looked at the localization of other nuclear envelope proteins in wild-type egg chambers, using antibodies directed against lamin or repeat-containing nucleoporins (mAb414) (Fig. 3). While anti-lamin antibodies clearly stained only the nuclear periphery (Fig. 3 *h*), the mAb414 against repeat-containing nucleoporins also stained the nuclear interior (Fig. 3 *g*), resembling the staining pattern seen with anti-Nup154 antibodies. This last finding is not surprising since intranuclear staining with mAb414 has been previously reported in yeast cells (Aris and Blobel, 1989).

Nup154 protein was present in our mutant alleles, indicating that *tlp1* and *tlp2* were not null, as also suggested by the hypomorphic phenotype of the mutations. However, in both *tlp1* and *tlp2* homozygous egg chambers showing morphological alterations (see below) the protein mainly accumulated in the cytoplasm of nurse and follicle cells and was only barely detected inside the nuclei (Fig. 3, *i* and *j*). Residual protein was still localized to the nuclear envelopes. Moreover, the level of anti-Nup154 staining was reduced in *tlp2* homozygous flies (Fig. 3 *i*).

We investigated whether mislocalization of Nup154 in mutant alleles could influence the localization of the nucleoporins recognized by mAb414. The results obtained

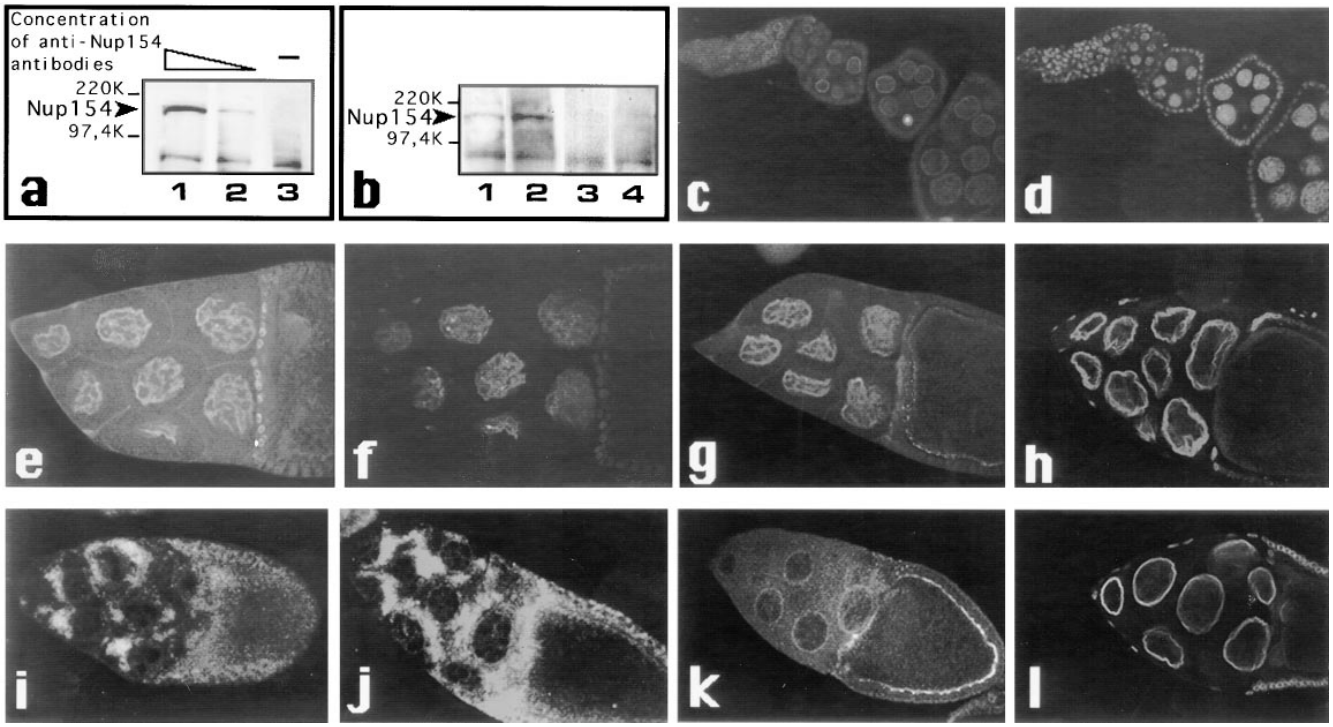

Figure 3. Western blot analyses and immunolocalization experiments in ovaries of wild-type and *tlp* homozygous flies. (*a* and *b*) Western blot analyses. Polyclonal antibodies directed against a recombinant fragment of the Nup154 protein recognize one protein of ~154 kD (*arrowhead*) in wild-type 0–6 h embryo (*a*, lanes *1* and *2*; *b*, lane *1*) and ovarian (*b*, lane *2*) extracts. In *a*, the specificity of the immunostaining was confirmed using two different concentrations of anti-Nup154 antibodies (1:100 for lane *1* and 1:500 for lane *2*). Lane *3* in *a*, lane *3* in *b* (both containing wild-type 0-6 h embryo extract), and lane *4* in *b* (wild-type ovarian extract) are additional controls where only secondary antibodies were used. Molecular weight standards are designated on the left in kD. (*c*, *e*, *i*, and *j*) Confocal microscopy of anti-Nup154 stained wild type (*c* and *e*), *tlp1* homozygous (*j*), and *tlp2* homozygous (*i*) egg chambers. In *d* and *f*, DAPI staining of the same egg chambers as in *c* and *e*, respectively, is shown. The ring of Nup154 protein around nurse cell nuclear membranes is evident in early stages, as well as the very bright central dot in the oocyte nucleus (*c*). The protein network inside nurse cell nuclei (*e*), that clearly overlaps with chromatin (*f*), is most evident at stage 10. A faint staining of the cell membranes is also observed. In *tlp1* (*j*) and *tlp2* (*i*) homozygous mutant egg chambers the protein mainly accumulates in the cytoplasm, in large aggregates close to nurse cell nuclei, but residual protein is still localized to the nuclear envelopes. (*g*, *h*, *k*, and *l*) Confocal microscopy of mAb414 (*g* and *k*) or anti-lamin (*h* and *l*) stained wild-type (*g* and *h*) and *tlp2* (*k* and *l*) egg chambers. Note how mAb414 signal is localized around the nuclear periphery as well as intranuclearly in wild type (*g*) while a certain degree of cytoplasmic mislocalization is observed in *tlp2* homozygotes (*k*). On the other hand, anti-lamin antibodies, which stain the nuclear rim in wild type (*h*), do not reveal any evident antigen localization defects in the mutant (*l*).

(Fig. 3 *k*) showed a prominent decrease in the nuclear mAb414 staining intensity and a concomitant increase in the cytoplasmic signal, compared with the wild type (Fig. 3 *g*). On the contrary, anti-lamin antibodies showed no gross differences in the overall antigen localization between wild type (Fig. 3 *h*) and mutant alleles (Fig. 3 *l*).

Effect of Mutations in Nup154 on Oogenesis

tlp1 and *tlp2* alleles affected oogenesis to different extents: *tlp2* homozygous females were almost completely sterile and produced very few eggs compared with *tlp1*. The majority of eggs produced by *tlp1* homozygous females (No. of eggs observed = 135) had fused, reduced or no chorionic dorsal appendages, and could be grouped in different phenotypic classes. The most striking group was represented by cup-shaped eggs (41%; Fig. 4 *c*); 42% showed a reduced A/P axis (Fig. 4 *b*), while in 13% the dorsal appendages were abnormal but length along the A/P axis was not grossly affected (not shown). The remaining 4% were similar to wild type (Fig. 4 *a*).

Despite their abnormal features, about half of the eggs (44 out of 87 scored) produced by *tlp1* homozygous mothers were fertilized and resumed meiosis as they were laid. Fig. 4, *d–i*, shows fertilized eggs during the metaphase of the second meiotic division. The *tlp1* egg was irregular in shape and smaller (Fig. 4 *e*) than the wild-type egg (Fig. 4 *d*). Nevertheless, the spindle apparatus (Fig. 4 *g*) and chromosome organization (Fig. 4 *i*) in the mutant egg looked very similar to those observed in wild type (Fig. 4, *f* and *h*) (Riparbelli and Callaini, 1996).

We examined egg chamber development in homozygous *tlp1* and *tlp2* as well as in heterozygous *tlp2/l(2)01501* females (Fig. 5), which showed the strongest ovarian defects. In the latter genetic combination, ovarioles appeared as strings of pearls in which egg chambers degenerated before entering vitellogenic stages (Fig. 5 *c*). *tlp2* homozygous egg chambers reached later developmental stages

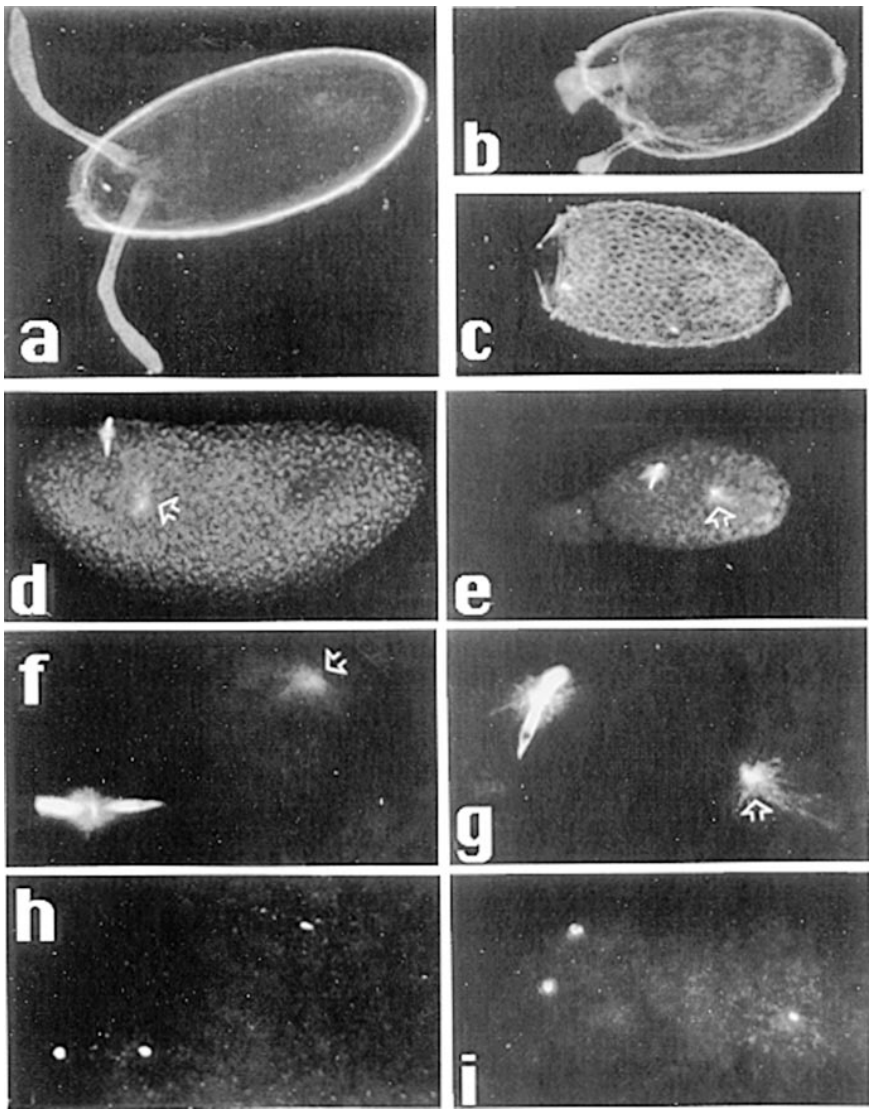

Figure 4. Egg morphology and meiosis in *tlp1* homozygotes. (*a–c*) Cuticle preparations showing egg morphology in (*a*) wild type and (*b* and *c*) *tlp1* homozygotes. (*d–i*) Meiosis in wild type (*left panels*) and *tlp1* homozygous (*right panels*) eggs as revealed by staining with an antibody against β-tubulin (*d–g*) and Hoechst dye (*h* and *i*). (*d* and *e*) Low magnification of fertilized eggs during metaphase of the second meiotic division; note the different dimensions. In *f* and *g*, details of the meiotic apparatus and the sperm aster (*arrow*) are shown.

(Fig. 5, *b* and *e*) but showed abnormal degrees of nurse cell chromatin condensation compared with wild type (Fig. 5, *a* and *d*). In the most extreme cases, nuclei retained the five blocks or even the polytene chromosome morphology (Fig. 5 *e*, and enlarged *insets*) characteristic of early developmental stages. Different levels of chromatin disorganization were observed even in nurse cell nuclei belonging to the same egg chamber. The failure to decondense nurse cell chromatin was barely detectable in *tlp1* homozygous egg chambers.

Other abnormal features, including altered oocyte growth rate and follicle cell movements, were observed in both *tlp1* and *tlp2* homozygous egg chambers, but especially in *tlp1*, which exhibited the weakest ovarian phenotype and allowed a greater number of cysts to reach more mature stages (Fig. 6). In a small fraction of egg chambers, abnormal nurse cell number (Fig. 6, *c* and *d*) or bicaudal-like phenotypes (not shown) were observed.

Effect of Mutations in Nup154 on Spermatogenesis

In *tlp1* and *tlp2,* male gametogenesis was affected in a more severe manner than oogenesis, as indicated by the fully male sterility conferred by both alleles. The spermatogenesis phenotypes of the two alleles were very similar and what follows applied to both. Testes from mutant pupae and adults were two to three times smaller than those from wild type. The most abundant germ cell stage observed in *tlp* testes from both pupae ($n = 74$) and adults ($n = 63$) were cysts of primary spermatocytes during the growth phase (Fig. 7 *b*). However meiotic figures were never observed. Two classes of accumulating cysts were easily distinguished by co-staining *tlp* testes ($n = 134$) with antibodies against β-tubulin (Fig. 7 *c*) and Hoechst dye (Fig. 7 *d*): cysts with microtubules forming elongated bundles, tapered at the extremities, which clearly contained only two nuclei, and round cysts containing spermatocytes (Fig. 7, *b* and *c*). The ratio between these two classes ranged from 1:1 to 2:1, respectively, in each testis. Several criteria indicated that the elongated cysts contained only the two somatic cyst cells and did not contain germ cells (see Discussion).

Although 16 spermatocytes were present in most of *tlp* cysts containing germ cells, in ~10% ($n = 27$) of the

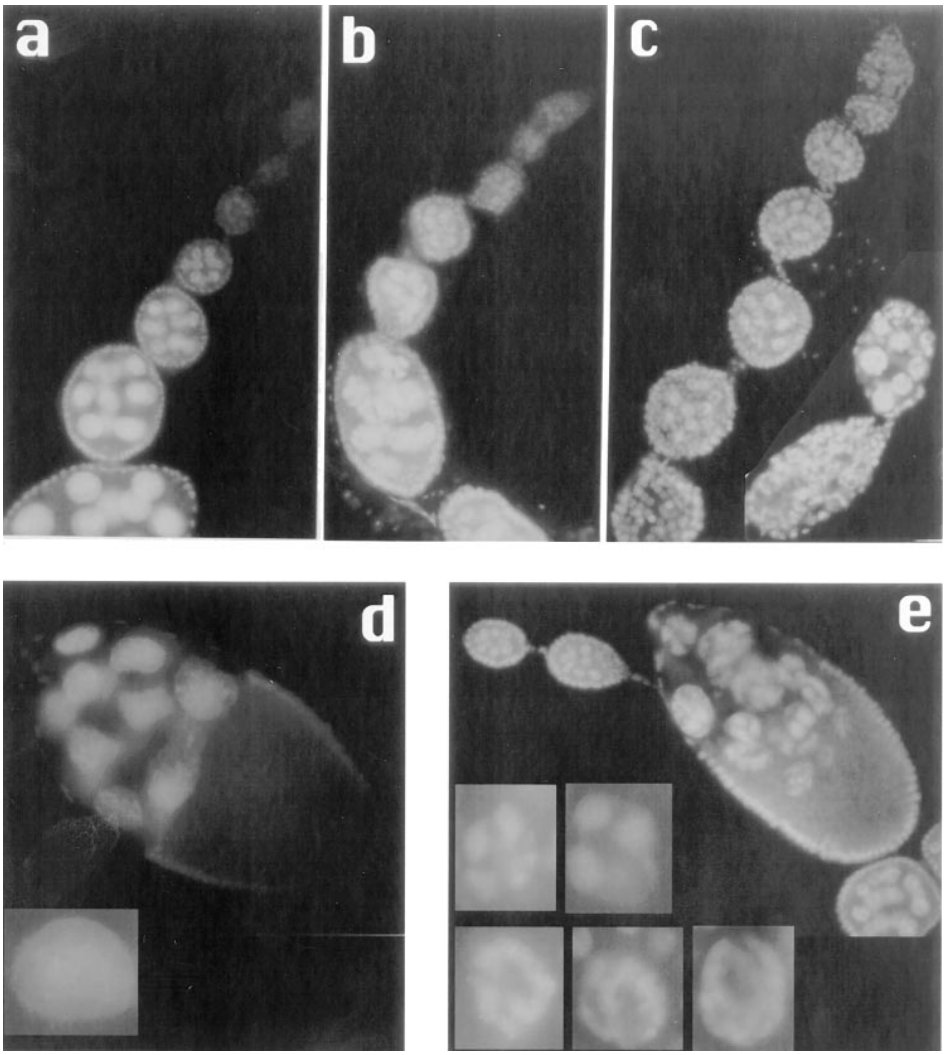

Figure 5. DAPI staining showing nurse cell nuclear morphology in wild-type and *tlp* homozygous ovaries. (*a*) Wild-type ovariole, progressively developing egg chambers, each containing 15 large nurse cell nuclei surrounded by small follicle cell nuclei, are easily recognized, while the oocyte nucleus is mostly undetectable. (*b*) *tlp2* homozygous ovariole showing abnormally condensed chromatin in growing egg chambers. (*c*) *tlp2/l(2)01501* ovariole with a "string of pearls" morphology resulting from an early arrest in egg chamber development. Alterations in chromatin decondensation are evident by comparing nurse cell nuclei in wild type (*d*) and *tlp2* (*e*) stage 10 egg chambers. In *d* and *e*, *insets*, single nurse cell nuclei from vitellogenic egg chambers are shown at higher magnification.

scored cysts (n = 291) lower numbers of primary spermatocytes, ranging from 2 to 8 were found (Fig. 7, *e* and *f*). These incomplete cysts always contained the pair of somatic cyst cells.

To define the meiotic arrest point in *tlp* testes, we analyzed centrosome behavior, spindle formation, chromosome condensation, nuclear envelope breakdown, and cyclin B localization (Figs. 8 and 9). In both wild-type (Fig. 8, *e* and *f*) and *tlp* (Fig. 8, *a* and *b*) testes, centrioles duplicated at the beginning of the spermatocyte growth phase and were associated with two small microtubule asters. At the end of prophase, centrioles elongated at least threefold in *tlp* spermatocytes (Fig. 8 *d*), and became associated with large asters (Fig. 8 *c*), as in wild type (Fig. 8, *g* and *h*). The interphase microtubule network completed reorganization at the end of prophase and broke down in the mutant, also as in wild type (Cenci et al., 1994). However meiotic spindles did not form in testes from *tlp* homozygous males. Whereas in wild-type testes a pair of centrosomes was inherited by each secondary spermatocyte at the end of the first meiotic division (Fig. 8, *k* and *l*), in *tlp* testes cells often contained four aster-like structures (Fig. 8 *i*) closely apposed to condensed chromatin masses (Fig. 8 *j*).

Although meiotic divisions did not appear to occur in *tlp* spermatocytes, some aspects of spermiogenesis seemed to continue. In older *tlp* cysts, the germ cell microtubule cytoskeleton appeared to have undergone spatial reorganization as if germ cells were attempting to perform postmeiotic differentiation. Microtubules elongated and arranged in circular bundles that formed a dense shell below the plasma membrane of round germ cells (Fig. 8 *m*) containing one or two chromatin masses (Fig. 8 *n*). Small anucleate cytoplasmic fragments similarly enveloped by round microtubule shells were also present. In addition, examination of old cysts by transmission electron microscopy revealed that irregular axonemes were sometimes present (not shown), confirming that differentiation program and meiosis had been uncoupled in the mutant.

An antibody previously demonstrated to recognize components of the nuclear membrane in *Drosophila* syncytial embryos (Riparbelli and Callaini, 1992) strongly stained the nuclear envelope of young primary spermatocytes in both *tlp* (Fig. 9 *a*) and wild type (Fig. 9 *e*). During late prophase the nuclear staining became weak (Fig. 9 *c*), suggesting that the nuclear envelope normally broke down in *tlp*. In wild-type testes, cyclin B was highly expressed dur-

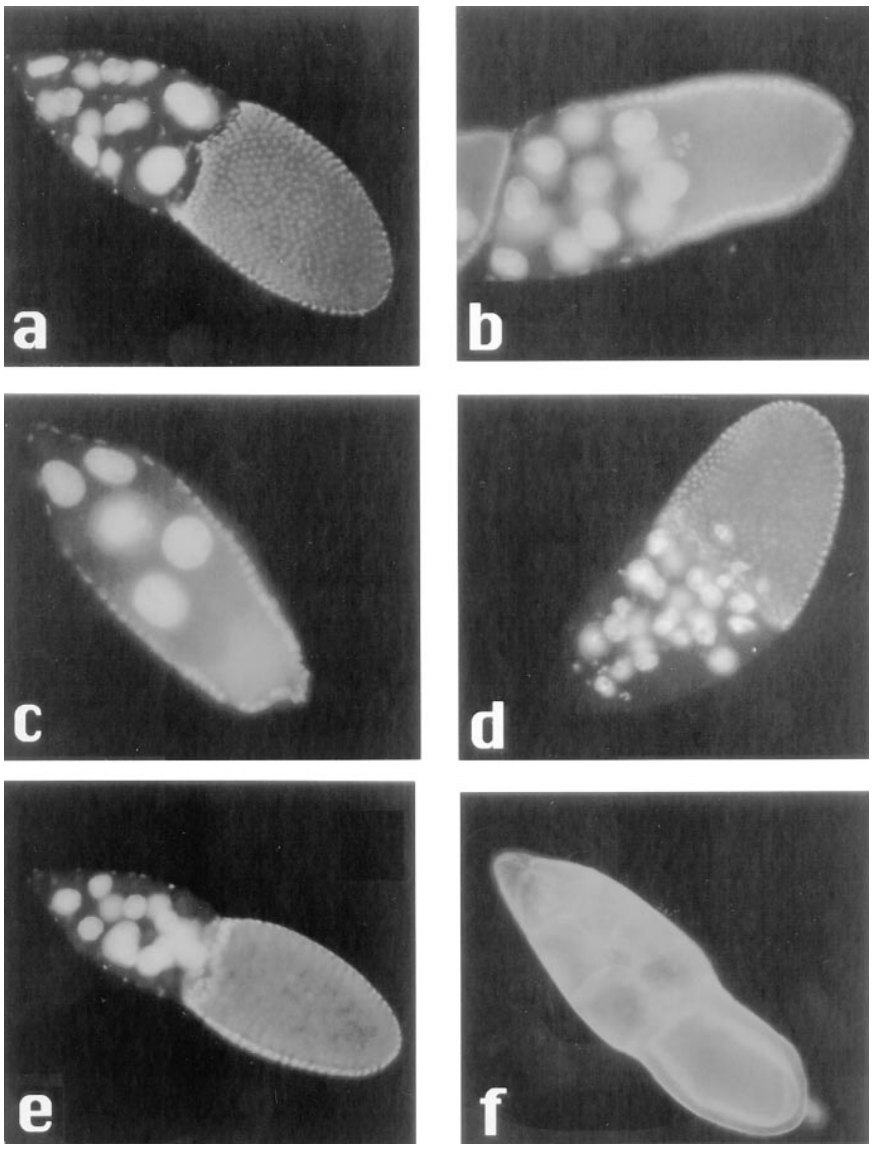

Figure 6. Overview of stage 10 egg chamber alterations in *tlp1* homozygous females. (*a–e*) DAPI staining. In *c* and *d* the number of nurse cell nuclei is lower or higher, respectively, than in wild type (*a*). In *b*, several rows of posteriorly migrating follicle cells remain in contact with the most posterior nurse cells, never reaching the undersized oocyte. They will therefore be unable to undergo the centripetal migration process between nurse cells and the oocyte that normally occurs. A likely consequence is the production of cup-shaped chorions, characteristic of both *tlp* alleles. In *e* and *f*, showing two different egg chambers stained with DAPI (*e*) or rhodamine-conjugated phalloidin (*f*), an excessive number of follicle cells is engaged in the centripetal migration process over the anterior pole of the oocyte. These altered cell movements result in the premature formation of a physical barrier impairing the transport of the nurse cell content into the oocyte, via the system of interconnected ring canals, in later stages of oogenesis. This defect could account for the production of mutant eggs characterized by reduced size and altered dorsal appendages.

ing prophase but underwent degradation starting from metaphase, and it was no longer visible at anaphase (Fig. 9, *i* and *j*). High levels of cyclin B were always detected in the mutant during prophase of the first meiotic division (Fig. 9 *h*) and at later stages of differentiation (not shown), indicating that cyclin B did not undergo normal degradation. We conclude from all these data that in mutant germ cells the meiotic block takes place at the transition between prophase and metaphase.

Nuclear Membrane Structure in tlp Alleles

Ultrastructural analyses of mutant spermatocytes showed that the nuclear membrane formed small evaginations, that presumably detached in the surrounding cytoplasm as distinct vesicles (Fig. 10 *a*). This peculiarity was never observed in wild-type spermatocytes (Fig. 10 *b*) and was reminiscent of the herniations described in nuclear envelopes of some nucleoporin yeast mutants (Wente and Blobel, 1993; Bastos et al., 1995). Examination of serial sections suggested that the nuclear envelope bulged out near nuclear pores (Fig. 10, *c* and *d*). When the nuclear envelope did not show surface blobs, the pore complexes of *tlp* spermatocytes (Fig. 10 *e*) looked very similar to those observed in wild type (Fig. 10 *f*).

Discussion

In an attempt to isolate female-sterile mutations in region 32 of the second chromosome of *D. melanogaster* (Gigliotti et al., 1993; Malva et al., 1994), we identified the first *Drosophila* mutants in a gene showing similarity with known nucleoporins. Mutation of the *Nup154* gene of *Drosophila* affects the spatial organization of the NPCs, resulting in nuclear membrane structural defects similar to those described for some yeast nucleoporin gene mutants (Wente and Blobel, 1993; Bastos et al., 1995). The various nucleoporins are found in distinct parts of the NPC, from the cytoplasmically disposed filaments to the nuclear bas-

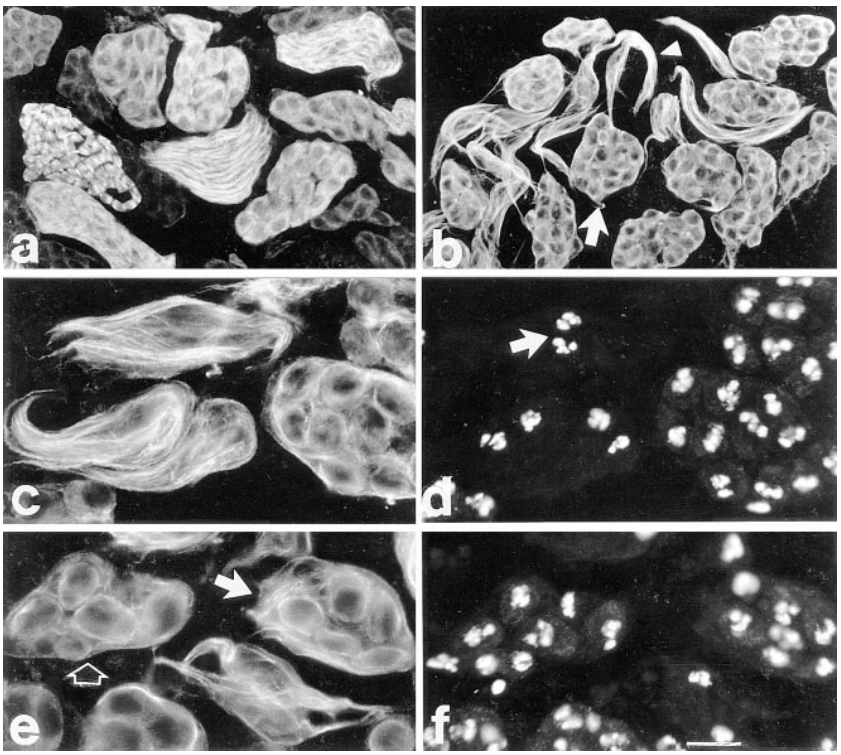

Figure 7. Cyst formation in *tlp* homozygous testes. Microtubule labeling in testes of wild-type (*a*) and *tlp* (*b*, *c*, and *e*) testes. In *d* and *f*, Hoechst staining of the same testes as in *c* and *e*, respectively, is shown. (*a*) Wild-type testis showing primary spermatocytes during the growth period or through meiosis, as well as clusters of elongating spermatids. (*b*) In most of *tlp* testes only primary spermatocytes clusters (*arrow*) and cyst cells (*arrowhead*) are visible, while meiotic figures are lacking. (*c* and *d*) *tlp* testis detail where cyst cell pairs lacking accompanying germ cells can be observed. The extensive network of microtubules (*c*) surrounding the pairs of somatic nuclei (*d*, *arrow*) is shown. A cyst containing primary spermatocytes with condensed chromatin is also visible. Note that cyst cell and spermatocyte nuclei show a similar degree of chromatin condensation. (*e* and *f*) Detail of a *tlp* testis showing the occurrence of cysts with an irregular spermatocyte number (*open* and *filled arrows*).

ket (Davis, 1995) and are involved in protein and RNA trafficking between nucleus and cytoplasm (Gerace, 1992; Fabre et al., 1995; Gorlich and Mattaj, 1996; Laemmli and Tjian, 1996; Nigg, 1997). Some proteins associated with the NPC have been demonstrated to be localized also to the nuclear interior (Cordes, 1993; Powers et al., 1995; Zimowska et al., 1997), suggesting that they could have additional functional role(s), which remain to be understood. One hypothesis predicts that the components of the NPC, together with the peripheral nuclear lamina, could be important players in the maintenance and the alteration of the three-dimensional genome structure during cell life (Blobel, 1985; Sukegawa and Blobel, 1993). The data reported in this paper suggest an intranuclear function also for the *Drosophila* Nup154 protein. Intranuclear localization has not been reported for rat Nup155 and yeast Nup170 and Nup157. Our observation could have been facilitated by the peculiar features of nurse cells. Because of the size and DNA content of their polytenic nuclei, nurse cells can be easily analyzed in more details than the majority of other cells, such as rat cells in culture or yeast cells or even the somatic follicle cells in the *Drosophila* ovary, where it is more difficult to look at Nup154 protein behavior. Alternatively, Nup154 of *Drosophila* has acquired, in *Drosophila*, new and more specialized functions.

Role of the Nup154 Gene during Egg Chamber Development

The most interesting aspect of the *tlp* ovarian phenotype in our hypomorphic alleles is the failure to decondense nurse cell chromosomes. Several genes are required to regulate nurse cell chromatin structure, including *cup*, *otu*, and *fs(2)B* (King et al., 1957; King and Storto, 1988; Keyes and Spradling, 1997). Cup is transiently associated with the nuclear membrane and has been proposed to indirectly influence chromatin structure by acting in a pathway that affects the nuclear membrane (Keyes and Spradling, 1997). By virtue of its dual nuclear localization, Nup154 could be an essential component of this pathway. In this hypothesis, the altered chromatin structure observed in *tlp* ovaries could be directly related to the mislocalization of Nup154. On the other hand, *tlp* mutations could have a more generalized impact on egg chamber physiology and development, by impairing the nucleo-cytoplasmic traffic of essential factors, including those required for chromatin organization. In both cases, the failure of Nup154 to attain a correct localization could produce alterations in nuclear pore assembly and structure and this interpretation is supported by the observed mislocalization of mAb414 immunoreactive proteins in *tlp* mutants. It has been demonstrated that yeast Nup170, to which Nup154 is structurally related, is required for normal stoichiometry of FG nucleoporins within the NPC (Kenna et al., 1996). Our results could suggest that the Nup154 *Drosophila* protein also plays a role in localizing specific FG nucleoporins within the NPC. However, a striking discrepancy can be found between mAb414 immunolocalization in *Drosophila tlp* mutants and in yeast *nup170* deletion mutants, where no evident differences in staining intensities or overall localizations of mAb414 could be detected compared with the wild type (Kenna et al., 1996). This discrepancy could indicate that *Drosophila* Nup154 is not the functional homologue of yeast Nup170. This hypothesis is supported by the observation that *Drosophila* Nup154 is essential for viability while yeast Nup170 is dispensable (Aitchison et al.,

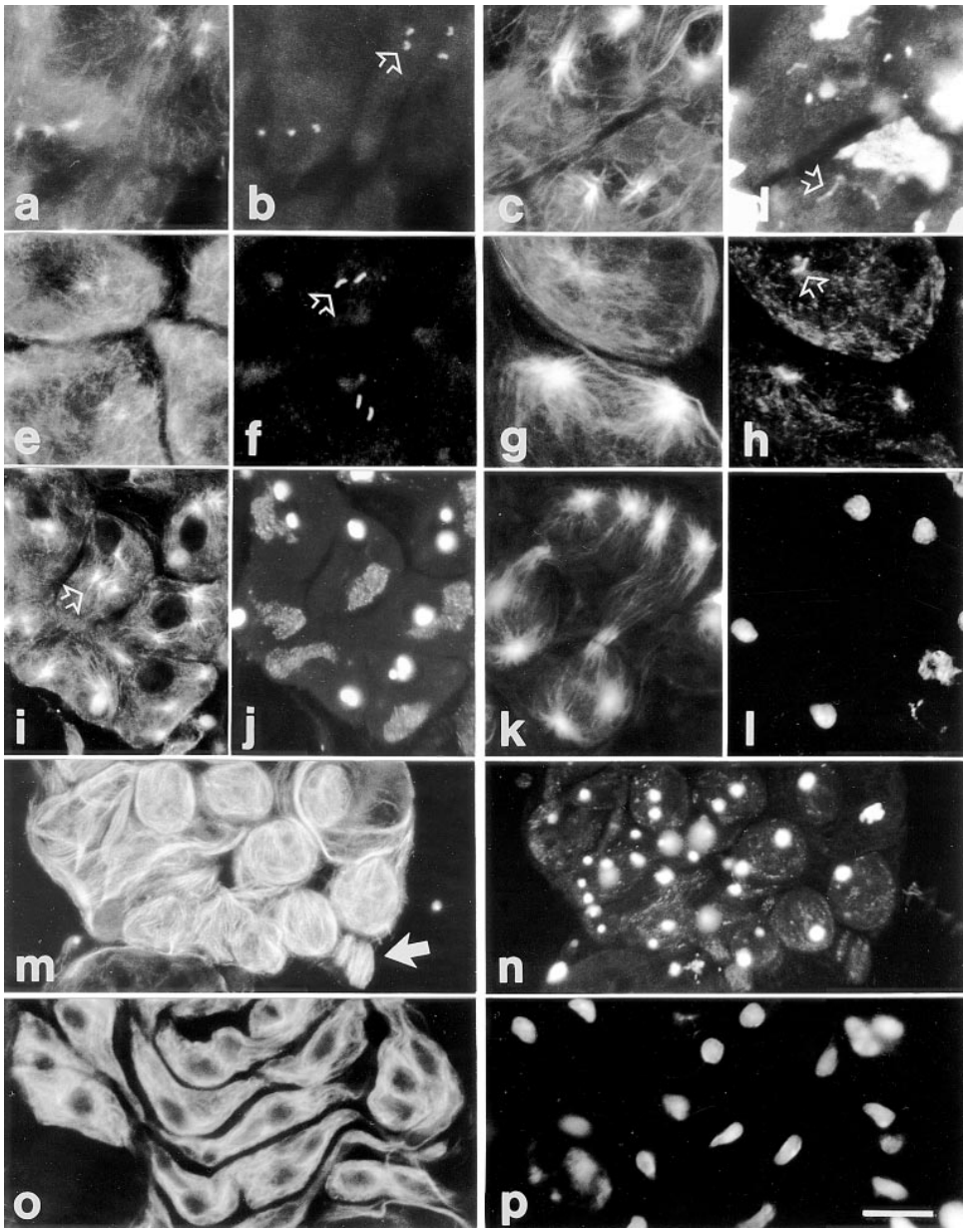

Figure 8. Spermatocyte development in *tlp* homozygous testes. *tlp* and wild-type spermatocytes were stained with antibodies against β-tubulin (*a*, *c*, *e*, *g*, *i*, *k*, *m*, and *o*), antibodies against γ-tubulin (*b*, *d*, *f*, and *h*), and Hoechst dye (*j*, *l*, *n*, and *p*). (*a–h*) Centriole and microtubule behavior in *tlp* (*a–d*) and wild-type (*e–h*) spermatocytes. Centrioles are short at the beginning of the growth phase (*b* and *f*, *arrows*), when they are associated with small asters (*a* and *e*) and become long at the end of prophase (*d* and *h*, *arrows*), when they are associated with large asters (*c* and *g*). (*i* and *j*) Mutant spermatocytes showing an abnormal number of centrosomes per cell (*arrow*). Mitochondria, that are also weakly stained by the Hoechst dye, because of their DNA content, appear as irregular masses in the cytoplasm of the germ cells. In wild type (*k* and *l*) a single pair of centrosomes is found in each secondary spermatid at the end of meiosis I. *tlp* testes show sometimes cysts where circular arrays of cortical microtubules are seen in the germ cells (*m*). Note that one or two condensed chromatin masses are present in these cells (*n*). Microtubules also surround anucleated cell fragments (*arrow*). In wild-type post meiotic germ cells, the growth of microtubule bundles leads to the formation of lens-shaped spermatids (*o*) containing only one chromatin mass (*p*). Bar: (*a–h*) 7 μm; (*i–p*) 10 μm.

1995*a*; Kenna et al., 1996) Furthermore, the obvious nuclear envelope ultrastructural abnormalities observed in *tlp* mutants are absent in *nup170Δ* mutants (Aitchison et al., 1995*a*).

Role of the Nup154 Gene during Early Stages of Male Gametogenesis

The most peculiar *tlp* testis phenotype is the occurrence of cysts that do not contain any developed germ cells but only the somatic cyst cell pair. The identity of these cells has been proven by several structural and molecular criteria: (*a*) the presence of large microtubule bundles; (*b*) the absence of any γ-tubulin spots; (*c*) the persistence of the nuclear envelope throughout development; and (*d*) the lack of cyclin B in the cytoplasm.

The presence of cysts containing only the two somatic cyst cells suggests that during gametogenesis cell division is independently regulated in somatic and germ line stem cells. This is consistent with the observation that cyst cells can form in testes of males in which pole cells have been ablated by UV (Aboim, 1945). However, it has been recently demonstrated that, when germ cells are missing, resulting from genetic ablation of pole cells, cyst cells do not withdraw from the cell cycle, suggesting that signals from germ cells are necessary to regulate their proliferation (Gonczy and DiNardo, 1996). These authors conclude that they cannot discriminate whether these signals arise from germ line stem cells or their proliferating daughters. Our data suggest that the appropriate signals are not produced in each single cyst in a direct cross talk between the germ line and the somatic components of the cyst itself, but probably represent an early event occurring before cyst formation.

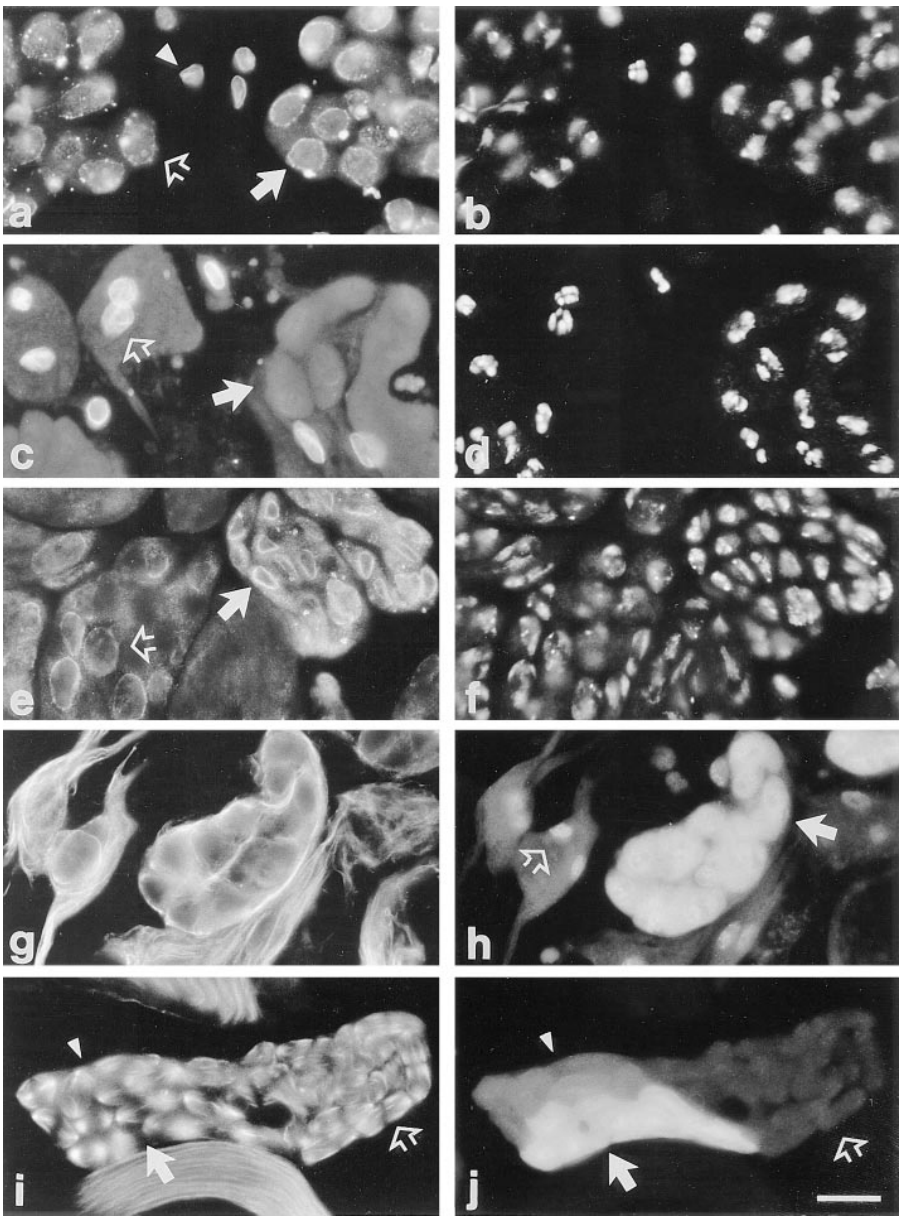

Figure 9. Nuclear envelope breakdown and cyclin B localization in *tlp* homozygous arrested spermatocytes. Immunostaining with antibodies against a nuclear envelope marker (*a*, *c*, and *e*) and DNA labeling with Hoechst (*b*, *d*, and *f*) in *tlp* (*a–d*) and wild-type (*e* and *f*) male germ cells. In both *tlp* (*a* and *b*), and wild type (*e* and *f*), the nuclear envelope is evident in cyst cells (*a*, *arrowhead*) and young spermatocytes (*a* and *e*, *arrow*), but becomes less evident in spermatocytes at later stages (*a* and *e*, *open arrow*). At the end of meiotic prophase, the nuclear envelope becomes barely detectable in *tlp* spermatocytes (*c*, *arrow*), while in cyst cells (*c*, *open arrow*), that do not divide, it is well evident. Microtubule (*g* and *i*) and anti–cyclin B (*h* and *j*) staining of *tlp* (*g* and *h*) and wild-type (*i* and *j*) testes. (*g* and *h*) Cyclin B persists in both nuclei and cytoplasm of *tlp* spermatocytes (*h*, *arrow*), whereas in cyst cells it is only localized in the nucleus (*h*, *open arrow*). In wild-type testis (*i* and *j*) cyclin B is expressed at high levels during prometaphase (*arrow*), undergoes degradation at metaphase (*arrowhead*) and is no longer visible during anaphase/telophase (*open arrow*). Bar, 10 μm.

Despite the high number (500) of male germ line cysts analyzed in pupae and adults, we never found spermatocytes completing meiotic division. This observation suggests that meiosis is prevented in those germ cells that escaped the early block induced by the mutation, and successfully underwent the four spermatogonial divisions. Comparison of centrosome behavior, nuclear envelope breakdown, and cyclin B localization in *tlp* and wild-type germ cells indicated that meiotic prophase is normal in *tlp* mutants. However, cyclin B is not abruptly degraded at metaphase but persists in arrested *tlp* spermatocytes until they degenerate or differentiate irregular axonemes. The simplest interpretation of our results is that the transition to metaphase does not occur in *tlp* mutants. Alternatively, we should postulate that impairment of Nup154 function leads to cyclin B stabilization and this in turn would explain the observed block in meiotic progression. This hypothesis could be supported by the finding that mutations in the yeast *cse1* gene, coding for the export factor for kariopherin α, prevent cell cycle–dependent degradation of B-type cyclins (Irniger et al., 1995). Molecular components of the nuclear import and export machinery, including Cse1 and Nup154, could therefore be part of a regulatory mechanism controlling cell transit through mitosis and/or meiosis.

Conclusions

The multiplicity of phenotypes observed in *tlp* mutants could be attributed to pleiotropic effects as well as to the hypomorphic nature of our alleles. In any case, *Nup154* is required not only for female and male germ line growth and development, as reported in this paper, but also in other tissues and developmental stages, as demonstrated

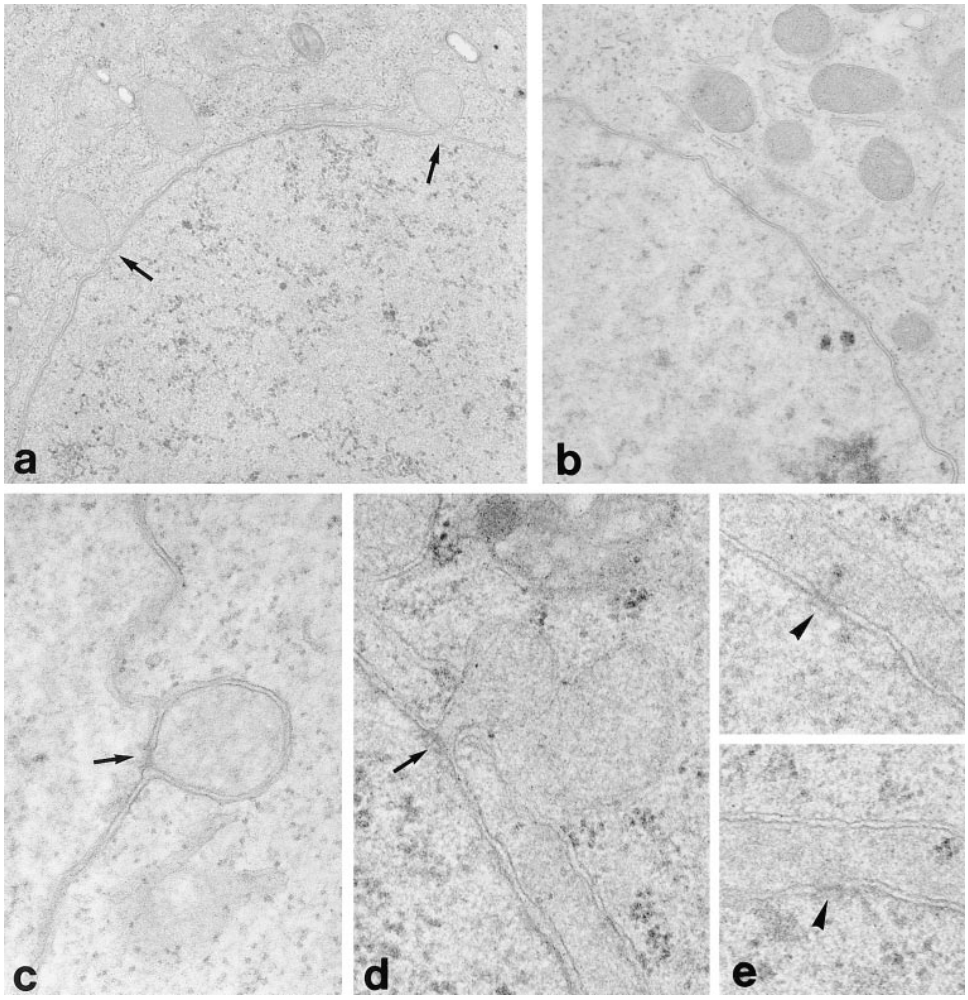

Figure 10. Nuclear membrane alterations in *tlp* homozygous spermatocytes. Ultrathin sections of *tlp* (*a, c, d,* and *e*) and wild-type (*b* and *f*) spermatocytes during prophase of the first meiotic division. The nuclear envelope of *tlp* spermatocytes (*a*) bulges out forming distinct evaginations (*arrows*); these structures are not found in wild-type testes (*b*). (*c* and *d*) Details of a *tlp* spermatocyte nucleus showing that the nuclear envelope protrudes near the pore regions (*arrows*). In the absence of surface blobs, pore complexes in *tlp* spermatocytes (*e*, *arrowhead*) look very similar to those observed in wild type (*f*, *arrowhead*).

by the existence of lethal alleles and the observation of complex embryonic phenotypes (Kiger, A., and M. Fuller, manuscript in preparation).

This notwithstanding, impairment of *Nup154* function affects male versus female gametogenesis to different extents. In addition, *Nup154* mutations have different consequences for germ line versus soma differentiation in males. These findings argue that distinct regulatory mechanisms, differentially involving *Nup154* function, underlie these processes. Several scenarios can be envisaged. On one hand, the residual activity of Nup154 protein in the mutants could be sufficient to go through some processes but not other ones. On the other hand, Nup154 could interact with sex- and tissue-specific proteins and this interaction could establish sex- and tissue-specific functions. One candidate for this interaction in the germ line could be the product of the *germ cell–less* gene, which acts early in the germ cell specification pathway, is a component of the NPC and requires for its function the presence of unidentified factors (Jongens et al., 1994). Finally, the data obtained so far cannot exclude the possibility that the *Nup154* gene is differentially spliced or gives rise to transcripts and translation products of slightly different lengths in different sex and/or tissues. These minor differences could have relevant consequences on protein function and protein–protein interactions. Clarification of the nature of the molecular mechanism(s) underlying the mutant phenotypes in our alleles may shed light on this point.

The studies presented here indicate that Nup154 is an essential protein involved in nuclear envelope structure. In addition, its dual localization, both at the nuclear envelope and in the nuclear interior, suggests multiple roles in nuclear functions. Its future study will be important for progress in understanding how nuclear pore components regulate nuclear and cellular physiology, also in relation to specialized developmental processes such as oogenesis and spermatogenesis.

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