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RESEARCH ARTICLE

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Autosomal control of the Y-chromosome *kl-3* loop of *Drosophila melanogaster*

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Abstract The Y chromosome of *Drosophila melanogaster* carries a limited number of loci necessary for male fertility that possess a series of unconventional features that still hinder a definition of their biological role: they have extremely large sizes; accommodate huge amounts of repetitive DNA; and develop prominent, lampbrush-like loops that bind a number of non-Y-encoded proteins. To obtain insight into the functional role of the loop-forming fertility factors, we characterized four autosomal male-sterile mutations that identify two loci we named *loop unfolding protein-1 (lup-1)* and *loop unfolding protein-2 (lup-2)*. Biochemical and ultrastructural analysis revealed that neither of them impairs the synthesis of the putative dynein subunit encoded by the ORF localized within the *kl-3* fertility factor. However, the stability of four dynein heavy chains is simultaneously affected in each mutant, together with the regular assembly of the axonemal dynein arms that are either absent or strongly reduced. These results indicate that the synthesis of the *kl-3*-encoded dynein can be uncoupled from the formation of the corresponding loop and suggest that this structure does not simply represent the cytological counterpart of a huge transcription unit, but must be regarded as a complex organelle serving some additional function necessary for male fertility.

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Introduction

The Y chromosome of *Drosophila melanogaster* is an entirely heterochromatic element that accounts for about 12% of the male genome and 35% of male heterochromatin (Pimpinelli et al. 1978). It carries a limited set of functions specifically required for male fertility; *X/0* males are in fact fully viable and phenotypically normal, but completely sterile (Bridges 1916). Complementation analyses and cytogenetic studies allowed the mapping along the Y chromosome of six genes necessary for male fertility—four on the long arm (*YL*) and two on the short arm (*YS*). Starting from the distal end of the long arm, they are designated as *kl-5*, *kl-3*, *kl-2*, *kl-1*, *ks-1*, and *ks-2* (Brosseau 1960; Kennison 1981; Hazelrigg et al. 1982; Gatti and Pimpinelli 1983). The existence of the *kl-4* locus, hypothesized by Brosseau (1960), was never confirmed by subsequent studies. Y-linked fertility factors are essential for normal sperm differentiation. In males deficient for one or more of these genes, spermatids elongate, but sperm degenerate before maturation (Lindsley and Tokuyasu 1980; Hardy et al. 1981; Lifschytz 1987). Extensive cytogenetic studies (Kennison 1981; Hazelrigg 1982; Gatti and Pimpinelli 1983) showed that each of the *kl-5*, *kl-3*, and *ks-1* fertility factors have extremely large physical dimensions, spanning chromosome areas containing up to 3–4 Mb of DNA, therefore being 100 times larger than an average euchromatic *Drosophila* gene. In situ hybridization studies have demonstrated that each of the *kl-5* and *ks-1* loop-forming regions contains several satellite sequences and transposable elements, while only a simple sequence satellite DNA was mapped within the *kl-3* region (Bonaccorsi and Lohe 1991; Pimpinelli et al. 1995). Another peculiarity of

these heterochromatic loci is that they develop giant, thread-like structures, analogous to the lampbrush loops of amphibian oocytes, that grow throughout spermatocyte development and eventually disintegrate prior to meiosis I (Bonaccorsi et al. 1988). Each loop consists of a DNA axis associated with huge repetitive transcripts, in turn associated with large amounts of proteins (Bonaccorsi et al. 1988, 1990; Pisano et al. 1993). The peculiar molecular structure of the loop-forming regions, consisting of large portions of satellite DNA interspersed with transposable elements, is maintained in different *Drosophila* species, although the DNA sequences involved are very different (Hennig et al. 1974; Vogt and Hennig 1983; Hujiser et al. 1988; Lankenau et al. 1990; Hochstenbach et al. 1994). Another conserved feature of the lampbrush-like loops is their association with a number of non-Y-encoded antigens, which include DNA-interacting proteins (Marhold et al. 2002), RNA-interacting proteins (Risau et al. 1983; Melzer and Glätzer 1985; Bonaccorsi et al. 1988; Eberhart et al. 1996; Cheng et al. 1998; Heatwole and Haynes 1996), and testis-specific antigens that are incorporated either in nuclei (Svensson et al. 2003) or in sperm tails (Hulsebos et al. 1984; Bonaccorsi et al. 1988; Pisano et al. 1993; Lu and Beckingham 2000) during late stages of spermiogenesis.

The observation that the Y loops accumulate high quantities of satellite transcripts and several autosome-encoded proteins led to the suggestion that they might have a structural role rather than a conventional coding function. In particular, it has been proposed that these huge structures might serve for storing proteins to be used after meiosis and/or modified upon their binding to the loops (Bonaccorsi et al. 1988; Hennig et al. 1989; Pisano et al. 1993). However, doubts on this hypothesis have been raised by observations carried out on *D. hydei*, showing that males with highly defective loops can be fertile (reviewed by Kurek et al. 2000). Moreover, evidence for a coding role of the loop-forming fertility factors *kl-3* and *kl-5* was first provided by Goldstein et al. (1982), who proposed that these loci contain the coding sequences for axonemal dynein heavy chain (Dhc) proteins. This hypothesis has been confirmed by Gepner and Hays (1993), who demonstrated the presence of an axonemal dynein heavy chain gene, designated as *Dhc-Yh3*, within the *kl-5* locus. More recently, Carvalho et al. (2000) developed a method for the identification of putative genes in the unmapped portion of the *Drosophila* genome and were able to demonstrate that the *kl-2* and *kl-3* fertility factors contain the coding sequences for a 1β-dynein and a γ-dynein heavy chain, respectively. Using this method, other five protein-coding sequences have been subsequently identified on the Y chromosome of *D. melanogaster*, two mapping to each of the *ks-1* and *ks-2* fertility regions and three representing novel genes that have not been genetically characterized (Carvalho et al. 2001).

Although most recent evidences favor a coding function of the Y-linked fertility factors, they still do not explain why giant, lampbrush loops are formed; what the biological significance of their protein-binding function is; and how this function relates to the presence of a conventional coding gene.

In order to get insight into the role of the Y loops, we have screened two collections of autosomal male-sterile mutations for those affecting loop morphology in primary spermatocyte nuclei. These mutations are likely to identify genes that encode either structural components of the loops or factors involved in the regulation of their unfolding. Thus, they should provide information not only on the molecular mechanisms underlying the development of these peculiar structures, but also on the functional relation existing between the loops, the transcription and translation of the genes they accommodate, and the correct assembly of mature sperm tails.

In this paper we report the identification of two autosomal loci that specifically interfere with the normal development of the *kl-3* loop. We have carried out a cytological, ultrastructural, and biochemical characterization of four mutations at these loci, showing that they not only affect the normal development of the *kl-3* loop, but also the stability of four high-molecular-weight polypeptides and the proper assembly of the dynein arms onto sperm axonemes.

Materials and methods

Drosophila stocks

All the stocks used in this work were reared at 25°C on standard *Drosophila* medium. *T(X;Y)V24* and *T(X;Y)W27* are fertile reciprocal translocations broken in the proximal X-chromosome heterochromatin, involving an X chromosome marked with *y w f* and a Y chromosome marked with B^s and y^+ (Kennison 1981; Hardy et al. 1984). Males deficient for the *kl-3* region were constructed by combining the Y-distal, X-proximal (Y^DX^P) element of *T(X;Y)V24* (marked with B^s) and the X-distal, Y-proximal element (X^DY^P) of *T(X;Y)W27* (marked with y^+; Bonaccorsi et al. 1988). All the mutations on the third chromosomes are maintained in stable stocks, using the multiply inverted balancer chromosome *TM6C*, marked with the dominant mutations *Tubby (Tb)* for the body shape and *Stubble (Sb)* for the bristles.

Statistical mapping has been performed using two multiply marked stocks, the first carrying *rughoid* (*ru*, 3-0.0), *hairy* (*h*, 3-26.5), *thread* (*th*, 3-43.2), *scarlet* (*st*, 3-44.0), *curled* (*cu*, 3-50.0), *stripe* (*sr*, 3-62.0), *ebony-soothy* (e^s, 3-70.7), and *claret* (*ca*, 3-100.7); the second carrying the same recessive mutations plus the dominant marker *Prickly* (*Pr*, 3-90.0). Both stocks are maintained over the *TM6C* balancer. (For further explanations about markers, see Lindsley and Zimm 1992.)

Deficiencies used for mapping were obtained from the Bloomington Stock Center. They have been rebalanced over *TM6C* and then crossed with mutant stocks. For cytological analysis, Tb^+ larvae, pupae, and Sb^+ adults have been selected and dissected as described below.

Testis immunofluorescence

Larval, pupal, or adult testes were dissected and fixed according to Pisano et al. (1993). Fixed slides were then washed twice in PBS (Dulbecco's modified formula, 5 min each) and incubated for 1 h in a humid chamber at room temperature with 20 µl of either the T53-1 antiserum (Pisano et al. 1993) diluted 1:10 in PBS, or the S5 monoclonal antibody (Saumweber et al. 1980) diluted 1:20 in PBS. Slides were then washed twice in PBS (5 min each) and incubated 1 h with the secondary antibody, a sheep–antimouse IgG conjugated with fluoresceine (FLUOS, Boehringer, Mannheim), diluted 1:20. After air drying, slides were mounted in 0.5 µg/ml Hoechst 33258 in PBS.

Light microscopy

Microscopy analysis and pictures were made using a Zeiss III RS photomicroscope equipped with HBO fluorescent light (100 W). For immunostained preparations with FLUOS-conjugated secondary antibodies, the Zeiss filter combination 09 was used.

Electronic microscopy

Testes dissected from adults were fixed with glutaraldehyde and tannic acid, postfixed with uranyl acetate, and embedded in an Epon/Araldite mixture according to Dallai and Afzelius (1990). Thin sections (about 50 nm), obtained with a Reichert Ultracut IIE ultramicrotome,

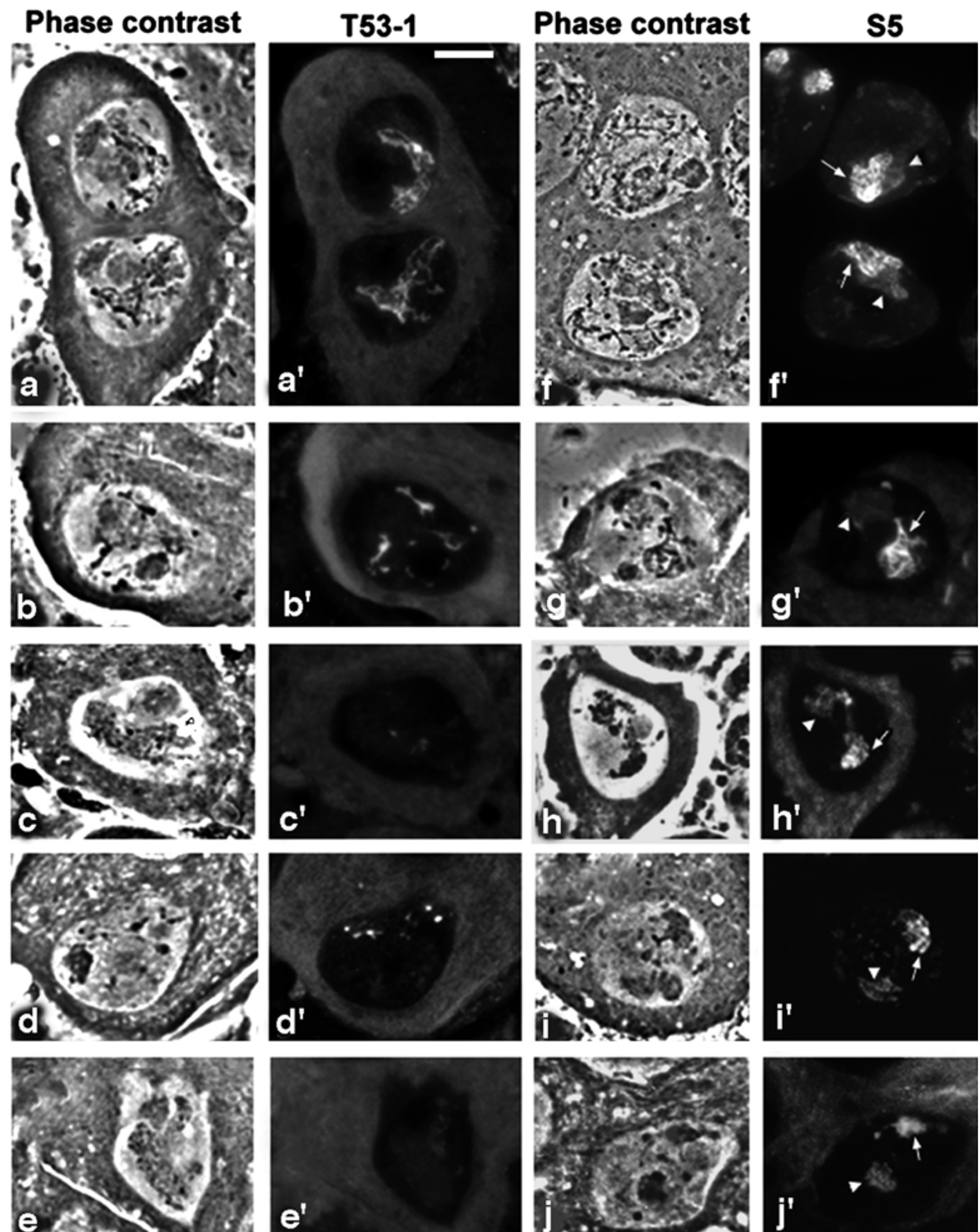

Fig. 1 Primary spermatocyte nuclei immunostained with either the T-53 or the S5 antibody and photographed under phase contrast (**a**–**j**) and fluorescence (**a′**–**j′**) microscopy. **a**, **f** Wild type; **b, g** *ms(3)HB933*; **c, h** *ms(3)HB267*; **d, i** *ms(3)155-34*; **e, j** *ms(3)HB223*. Note that the T53 staining of *kl-3* loop observed in wild-type nuclei (**a′**) is significantly reduced (**b′**, **d′**) or completely absent (**c′**, **e′**) in mutant spermatocytes. Conversely, S5 immunostaining appears unaffected in mutants (**g′**–**j′**) as compared to wild type (**f′**). *Arrows* point to the brightly fluorescent *kl-5* loop; *arrowheads* point to the dull fluorescent *ks-1* loop. *Bar* = 10 µm

were routinely stained with uranyl acetate and lead citrate and observed with a Philips CM10 TEM at 80 kV.

Biochemical characterization of mutants

The testis polypeptide pattern of mutant as well as wild-type males was analyzed by electrophoresis on 3–5% SDS–polyacrylamide gels (8.3×5.5×0.75 cm) according to Laemmli (1970). Samples were electrophoresed immediately after denaturation at a constant amperage of 7 mA in the stacking gel and 15 mA in the separation gel. Gels were silver stained by the method of Wray et al. (1981).

Labeling of protein synthesis was performed as described by Goldstein et al. (1982). Briefly, ten testes from each mutant and from the wild type were incubated in serum-free modified Eagle's medium (2 µl/testis) containing [^{35}S]-methionine (5 mCi/ml) for 4 h. Labeled samples were denatured in Laemmli sample buffer. After electrophoresis, gels were fixed in 50% methanol 12% acetic acid, washed for 30 min in 10% acetic acid, dried, and then analyzed with a PhosphorImager (Molecular Dynamics).

Results

Isolation of male-sterile mutants affecting the *kl-3* loop

In order to identify mutations in genes required for Y-loop development, we have screened two collections of EMS-induced male-sterile mutants for the presence and normality of these structures in mature spermatocyte nuclei: 23 putative loop-defective mutants selected from a collection of 400 autosomal male steriles (Hackstein 1991) by in vivo analysis of spermatocyte nuclei and a group of approximately 600 male-sterile mutants kindly provided by Dan Lindsley (University of California, San Diego) and B. Wakimoto (University of Washington, Seattle), who isolated them from a collection of vital autosomal mutations induced in C. Zuker's laboratory (University of California, San Diego).

Larval, pupal, and adult testes dissected and immunostained as described in Materials and methods were analyzed for the presence and normality of the *kl-5*, *kl-3*, and *ks-1* loops. This analysis enabled us to isolate four mutations, three [*ms(3)HB-223*, *ms(3)HB-267*, and *ms(3)HB-933*] from the first collection and one [*ms(3)155-34*] from Dr Zuker's collection, that dramatically affect the morphology of the *kl-3* loop (Fig. 1). In primary spermatocyte nuclei of flies homozygous for these mutations, both the *kl-5* and *ks-1* loops are morphologically normal, as revealed by immunostaining with the S5 antibody (Fig. 1, f′–j′) while there is a clear defect in the unfolding of the *kl-3* loop at any stage of spermatocyte growth. The absence of some thread-like intranuclear material is already detectable after phase-contrast observation of mutant testis preparations (Fig. 1a–e) and becomes particularly evident after immunostaining with the T53-1 antibody, which specifically decorates the *kl-3* loop in wild-type testes (Fig. 1a; Pisano et al. 1993). Despite the presence of a normal Y chromosome in all mutant stocks, only small fragments of immunostained *kl-3* loop are visible within the mature spermatocyte nuclei of *ms(3)HB-933* (Fig. 1b) and *ms(3)155-34* (Fig. 1d) mutants, while the T53-1 antibody does not evidentiate any intranuclear structure within either *ms(3)HB-223* (Fig. 1c) or *ms(3)HB-267* (Fig. 1e) testes.

Complementation tests performed among the four mutants revealed that they identify two complementation groups, one defined by *ms(3)HB-223* and *ms(3)155-34* [from now on, called *loop unfolding protein-1* (*lup-1*)], and the other by *ms(3)HB-267* and *ms(3)HB-933* [from now on, called *loop unfolding protein-2* (*lup-2*)]. Males homozygous for each of the four mutations are completely sterile. In addition, in flies belonging to the first complementation group female fertility is also affected: homozygous *ms(3)HB-223* females are 75% less fertile than their heterozygous siblings, while *ms(3)155-34* females are completely sterile.

Mapping of the *lup-1* and *lup-2* loci

We first performed a series of statistical mapping experiments, using the multiply marked chromosomes described in Materials and methods. This permitted us to place *lup-1* between *hairy* and *thread*, at 42.5 of the statistical map, while *lup-2* was mapped at 58.1, between *curled* and *stripe*.

Based on these recombination data, we could grossly localize *lup-1* and *lup-2* in regions 69 and 89 of the cytological map, respectively. We then undertook a deficiency mapping of these loci, using all the deficiencies in these two regions available from the Bloomington deficiency kit. We found that both *lup-1* alleles were complemented by deletions spanning from 67F2 to 69E8 and from 70A2 to 73B, strongly suggesting that this locus maps within the 69E8–70A1 interval, which, unfortunately, is not uncovered by any available deficiency stock.

As to *lup-2*, we were able to restrict its mapping to 89C7-D1. It is in fact complemented by *Df(3R)sbd26* (89B9;89C7), *Df(3R)Ubx109* (89D1-2;89E1), *Df(3R)P2* (89D9-E1;89E2-3), and *Df(3R)P9* (89E1;89E5) but not by *Df(3R)P10* (89C1-2;89E1-2). Interestingly, males carrying the *lup-2*^{HB933} allele over *Df(3R)P10* exhibit a complete absence of the *kl-3* loop, a cytological phenotype stronger than that of *lup-2*^{HB933} homozygotes and comparable to that observed in both *lup-2*^{HB267}/*lup-2*^{HB267} and *lup-2*^{267}/*Df(3R)P10* flies. Thus, the *lup-2*^{HB933} and the *lup-2*^{HB267} alleles are likely to represent a hypomorph and a null mutation at the *lup-2* locus, respectively.

Biochemical and ultrastructural analysis of mutants

To test whether the defect in *kl-3* loop morphology observed in our mutants is accompanied by a defect in the synthesis of the dynein heavy chain encoded by the *kl-3* fertility factor (Goldstein et al. 1982; Carvalho et al. 2000), we compared the electrophoretic profile of wild-type and mutant testis extracts.

Dynein heavy chains have been identified by comparing the testis polypeptide pattern obtained from wild-type and mutant males carrying the $Y^D X^P$ element of *T(X;Y)V24* and the $X^D Y^P$ of *T(X;Y)W27* (see Materials and methods for details on the construction of these males); the latter lack the *kl-3* region and a high-molecular-weight band that, according to Goldstein et al. (1982), should correspond to the putative dynein heavy chain 3. This allowed us to unambiguously identify the electrophoretic bands previously recognized as dynein heavy subunits 2′, 3, 5, and 2 (Fig. 2). In addition, a very faint band, which shows an electrophoretic migration very close to that of band 3, is present in the electrophoretogram of the mutant males; it is likely to consist of a distinct protein that has not been previously identified, possibly due to the different electrophoretic conditions that we have employed (cf. Goldstein et al. 1982).

When compared to the wild type, testis extracts from males homozygous for any of the mutant alleles or from *lup-2/Df(3R)P10* males consistently exhibited a modified electrophoretic pattern, consisting in a strong reduction of both bands 3 and 5 and in the complete absence of bands 2 and 2′ (Fig. 3).

In order to establish whether such alterations of the electrophoretic profile are due to a reduced or absent protein synthesis or to some post-translational process affecting protein stability, testes dissected from both wild-type and mutant flies were incubated with $[^{35}S]$methionine. Protein extracts were then analyzed by electrophoresis and autoradiography. As shown in Fig. 4, wild-type and mutant extracts do not exhibit any detectable difference in their dynein heavy chain-labeling patterns. Taken together, these results indicate that our mutants do not affect the rate of synthesis of these polypeptides but significantly reduce their stability.

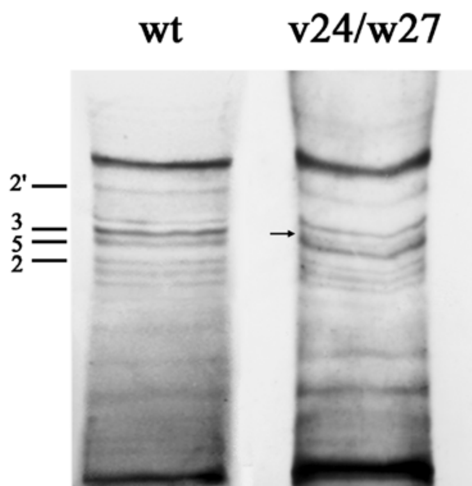

Fig. 2 High-molecular-weight testis polypeptides from wild-type (*wt*) and *kl-3*-deficient males (*V24/W27*). *Numbers* mark the position of dynein heavy chains, as they have been previously named by Goldstein et al. (1982). *Arrow* indicates the position of dynein heavy chain 3, which is not expressed in *kl-3*-deficient males

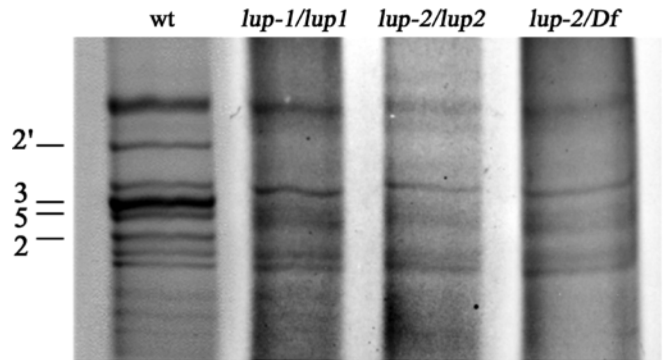

Fig. 3 High-molecular-weight polypeptides from wild type (*wt*), homozygous *lup-1*^{HB223}, homozygous *lup-2*^{HB267}, and hemizygous *lup-2*^{HB267} testes. *Numbers* indicate dynein heavy chains. See text for further details

At the ultrastructural level, the main defect occurring in the axonemes of the four mutants concerns the assembly of the outer dynein arm, which is drastically reduced (Fig. 5). Only in a few instances, a very short protuberance or a slender projection can be observed on the subfiber A (arrows in Fig. 5d). The assembly of the inner dynein arm seems less affected by these mutations; sometimes, however, the inner arm complement is incomplete, and a variable number of doublets are deprived of this structure (arrowheads in Fig. 5d, f). In addition to the defects concerning the dynein arms, other structural aberrations sometimes occur during spermatogenesis of these mutants. They consist mainly (1) in a tendency of the axoneme to fragment longitudinally and in the presence of laminae of microtubular doublets that are not organized around the central microtubular complex (Fig. 6a, b); (2) in the ectopic assembly of microtubular doublets and their associated accessory tubules, which are randomly distributed in the spermatid cytoplasm (arrows in Fig. 6b); and/or (3) in the presence of supernumerary central

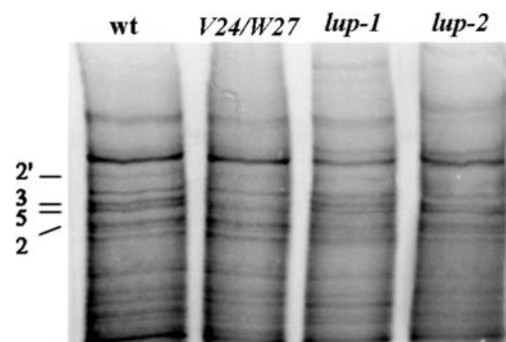

Fig. 4 $[^{35}S]$methionine labeling of high-molecular-weight polypeptides from wild-type (*wt*), *lup-1*^{HB223}, and *lup-2*^{HB267} testes. *Numbers* mark the position of dynein heavy chains

tubules, which appear to assemble from the wall of the pre-existing central microtubules (Fig. 6c–e).

Taken together, these results indicate that our mutants substantially affect both the stability of different dynein subunits and their ability to assemble into a complete dynein arm.

Discussion

The biological role of the Y-chromosome fertility factors has been the subject of a long-lasting debate between two hypotheses: one suggesting that they code for structural components of the axoneme (Goldstein et al. 1982; Hackstein and Hostenbach 1995) and another that considers an unconventional protein-binding function more compatible with the peculiar genetic and molecular organization exhibited by these heterochromatic loci (Hennig 1993; Pisano et al. 1993). Strong support to the first hypothesis has been provided by a series of recent studies that have identified three distinct dynein heavy chain-encoding sequences within each of the *kl-5*, *kl-3*, and *kl-2* fertility regions (Gepner and Hays 1993; Carvalho et al. 2000). The genomic organization of the dynein sequences localized within the *kl-5* fertility factor of *D. melanogaster* and its orthologue *Thread* of *D. hydei* has been characterized. Both these loci were shown to contain a few exons interspersed among several huge introns composed of degenerated transposable elements and large clusters of simple repetitive sequences (Kurek et al. 1996, 1998, 2000; Reugels et al. 2000; R. Kurek, personal communication). Thus, it has been argued that this peculiar molecular organization could account for the unusual size of the fertility genes, and that the lampbrush-like loops visible in mature primary spermatocytes of both species can be considered as the cytological counterpart of extremely large transcription units. However, no evidence of a direct relationship between the transcription of the Y-linked genes and the transcription units appearing as lampbrush loops has been provided to date.

Here we have shown that the synthesis of the putative dynein polypeptide encoded by the *kl-3* fertility region can be uncoupled from the formation of the corresponding loop. In four mutants that severely affect the formation of the *kl-3* loop, in fact, the *kl-3*-related, high-molecular-weight polypeptide (Goldstein et al. 1982) is normally synthesized, as demonstrated by [^{35}S]methionine incorporation experiments. Notwithstanding, the four mutants all exhibit a peculiar pleiotropic phenotype that eventually leads to the production of unmotile sperm: a destabilization of the four putative dynein heavy subunits described by Goldstein et al. (1982) and a defect in the assembly of the outer and, to a lesser extent, of the inner dynein arms of sperm axonemes.

Thus, our results suggest that, at least in the case of the *kl-3* loop, this peculiar structure cannot simply represent the cytological manifestation of the transcription of a coding sequence, but must be regarded as a complex organelle that requires the activity of several autosomal loci for its correct assembly and that most likely serves some additional function necessary for male fertility. In this context, it is worth noting that while the *kl-5* and *ks-1* loops exhibit striking similarities both in their morphology and in their biochemical composition, the *kl-3* loop possesses a series of distinctive cytological features and appears to associate with a functionally distinct class of proteins. The *kl-5* and *ks-1* loops start to develop simultaneously within young primary spermatocyte, are

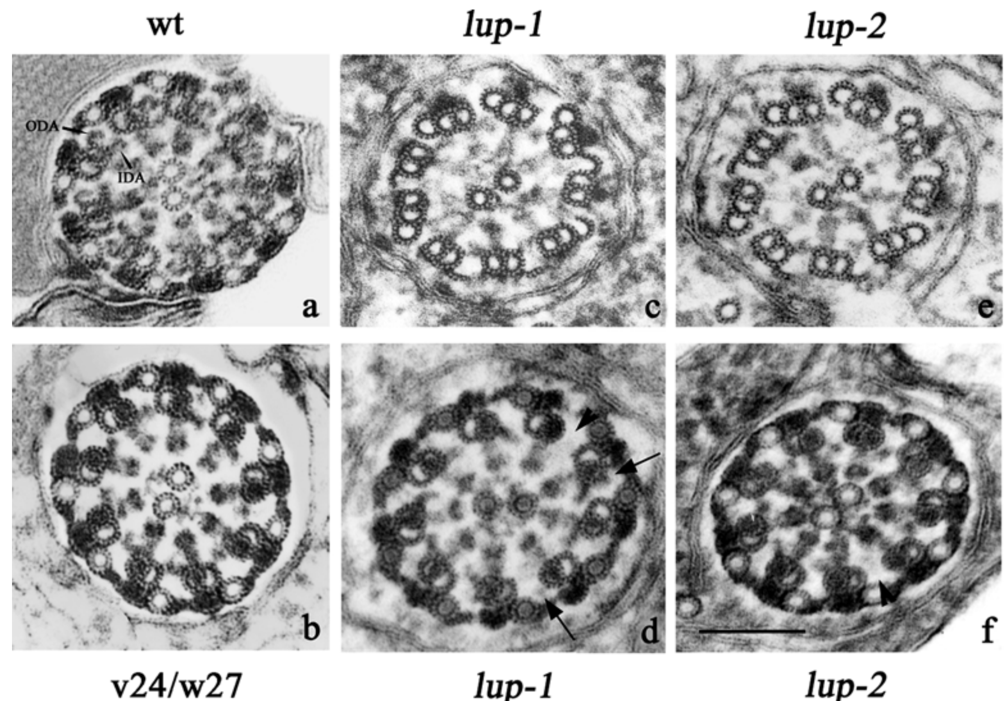

Fig. 5 Axonemal cross-sections from wild-type (**a**), Y^DX^PV24/X^DY^PW27 (**b**), *lup-1* (**c**, **d**) and *lup-2* (**e**, **f**) sperm flagella. *ODA* and *IDA* indicate the position of the outer and inner dynein arms, respectively, in wild-type axonemes (**a**). In mutant testes, outer dynein arms are generally absent (**e**, **f**); only in a few instances, a very short protuberance is visible (*arrows* in **d**). Inner dynein arms are less affected; sometimes, however, they may be absent (*arrowheads* in **d**, **f**). See text for further details. *Bar* = 0.1 µm

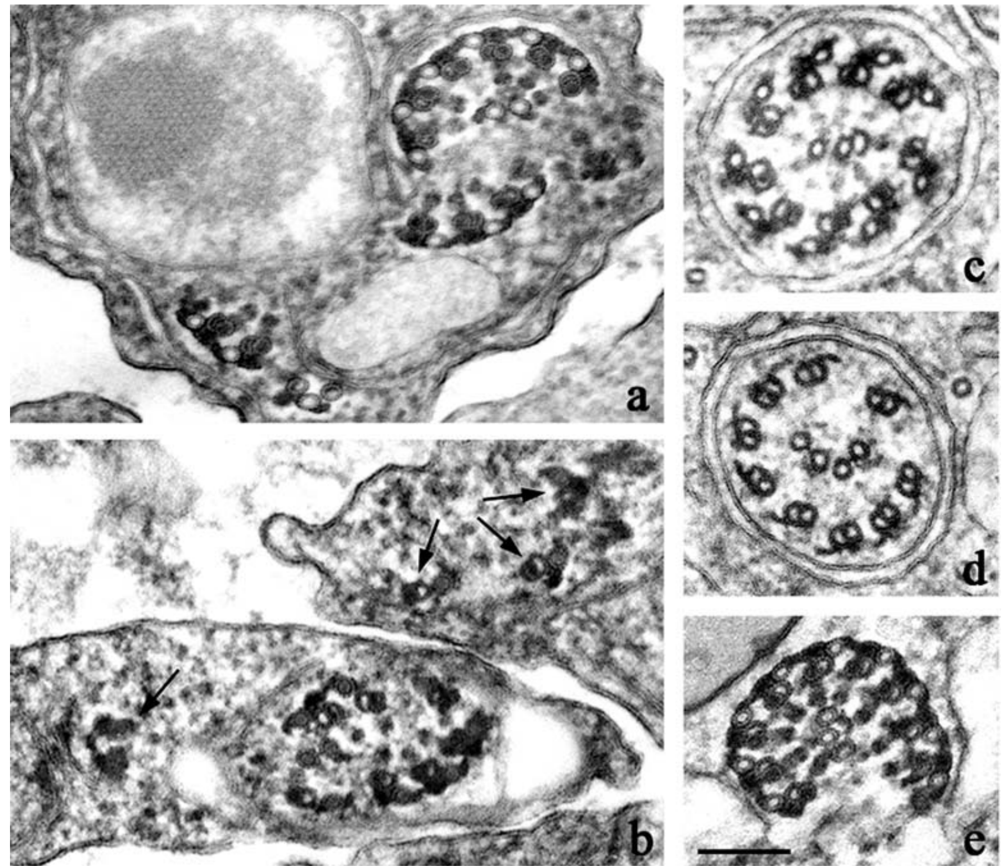

Fig. 6 Testis ultrathin sections showing defects occasionally occurring in the axonemal organization during spermatogenesis of *lup-1* and *lup-2* mutants. *Arrows* point to microtubular doublets that are ectopically distributed within the cytoplasm. See text for further details. *Bar* = 0.1 μm

made of a thick filament (Bonaccorsi et al. 1988), abundantly transcribe satellite sequences (Bonaccorsi et al. 1990), and appear to accumulate mainly proteins implicated in RNA processing and/or function: a minor hnRNA-associated protein recognized by the S5 antibody (Risau et al. 1983) that decorates both the *kl-5* and the *ks-1* loops (Bonaccorsi et al. 1988), RB97D and Boule, that specifically accumulate on the *ks-1* loop and belong to the RRM family and the DAZ family of RNA-binding proteins, respectively (Heatwole and Haynes 1996; Cheng et al. 1998). The *kl-3* loop develops later than the other two loops, consists of a thinner and more extended filament, and was shown to accumulate several testis specific proteins that are subsequently incorporated into the sperm tail: the antigen recognized by the sph-155 antiserum raised against a testis specific protein of *D. hydei* (Hulsebos et al. 1984; Bonaccorsi et al. 1988), a 53-kDa protein homologous to a leucine aminopeptidase (Pisano et al. 1993; Gatti 1995), and a calmodulin-related protein, specifically expressed in the male germ line, denominated Androcam (Lu and Beckingham 2000). In addition, no evidence of satellite-DNA transcription along this loop has been obtained to date (our unpublished results), strongly supporting the hypothesis that the *kl-3* locus possesses a distinctive molecular and functional organization with respect to the other loop-forming fertility factors.

Currently, we have no clues about the molecular nature of the products encoded by *lup-1* and *lup-2*. They could be regulatory molecules that control the unfolding of the *kl-3* loop. The absence of this loop in primary spermatocytes of *lup-1* and *lup-2* mutants would not impair the synthesis of the dynein polypeptide encoded by the *kl-3* region, but substantially affect its stability. However, the simultaneous instability of both the *kl-2* and *kl-5* high-molecular-weight polypeptides observed in both *lup-1* and *lup-2* mutants cannot simply be the consequence of the absence of the *kl-3* loop, as these effects are not observed in males carrying a deficiency of the *kl-3* region (cf. Goldstein et al. 1982). Thus, it seems more conceivable that the pleiotropic phenotype of our mutants is the consequence of a defect in proteins that can act as mediators in two processes: (1) the assembly of the complex cytochemical structure of the loop and (2) the assembly of the dynein arms in the axoneme.

The mechanism by which dynein arms are assembled and targeted to their specific location within the axoneme is not yet completely understood. Current knowledge indicates that the correct formation of dynein arms is achieved through the interaction of several complexes that are preassembled in the cytoplasm and are subsequently transported to their binding sites on the axonemal doublets. In *Chlamydomonas* at least three distinct complexes are required, two consisting of different dynein subunits and one formed by proteins that function as docking structures involved in the positioning and binding of the arm on the microtubule (Fok et al. 1994; Fowkes and Mitchell 1998; Wakabayashi et al. 2001). The stability

of dynein polypeptides depends on their correct association, and dynein subunits that are not bound to doublet microtubules do not accumulate in the cytoplasm; therefore, mutations that affect proteins involved in the assembly process may result in a diminished stability of several dynein subunits other than the mutated protein. In this context, a possible explanation for the pleiotropic phenotype elicited by our mutants is that *lup-1* and *lup-2* products are proteins that would function in mediating one of the different steps that contribute to the correct assembly of the dynein arms, thus ensuring the stability of the several polypeptides involved in this process. *lup-1*- and *lup-2*-encoded proteins would accumulate along the *kl-3* loop in primary spermatocytes, where they could contribute to its morphology by allowing the binding of other polypeptides to loop-associated transcripts. Later in spermiogenesis, they would be transferred to the elongating sperm tail, where they would be involved in the achievement of the correct axonemal organization. According to this model, the *kl-3* loop should therefore be regarded as a highly complex structure specifically designed to fulfill a dual role (cf. Hackstein et al. 1991): accommodating the coding sequence of a dynein subunit and allowing the storage and preassembly of axonemal components. We believe that further work aimed at identifying the *lup-1* and *lup-2* products will help to verify this hypothesis and to get further insight into the elusive nature of the Y-chromosome fertility factors.

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