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γ-Tubulin is required for the structure and function of the microtubule organizing centre in *Drosophila* neuroblasts

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We report that in *Drosophila*, γ-tubulin is required for the structure as well as the function of microtubule organizing centres (MTOCs). This conclusion is based on the identification and phenotypic characterization of a mutant allele of the γ-tubulin gene located at region 23C of the polytene chromosome map. This mutation, which we have called γ-*tub23C*^{PI}, is caused by the insertion of a P-element within the 5′ untranslated leader of the γ-tubulin transcript. Northern and Western analyses show that γ-*tub23C*^{PI} is either a null or a very severe hypomorph as no γ-tubulin mRNA or protein can be detected in mutant individuals. Visualization of DNA, MTOCs and microtubules by confocal laser scanning microscopy of cells from individuals homozygous for γ-*tub23C*^{PI} reveals a series of phenotypic abnormalities. Some of these are similar to those observed after disruption of γ-tubulin function in other organisms, including mitotic arrest and a dramatic decrease in the number of microtubules, but, in addition, we have observed that mutation in this gene also results in highly abnormal MTOCs which show a variety of shapes and sizes which we never observed in wild type cells. These results show that γ-tubulin is required for both structural and functional roles in the MTOCs.

Key words: *Drosophila*/mitosis/MTOC/mutant/γ-tubulin

Introduction

Gamma-tubulin (γ-tubulin), a member of the tubulin superfamily, was identified initially in *Aspergillus nidulans* as the product of the *mipA* gene (Weil *et al.*, 1986; Oakley and Oakley, 1989). It was shown to share some 30% amino acid identity with either α- or β-tubulin and it appears to account for ~1% of the total tubulin pool in *Xenopus* (Sterns *et al.*, 1991). Subsequently, γ-tubulin has been shown to be conserved in many other species including *Schizosaccharomyces pombe* (Horio *et al.*, 1991; Sterns *et al.*, 1991) *Xenopus laevis*, *Homo sapiens* and *Drosophila melanogaster* (Zheng *et al.*, 1991), and in the malaria parasite *Plasmodium falciparum* (Meassen *et al.*, 1993).

Immunolocalization of γ-tubulin has generally supported the view that it performs essential functions in both cytoplasmic and mitotic microtubule organizing centres (MTOCs). γ-Tubulin has been reported to be a component of MTOCs associated with the formation of spindle microtubules during mitosis (Oakley *et al.*, 1990; Horio *et al.*, 1991; Sterns *et al.*, 1991; Zheng *et al.*, 1991; Joshi *et al.*, 1992) even in the absence of centrioles (Gueth-Hallonet *et al.*, 1993). Also, in *S.pombe* (Horio *et al.*, 1991) and mammalian cells (Joshi *et al.*, 1992) γ-tubulin has been shown to be associated with cytoplasmic MTOCs during interphase. Furthermore, γ-tubulin has been found to be present at the centre of taxol induced asters in cell-free extracts (Sterns and Kirschner, 1994). It has also been reported to localize to the midbody of mammalian cells during cytokinesis (Julian *et al.*, 1993).

Genetic analysis of γ-tubulin genes has been performed in at least two species, showing that this essential gene is required for mitosis. In *A.nidulans*, cells mutated for the *mipA* gene cannot undergo nuclear division, display a reduction in number and length of cytoplasmic microtubules, and have virtually a complete absence of mitotic apparatus. These results are consistent with the hypothesis put forward by these authors that γ-tubulin might be required for microtubule nucleation and/or attachment to the spindle pole body (Oakley *et al.*, 1990). Similar results have been obtained after mutation of the homologous gene in *S.pombe*. Disruption of *gtb1*$^+$ causes a mitotic block with overcondensed chromosomes that never separate their sister chromatids. However, unlike *mip*A cells, *gtb*1 cells display a range of abnormal mitotic apparatus (Horio *et al.*, 1991).

In vivo microinjection of mammalian cells with anti-γ-tubulin antibodies has been shown to prevent the formation of both cytoplasmic and spindle microtubules suggesting that also in mammalian cells, γ-tubulin might be required for microtubule nucleation (Joshi *et al.*, 1992). More recently, *in vitro* reconstitution of centrosome assembly and function in *Xenopus* cell-free extracts has suggested the possibility that γ-tubulin might not only be required for centrosome function but also for centrosome formation (Felix *et al.*, 1994; Sterns and Kirschner, 1994).

Here we report the identification of a P-element mutant allele of the *Drosophila* γ-tubulin gene located at polytene chromosome region 23C which by molecular analysis we show to be a null or severe hypomorph. Immunofluorescence of mutant neuroblasts shows metaphase cells with various degrees of aberrant spindle morphology. However, the most significant observation reported here indicates

that MTOC structure, as determined by spindle pole shape and the distribution of the centrosomal antigen BX63, is highly abnormal in mutant cells. These results provide strong support for the hypothesis that γ-tubulin is required for both the structure and function of the MTOC in *Drosophila*.

Results

Molecular characterization of the P-element insertion at 23C

We have identified a mutation induced by the P-element which localizes to the region 23C1–4 region known to contain one of the two *Drosophila* γ-tubulin genes (Zheng *et al.*, 1991). In order to determine the molecular nature of the lesion we cloned and sequenced the insertion site. The results are shown in Figure 1. The genomic sequences adjacent to the P-element insertion site were cloned using both plasmid rescue and inverse PCR. A 2480 bp fragment (pRG.1) containing one side of the insertion was cloned and sequenced. It was found to contain a P-element repeat followed by nucleotide 75 of the 5′ untranslated leader of the transcript that codes for the γ-tubulin gene located in 23C previously reported (γ-tubulin23C) (Zheng *et al.*, 1991). The sequence of pRG.1 is the same as that of the γ-tubulin23C cDNA up to nucleotide 244 where homology is lost and which coincides with a conserved sequence for a donor splice site. The genomic organization of γ-tubulin23C was later determined after isolation of both genomic and cDNA clones (Figure 1a).

Germline transformation was used to show that this P-element insertion was responsible for the lethality and mitotic phenotype (see next section). A 9 kb genomic fragment including the complete coding region was inserted into the pw8 transformation vector (Klemenz *et al.*, 1987) and two independent transformants isolated. As a negative control a 5 kb fragment truncated at the 3′ end of the gene was also used for transformation. Both transformants containing the wild type γ-tubulin23C gene p[w-γ-23C] were able to complement both the lethality and mitotic phenotype, while the transformants containing the truncated gene p[w-γ-23CΔ] did not (Figure 1a). Therefore, we have designated the mutant as γ-*tub23C*^{PI}.

In order to determine the level of expression of the γ-*tub23C*^{PI} allele, total protein extracts from wild type ovaries and neuroblasts, as well as from homozygote γ-*tub23C*^{PI} neuroblasts, were probed with an anti-γ-tubulin polyclonal antibody (Joshi *et al.*, 1992) by Western blotting. The antibody recognizes one polypeptide of 53 kDa in wild type ovaries, two polypeptides of 53 and 70 kDa in wild type brain extracts and only a 70 kDa band in γ-*tub23C*^{PI} brain extracts (Figure 1b). The 53 kDa protein band corresponds in size to the expected molecular weight of the conceptual translation of the γ-tubulin23C cDNA (Zheng *et al.*, 1991). The identity of the 53 kDa band from ovaries and brains was confirmed using antibodies from the polyclonal serum immunopurified against the MAL–γ-tubulin23C fusion protein (Figure 1c). Similar results were obtained using a polyclonal antibody raised against the complete *Drosophila* γ-tubulin23C protein (W.G.F.Whitfield, D.M.Glover and C.E.Sunkel, unpublished results). The 70 kDa protein appears to have a closely related epitope to that of γ-tubulin which is

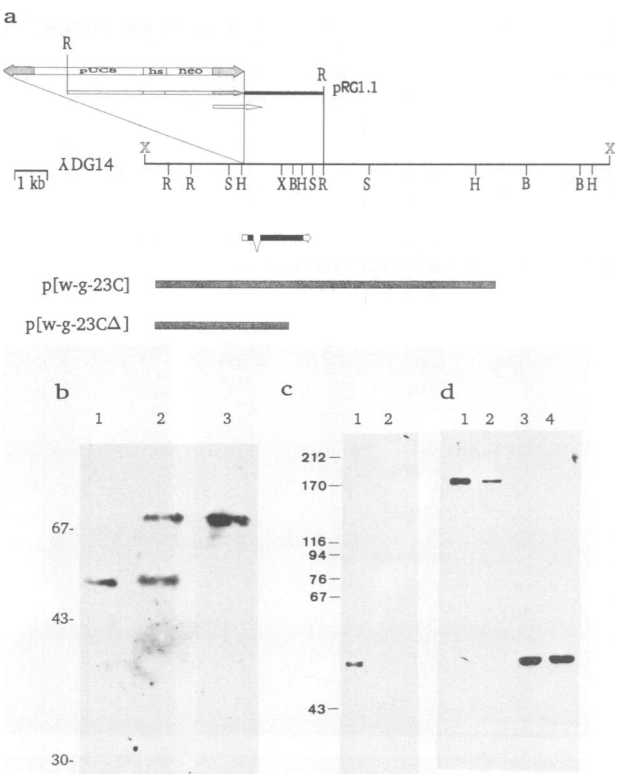

Fig. 1. Molecular characterization of the P-element insertion within the γ-tubulin23C gene and detection of the γ-tubulin protein in wild type and mutant extracts. (**a**) In the top part the molecular structure of the P-element inserted at 23C is represented. It contains sequences from the P-element repeats (inverted shaded arrows), sequences from pUC8, the heat shock promoter (HS) and the neomycin-resistance gene (neo). Below, the pRG1.1 plasmid is shown. This plasmid was obtained after the *Eco*RI (R) digest of the γ-*tub23C*^{PI} genomic DNA was ligated and transformed into *Escherichia coli*. The black fragment represents part of the adjacent genomic DNA cloned into pRG1.1. The white arrow below indicates the direction and extent of the region which was sequenced. The genomic map to which the insertion was mapped is shown below covering some 15 kb. Most of this region is contained within the λDG14 clone. The single transcript identified within this genomic region is shown below and the coding region indicated by the black bars. The direction of transcription is indicated by the arrowhead and comparison of cDNAs with the genomic region indicates that there is a single intron near the 5′ end. p[w-γ-23C] was used for germline transformation with the complete coding region, while p[w-γ-23CΔ] was truncated at the C-terminal end. (**b**) Total protein extracts from wild type ovaries (lane 1), wild type third larval instar brains (lane 2) and homozygote mutant γ-*tub23C*^{PI} third larval brains (lane 3) were separated by SDS−PAGE, transferred to nitrocellulose and probed for γ-tubulin. (**c**) Wild type (lane 1) and γ-*tub23C*^{PI} (lane 2) brain extracts were probed with immunopurified antibodies against a MAL−γ-tub23C fusion protein. (**d**) Total protein extracts from either wild type (lanes 1 and 3) or γ-*tub23C*^{PI} mutant (lanes 2 and 4) third instar larval brain were probed either with the polyclonal serum RB188 (lanes 1–2) which recognizes the BX63 antigen or a monoclonal anti-γ-tubulin antibody (lanes 3–4). Molecular weights are the same in (c) and (d).

not recognized by the immunopurified serum. Since the immunopurified anti-γ-tubulin antibodies failed to detect any proteins in homozygote mutant brain extracts, it can be suggested that the γ-*tub23C*^{PI} allele is either a null or a severe hypomorph. The same conclusion was derived from Northern analysis of poly(A)$^+$ RNA isolated from mutant brains which failed to show the presence of γ-tubulin23C mRNA (data not shown). Also, it is clear that

Table I. Quantification of mitotic activity in wild type and γ-*tub23C*^{PI} mutant neuroblasts

Genotypes	No. of fields	No. of mitotic figures[a]	No. of metaphases	No. of anaphases	Mitotic index	Frequency of metaphase	Frequency of anaphase	2*n*		>2*n*	
								No.	%	No.	%
$\gamma^{PI}/+$	521	685	545	140	1.31	1.05	0.27	545	100	0	0
γ^{PI}/γ^{PI}	437	297	294	3	0.67	0.67	<0.01	87	30	207	70

γ^{PI}, γ-*tub23C*^{PI}; +, Oregon R; %, percentage over total number of metaphases.
[a]Ten brains were scored for each genotype.

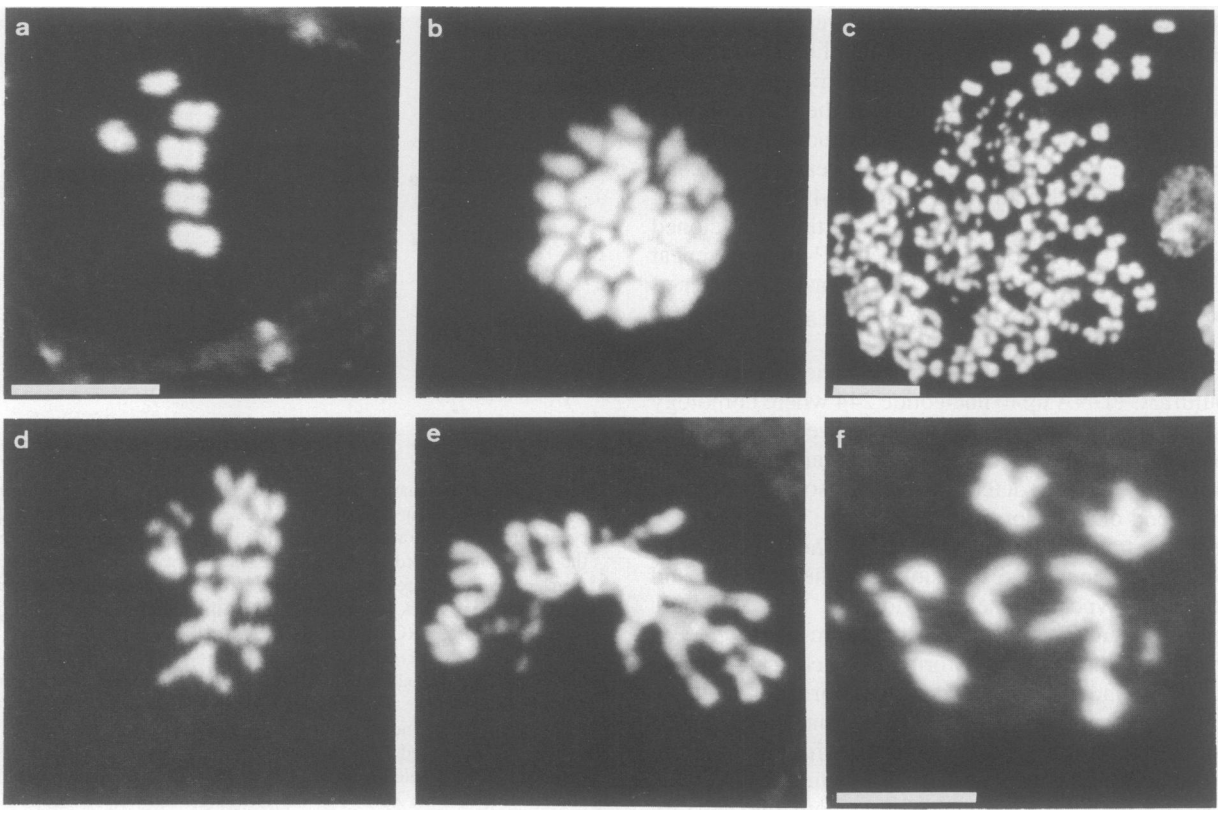

Fig. 2. Propidium iodide staining of squashed third instar larval γ-*tub23C*^{PI} neuroblasts. (**a**) Mutant cell showing highly condensed chromosomes with no metaphase plate organization. (**b**) Polyploid mitotic figure with condensed chromosomes closely packed together. (**c**) Highly polyploid mitotic figure with high chromosome condensation and scattered over a large area. (**d**) Aneuploid cell. (**e**) cell showing abnormal chromosome number and irregular chromosome condensation. (**f**) Cell showing a very abnormal anaphase-like figure. Amplification is the same in (a), (b), (d) and (e). Bars in (a) and (c) are 5 μm; in (f), 2.5 μm.

the γ-tubulin protein present in ovaries does not remain in any significant amounts in third instar larval mutant brains since the antibodies failed to detect it. Nevertheless, the absence of γ-tubulin protein does not seem to affect significantly the level of other components of the mitotic apparatus such as β-tubulin (Figure 1d). However, while the same amount of total brain extracts from either wild type or mutant individuals was used for detection of BX63, the intensity of the band in mutant extract is about one-third. This does not appear to be a specific effect upon BX63 since the same relative abundance was obtained when the same blot was reprobed with an antibody against the POLO kinase (data not shown). Therefore, the reduction in the BX63 antigen in the mutant cells probably reflects the low mitotic index of the mutant brains.

Mitotic phenotype

Homozygote γ-*tub23C*^{PI} mutant individuals die during late larval stage and have reduced brain and imaginal discs. Mitotically active tissue from individuals homozygous for this mutant chromosome was analysed to determine a number of quantitative mitotic parameters. The results summarized in Table I show that these mutant cells display a low mitotic index, high frequency of polyploid cells and very low frequency of anaphases. Cytological analysis indicates that these cells show a variety of abnormal mitotic phenotypes including abnormal chromosome condensation, various degrees of polyploidy, alterations in the arrangement of the metaphase plate and highly disorganized anaphase figures (Figure 2). These results suggest that this mutation affects an essential gene required to complete normal mitosis.

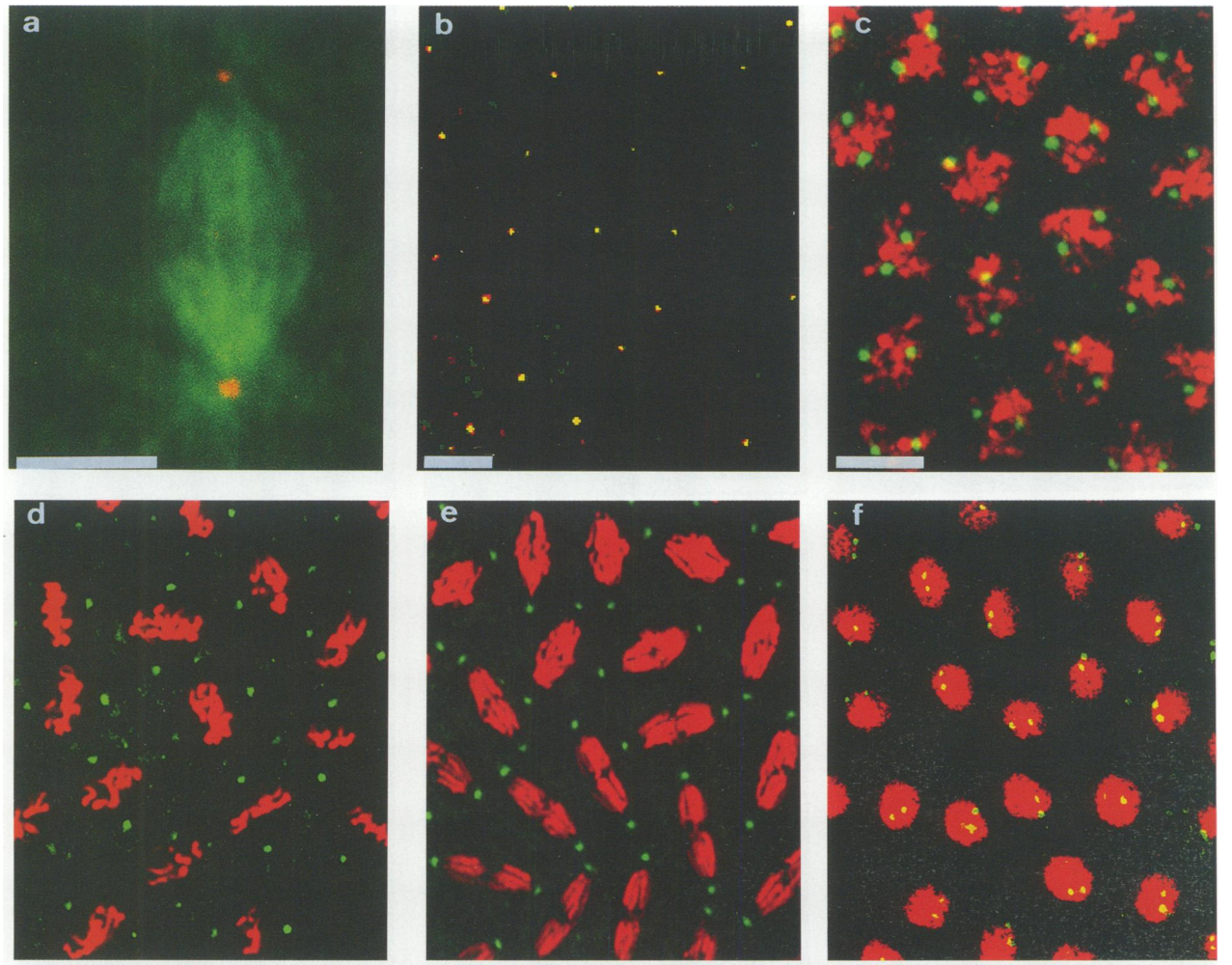

Fig. 3. Immunolocalization of γ-tubulin during the early nuclear multiplication stages and co-localization with the BX63 centrosomal antigen. (**a**) Double labelling of an early embryo to visualize β-tubulin (green) and γ-tubulin (red). The difference in size of the two centrosomes is due to their location in slightly different focal planes. (**b**) Double labelling of an early embryo to visualize the BX63 antigen (green) and γ-tubulin (red); note that overlapping colours will appear in yellow. (**c**) Early embryo at prophase stained for γ-tubulin (green) and DNA (red). (**d**) Early embryo at metaphase stained as in (b). (**e**) Early embryo at anaphase stained as in (b). (**f**) Early embryo at telophase stained as in (b). For description see text. Amplification is the same in (c), (d), (e) and (f). Bar in (a) is 2.5 μm and all others are 5 μm.

Immunolocalization of γ-tubulin to the MTOC

Immunopurified antibodies against γ-tubulin were used to determine its localization during early stages of nuclear multiplication in relation to other components of the mitotic apparatus. The results are summarized in Figure 3. In the first place we wished to determine whether γ-tubulin localizes to the MTOC. Wild type embryos were stained simultaneously for γ-tubulin and β-tubulin. The immunofluorescence results show that during metaphase γ-tubulin localizes to the spindle poles, although some signal is also detected over the spindle region near the poles (Figure 3a). Co-localization of γ-tubulin with the centrosomal associated antigen BX63 was also determined. Embryos were stained simultaneously with a monoclonal antibody against BX63 (Frash *et al.*, 1986) and an anti-γ-tubulin polyclonal antibody. The results are shown in Figure 3b. The co-localization of both antigens during metaphase (Hoechst staining, not shown) is almost perfect. However, careful observation indicates that in most centrosomes γ-tubulin staining extends further than BX63 staining.

The pattern of γ-tubulin centrosomal staining during different stages of the nuclear mitotic cycle was also determined. The results show that γ-tubulin is always present in the early embryonic centrosome. Staining appears as a relatively large dot during prophase (Figure 3c), condenses to a smaller and brightly stained dot during metaphase (Figure 3d), elongates during early anaphase (Figure 3e) and duplicates by late anaphase, so that it can be seen to separate during early telophase (Figure 3f). Since the size and shape of the γ-tubulin signal change during mitosis we have not been able to determine whether the total amount varies according to the stage of the mitotic cycle. The pre-immune serum was used as a control for specific γ-tubulin staining and no specific labelling was detected (data not shown).

γ-tub^{Pl} mutant cells have highly abnormal mitotic spindles

In order to determine whether γ-*tub23C^{Pl}* mutant neuroblast cells display mitotic apparatus, these cells were stained with an anti-β-tubulin antibody. There were essen-

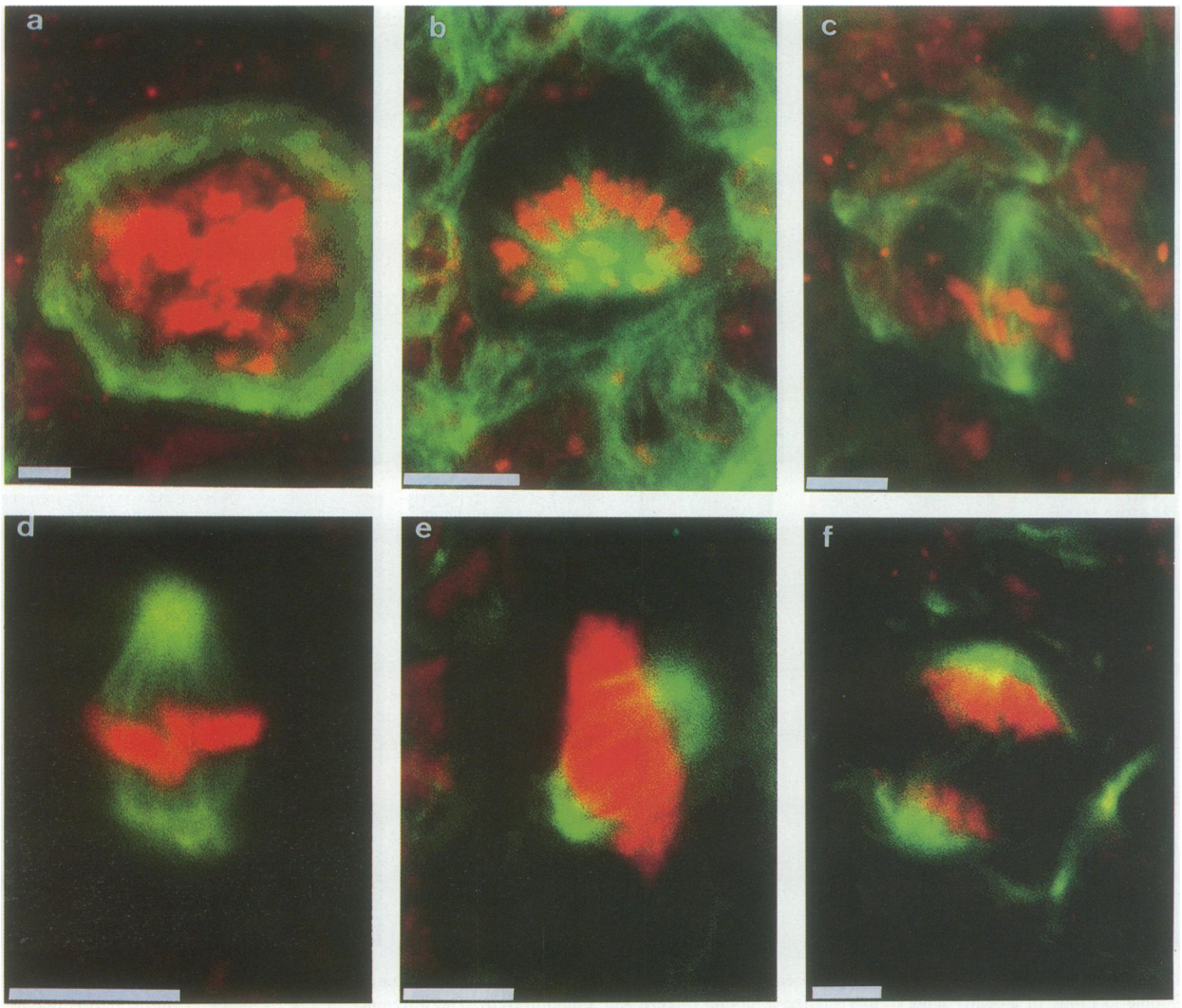

Fig. 4. Immunolocalization of γ-tubulin in whole mount third instar brains of γ-*tub23C*^{PI} homozygotes. All preparations where stained for β-tubulin (green) and DNA (red). Confocal images were constructed from an average of 10 optical sections. (**a**) Mutant cell showing a broad band of β-tubulin with no signs of microtubule organization and condensed chromosomes at the centre. (**b**) Mutant cell showing a monopolar spindle with polar microtubules running between the condensed chromosomes. (**c**) Mutant cell with an almost normal bipolar spindle but abnormal metaphase plate organization. (**d**) Mutant cell with a near normal bipolar spindle and metaphase plate but with very abnormal poles. (**e**) Highly polyploid cell showing a short bipolar spindle with very broad poles. (**f**) Abnormal anaphase in progress showing irregular chromosome distribution. All bars are 5 μm.

tially four different staining patterns: cells with no obvious microtubule organization, cells in which β-tubulin is localized in a broad band around the condensed chromosomes (Figure 4a), cells with a monopolar spindle clearly organized from a discrete organizing region (Figure 4b) and cells containing a bipolar spindle (Figure 4c–f). The spindle poles of the majority of mutant cells with near normal ploidy are abnormal in size and morphology (Figure 4c and d). Highly polyploid cells were also found to have very short bipolar spindles with very broad poles and a compact mass of condensed chromosomes (Figure 4e). None of the monopolar or bipolar spindles present in these mutant cells appear to be associated with clearly defined asters. There are no microtubules beside those going to the metaphase plate. Although very few anaphase-like figures were found, we were able to observe one case in which irregular chromosome or chromatid distribution is very clear (Figure 4f). From these results we can conclude that this mutation results in abnormal microtubule nucleation, as well as irregular organization of spindle poles. Nevertheless, it is significant that some MTOC function still remains even in the absence of γ-tubulin.

γ-*tub*^{PI} mutant cells have abnormal MTOC morphology

In the previous section we showed that many γ-*tub23C*^{PI} cells have either monopolar or bipolar spindles. Most of these spindles appear to have very abnormal spindle poles both in terms of structure and microtubule nucleation capacity. To assess further the effects of the γ-*tub23C*^{PI} allele upon MTOC morphology, mutant cells were stained for the *Drosophila* centrosomal associated antigen BX63. In wild type cells this antigen appears as a highly regular circular structure of ~0.4 μm in diameter (Figure 5a). In mutant cells, the organization and disposition of the BX63 reacting material are severely affected (Figure 5b–f). Most of the structures stained by the antibody have lost their circular shape and appear as elongated and irregular

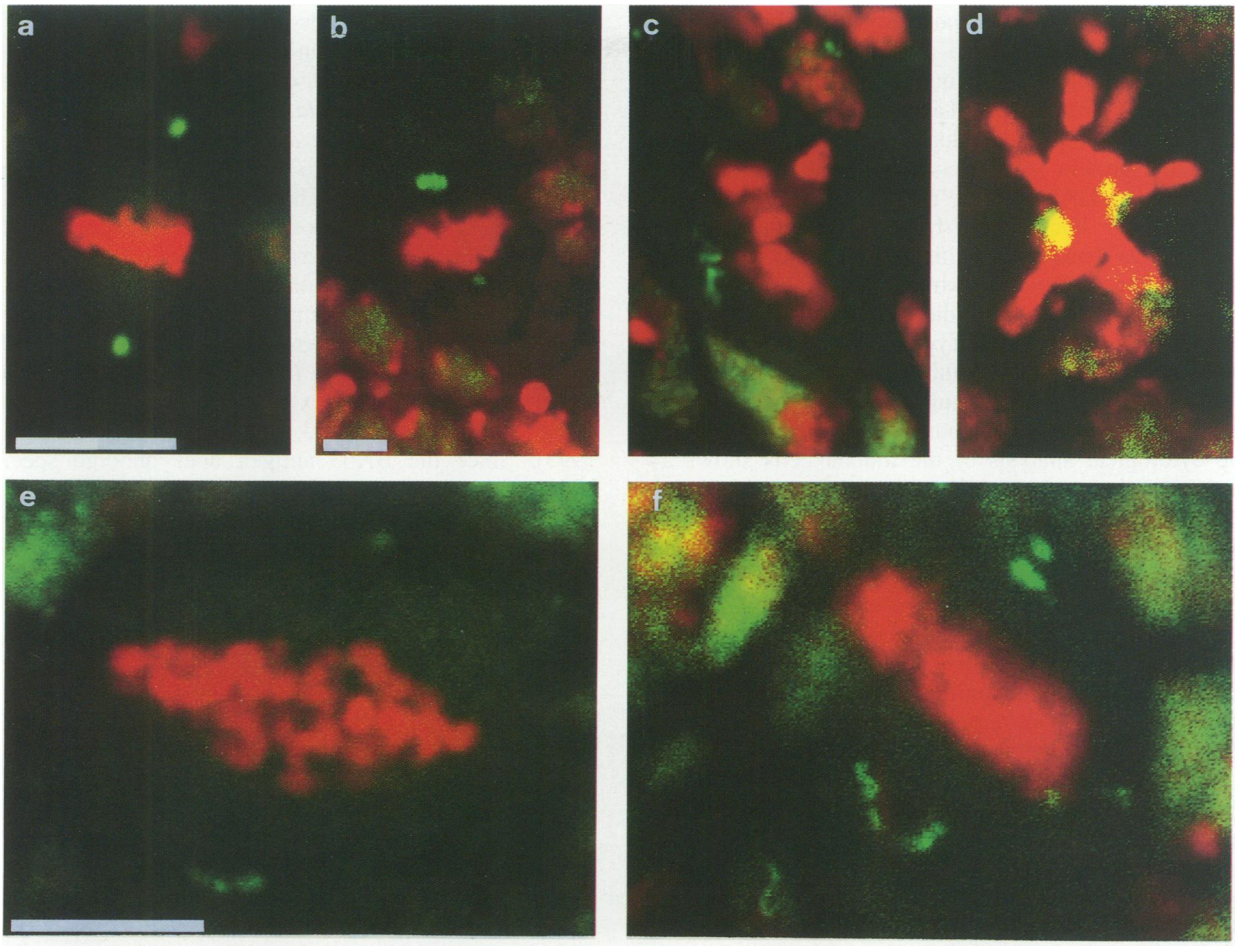

Fig. 5. Immunolocalization of MTOCs in wild type and γ-*tub23C*^{PI} mutant cells. (**a**) Wild type neuroblast undergoing metaphase stained for BX63 (green) and DNA (red) showing the very regular size and shape of the MTOCs and their location at either side of the metaphase plate. All other figures (b–f) show γ-*tub23C*^{PI} mutant cells stained with RB188 (green) and DNA (red). (**b**) Polyploid mutant cell showing very abnormal MTOCs at both poles. Note the significant difference in size and shape of the two MTOCs. (**c**) Mutant cell showing string-like MTOC associated with one side of the condensed chromosomes. (**d**) Polyploid mutant cell showing abnormal MTOCs associated with the chromosomes. The yellow signal indicates complete co-localization of the BX63 and DNA signal. (**e**) Mutant cell showing a string-like MTOC at one pole, while the other pole has a very diffuse round MTOC. (**f**) Polyploid cell with well organized metaphase plate showing at least two very abnormal MTOCs at either pole. The two at the top are strongly stained while those at the bottom of the mitotic figure have a string-like structure. Amplification is the same in (a) and (c), and in (d), (e) and (f). All bars are 5 μm.

string-like structures. These alterations appear to follow a random pattern, since even the MTOCs located at each pole within a single cell are nearly always different from one another, both with respect to shape and size. In some instances these abnormal structures are not located at each side of the metaphase plate but found together at either the centre or the periphery of the cell. Quantification of these phenotypic traits indicates that cells displaying abnormal distribution of the BX63 antigen with respect to shape and size account for >70% of the total mitotic figures.

Discussion

γ-Tubulin has been shown to be a component of MTOCs in all species in which it has been analysed. MTOCs are the sites of microtubule nucleation responsible for the formation of the mitotic spindle and also for the nucleation of cytoplasmic microtubules during interphase. In this paper we provide evidence which indicates that γ-tubulin is not only required for the proper organization of functional microtubule arrays of the mitotic spindle but also for the structure of MTOCs.

We have identified a P-element induced late larval lethal mutation which localizes to the region 23C1–4 of the *Drosophila* genome. Previous data had indicated that *D.melanogaster* contains two γ-tubulin genes, one of which was mapped by *in situ* hybridization to the 23C region (Zheng *et al.*, 1991; Y.M.Zheng, K.Jung and B.Oakley, unpublished data). Molecular analysis of the mutant chromosome indicates that the P-element is inserted within the 5′ untranslated leader 75 bp from the 5′ end of the transcript. We were unable to detect any normal or abnormal γ-tubulin transcripts in poly(A)$^+$ RNA from homozygote mutant brains. Germline transformation with a genomic fragment which contains the complete γ-*tub23C* gene showed that the lethality and mitotic phenotype are due to the P-element insertion. Therefore, we have designated the mutation as γ-*tub23C*^{PI}. Since the mutation causes late larval lethality it is clear that γ-tubulin23C is

required for zygotic development. Whether this gene is also expressed maternally, and is thus required for early embryonic development, remains to be determined.

A polyclonal serum prepared against a conserved region of various γ-tubulin proteins (Joshi *et al.*, 1992) was used to detect the protein in extracts from wild type ovaries and third instar larval brains, as well as from extracts from γ-*tub23C*^{PI} homozygote brains. The results show that a 53 kDa band is specifically recognized by the immunopurified antibody in extracts from both wild type ovaries and brains. This indicates that the antibody recognizes both maternal and zygotic γ-tubulin. The same antibody did not detect any γ-tubulin in the extracts from γ-*tub23C*^{PI} homozygote cells, suggesting that the mutation is either a null or a severe hypomorph.

The cytological analysis of the mutant cells shows a low mitotic index suggesting that viability is significantly affected. The phenotypic analysis indicates that a large proportion of the mitotic figures observed are polyploid with different degrees of chromosomal condensation. The results suggest that these cells are unable to segregate their chromosomes properly. This is confirmed by the very low frequency of anaphases or sister chromatid separation detected. However, it is significant that in the abnormal anaphase-like figures observed, we could find chromosomes which had separated, as well as intact chromosomes. This could explain the irregular distribution of DNA observed in Figure 4f. In this regard, the mitotic phenotype caused by γ-*tub23C*^{PI} differs to some extent from that described for *mipA* (Oakley *et al.*, 1990) and *gtp*1 (Horio *et al.*, 1991). In either case the mutations do not lead to the formation of highly polyploid cells and sister chromatid separation was never observed.

The immunolocalization of γ-tubulin in early *Drosophila* embryos shows that it is localized mostly to the mitotic spindle poles as has been shown for many other cell types (Oakley *et al.*, 1990; Horio *et al.*, 1991; Sterns *et al.*, 1991; Zheng *et al.*, 1991; Joshi *et al.*, 1992; Gueth-Hallonet *et al.*, 1993). We also detected a low level of staining within the spindle very near the poles. This would suggest that γ-tubulin might also be present associated with spindle microtubules. This observation is supported by recent data which indicate that γ-tubulin associates with the minus ends of microtubules nucleated by asters produced in cell-free extracts (Sterns and Kirschner, 1994). The authors suggest that the γ-tubulin staining within the spindle might be due to its association with the minus ends of microtubules which do not reach the centrosome. Double labelling of early embryos with antibodies raised against γ-tubulin and the centrosomal antigen BX63 clearly indicates that the two proteins co-localize. However, we found that γ-tubulin staining occupies a larger area than that for BX63. This suggests that γ-tubulin might extend to the pericentrosomal area in *Drosophila* centrosomes, as shown previously for *Xenopus* (Sterns *et al.*, 1991).

The localization of γ-tubulin during the different stages of the early nuclear cycle was also determined. The results show that γ-tubulin is always present in the centrosome, unlike the BX63 antigen which during interphase associates with the chromatin. We have not been able to detect any consistent change in the signal intensity of γ-tubulin during different stages of mitosis. The only detectable changes are those caused by the changes in the shape of the centrosomes during its replication. The staining appears larger during prophase and condenses somewhat during metaphase and telophase. This would suggest that in *Drosophila*, as has been described for other cell types (Sterns *et al.*, 1991), γ-tubulin does not accumulate preferentially to the mitotic centrosome. Since microtubule nucleation by the centrosome occurs preferentially during mitosis, its participation in this process could either be determined by cell cycle-dependent post-translational modifications of the γ-tubulin already present or be due to other proteins that associate with γ-tubulin in a cell cycle-dependent manner. In this regard it is worth mentioning that in *Drosophila*, γ-tubulin has been shown to form a complex with two other microtubule associated proteins, BX63 and DMAP60 (Raff *et al.*, 1993). Since the BX63 antigen does undergo specific localization to the centrosome during the early stages of mitosis it is tantalizing to suggest that this protein either on its own or in association with γ-tubulin might be directly involved in the nucleation of spindle microtubules. Furthermore, the results presented above show that γ-tubulin does not seem to be required for the centrosomal localization of BX63.

In *S.pombe*, *gtp*1 cells have a range of abnormal mitotic apparatus (Horio *et al.*, 1991). In order to determine whether γ-*tub23C*^{PI} mutant cells are also capable of organizing a mitotic apparatus we stained these cells with anti-β-tubulin antibodies. The results show that a large number of mutant cells have either monopolar or bipolar mitotic apparatus. These spindles are generally nucleated from broad or diffused poles without any asters. The bipolar spindles appear to have kinetochore as well as non-kinetochore microtubules and the organization of the metaphase plate in cells with low chromosome number is clearly abnormal. The organization of the metaphase plate in highly polyploid cells which have bipolar spindles cannot be clearly defined due to the large number of chromosomes. The presence of kinetochore microtubules can be inferred from the fact that chromosome or chromatid segregation can be seen at a very low frequency and also because the chromosomes associated with monopolar spindles are not dispersed but arranged around the spindle microtubules. The presence of residual amounts of microtubules which in some cells assume a clear bipolar organization is similar to that reported for *S.pombe* γ-tubulin mutant cells (Horio *et al.*, 1991). Whether these microtubules are nucleated by undetectable levels of γ-tubulin or independently of γ-tubulin remains to be determined. However, the most significant finding is that the majority of these bipolar microtubule arrays are not functional, clearly indicating that γ-tubulin is required for the normal function of the mitotic spindle.

The immunostaining of abnormal mitotic apparatus of γ-*tub23C*^{PI} mutant cells shows that the poles of either monopolar or bipolar spindles are mostly abnormal. They appear to be very broad and, in many cases, highly irregular in shape. In order to determine the structure of the MTOCs, these mutant cells were stained to visualize the centrosomal associated antigen BX63. The results indicate that there are cells with no MTOCs or one or more MTOCs. Amongst the cells which have MTOCs, it became clear that a significant proportion has a very abnormal structure. There are cells with very large and

diffused BX63 staining, as well as MTOCs which display a ribbon-like elongated structure. The abnormal distribution of the BX63 antigen does not appear to follow a specific pattern since individual cells might have one almost normal MTOC while the other is completely abnormal. These results show that γ-tubulin is required for the structure of the normal MTOC which in *Drosophila* is known to contain a complex of at least three proteins including BX63, DMAP60 and γ-tubulin (Raff *et al.*, 1993). Recent reports (Felix *et al.*, 1994; Sterns and Kirschner, 1994) have shown that γ-tubulin is required for the formation of mature centrosomes in a cell-free system and they have proposed that γ-tubulin is an integral component of the MTOC. Our observations provide strong support for this model and suggest, furthermore, that γ-tubulin might have a structural role within the MTOC. Taken together, the analysis of the γ-*tub23C*^{PI} mutant shows that the function of this gene is essential not only for normal microtubule nucleation and function of the mitotic spindle but also for the structure of the MTOC.

Materials and methods

Strains, culture conditions and cytological preparations

The P-element mutant lines were obtained from Bloomington *Drosophila* Stock Center (Indiana) and their construction has been previously described (Cooley *et al.*, 1988). The γ-*tub23C*^{PI}/Cy,O stock was later balanced over T(3;2)Cy,O Ch Tb/TM3, *Sb e*, so as to identify homozygote larvae carrying the second chromosome insertion element. All stocks were grown at 25°C under standard conditions and media. Cytological preparations were made from third instar larvae as described previously (Gonzalez and Glover, 1993).

Molecular cloning of pRG1.1, DNA sequencing and Western blotting

pRG1.1 was initially cloned by plasmid rescue essentially as previously described (Gatti and Goldberg, 1991). Two identical clones pRG1.1 and pRG1.2 were obtained and mapped to polytene chromosomes by *in situ* hybridization. Subsequently, the insertion site of γ-*tub23C*^{PI}/Cy,O was also isolated using inverse PCR. Genomic DNA from the stock was isolated, digested and re-ligated as described above. 100 ng of the re-ligated DNA were subjected to PCR amplification essentially as described elsewhere (Ochman *et al.*, 1988; Gatti and Goldberg, 1991). The primers used for amplification were the 16mer reverse primer from pUC8 (5′-AACAGCTATGACCATG-3′) and PI which hybridizes to one of the P-element repeats (5′-CTGGTGGAATACAATAAAGT-3′). A single band of 2480 bp was obtained and mapped to polytene chromosomes by *in situ* hybridization as previously described (Pardue, 1986). pRG1.1 was sequenced using the PI oligo and the Sequenase kit (USB, Biochemicals) according to the manufacturer's instructions. The sequence we obtained was compared with EMBL and GenBank using the PCGENE software (Intelligenetics, USA). The P-element probe was isolated from pπ25.1 (O'Hare and Rubin, 1983).

Screening of genomic and cDNA libraries. A wild type genomic partial *Sau*3AI *Drosophila* library cloned into λDash (C.Gonzalez, personal communication) was screened using the genomic fragment isolated from pRG1.1 labelled with digoxigenin (Boehringer Mannheim, Germany). Four positives clones were isolated. One of the clones λDG14 spans over 15 kb including the whole γ-*tub23C* locus. The genomic fragment cloned in pRG1.1 was also used to screen a testis cDNA *Drosophila* library constructed into the λZAPII vector (Stratagene, USA). Three positive clones (λZe1, λZe2, λZe15) were isolated. The MAL–γ-tub23C fusion protein was obtained by inserting λZe2 which as a deletion for the first 19 N-terminal amino acids in-frame into the *Stu*I site of the pMALc vector series (New England Biolabs, USA).

Western blotting. Total brain protein extracts from either γ-*tub23C*^{PI} homozygotes or Oregon-R wild types were prepared as follows. The brains were individually dissected in saline (0.7% NaCl) and placed in 25 μl of cold (4°C) SDS–PAGE running buffer, boiled for 3 min and centrifuged for 1 min at top speed to clear the lysate. The total amount of protein in individual samples was quantified according to the method of Bradford (Stratagene, USA). Prior to immunoblotting, samples were run in parallel and stained with Coomassie blue to assess total protein content and the quality of the preparation. Total protein per lane was 50 μg. The primary antibodies used were: anti-γ-tubulin polyclonal sera (Joshi *et al.*, 1992) at 1:300 dilution, anti-β-tubulin according to the manufacturer's instructions (Amersham, UK) and RB188 (Whitfield *et al.*, 1988) which recognizes the *Drosophila* centrosomal antigen BX63 at 1:500 dilution. The immunopurified anti-γ-tubulin antibody was isolated from nitrocellulose blots containing the pMAL–γ-tubulin fusion protein according to published protocols (Sambrook *et al.*, 1989). Appropriate peroxidase linked second antibodies were from Amersham and used according to the manufacturer's instructions. All immunoblots were incubated for 1.5 h with end-to-end mixing. Detection of antibody signal was carried out using the ECL chemiluminescent detection system (Amersham, UK)

Immunofluorescence

Whole third instar larval brains or early embryos were prepared for immunostaining as previously described (Gonzalez and Glover, 1993). Either Hoechst or propidium iodide were used to visualize DNA. Anti-γ-tubulin antibodies (Joshi *et al.*, 1992) and the BX63 monoclonal antibody (Frash *et al.*, 1986) were used to co-localize the two proteins. All other immunostainings of the BX63 antigen were done using the RB188 polyclonal antibody (Whitfield *et al.*, 1988). Second antibodies used were FITC or Texas Red conjugated anti-rabbit or mouse IgG (Vector Laboratories, UK) and observed with a Bio-Rad MRC600 confocal microscope. FITC and Texas Red images were obtained simultaneously. An average of five consecutive optical planes were obtained and then added up to compose the final figure. Merging of the images was done using the COMOS software (Bio-Rad).

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