ELSEVIER

# Proliferative Genes Induce Somatic Pairing Defects in *Drosophila melanogaster* and Allow Replication

**Isolde Riede**

**ABSTRACT:** Drosophila *tumor forming lines (malignant brain tumor, lethal giant larvae, discs large, brain tumor, and tumor suppressor gene) exhibit incomplete somatic pairing of specific regions in the salivary gland chromosomes, indicating that excessive cell proliferation correlates with somatic pairing defects in* Drosophila. *Alleles of malignant brain tumor enhancing the frequency of cell divisions exhibit melanizing tumors in the larvae. The giant chromosomes are defective in somatic pairing, indicating that a functional component of the chromosomes is influenced. Genes at different sites are affected, but the similarity of the phenotypes and complex complementation pattern reveals that their functions are interrelated. In the brain of malignant brain tumor recombinants and mutants in proliferative genes, polytene cells appear; wildtype does not amplify DNA in brain tissue cells. Thus, mutant proliferative genes induce the S-phase and allow replication of DNA.* © *Elsevier Science Inc., 1997*

## INTRODUCTION

Oncogenes are instrumental in developmental processes such as cell communication, signal transduction, and regulation of gene expression [1]. No single oncogene mutation has been found to be capable of inducing a tumor. Mutation or loss of both copies of tumor suppressor genes is associated with a number of human cancers. Their protein products keep in check an otherwise uncontrollable ability of cells to proliferate. Both oncogenes and tumor suppressor genes appear to modulate the G1 phase and restrict or promote cell cycle progression at a point in the late G1 phase. Two wide-ranging tumor suppressor proteins, P53 and Retinoblastoma, are nuclear phosphoproteins. The *retinoblastoma* gene, which is responsible for the hereditary predisposition to retinoblastoma, is ubiquitously expressed in most normal cells of vertebrates. *p53* is, by far, the most frequently mutated gene in human cancers [2, 3].

At the present time, more than forty different *Drosophila melanogaster* lines inducing tumors are known. Apparently recessive mutations are related to tumorous growth of a number of cells and tissues. Three lines have been isolated that can cause malignant transformation of the neuroblasts in the larval brain: lethal giant larvae (LGL) [4, 5], lethal brain tumor (BRAT), and lethal malignant brain tumor (MBT) [6]. Nonmalignant overgrowth of the brain has been observed in lines discs large (DLG) [7, 8] and FAT [9]. dlg has been mapped to the first, *lgl*, *fat*, and *brat* to the second, and *mbt* to the third chromosome (for review see [10]). None of the identified *Drosophila* genes belongs to one of the families of oncogenes or tumor suppressor genes already known.

As in humans, brain tumor formation in MBT is a polygenic event [11]. Genetic analysis of MBT has revealed three classes of genes being involved in tumor formation. (1) Oncogenes have been identified within the alleles of the dorso-ventral pathway. *Toll* encodes a transmembrane protein, homologous to the human interleukin-1 receptor [12], *pelle* encodes a protein kinase [13]. Alleles of *Toll* or *pelle* act as oncogenes over MBT. (2) As tumor suppressor gene of MBT, a *tolloid* (*tld*) allele has been identified. Tld is homologous to human bone morphogenetic protein 1, interacting with members of the TGFβ superfamily. This family of extracellular factors can stimulate or inhibit cell growth or differentiation, depending on the cell type involved [14]. Neither the oncogenes nor the tumor suppressor gene *tld* alone induce cell overgrowth, breaking the restriction of the cell cycle. (3) Hyperplasic growth of cells is coupled to mutations in a different class of genes, the proliferative genes.

Diptera have an intimate synapsis of homologous chromosomes from early prophase to metaphase, called somatic pairing (for review see [15]). In the salivary gland cells of *Drosophila*, homologous chromosomes are usually completely paired. Thus, only a haploid set of polytene chromosomes appears. The pairing process is initiated during the S-phase [16]. Pairing defects are known from hybrids of sibling species. There, the homology of the DNA in a chromosome band is responsible for the frequency of somatic pairing [17].

*From the BL-Institut für Neurobiologie, Magdeburg, Germany.*
*Address reprint requests to: Dr. Isolde Riede, Pappelweg D-55291 Saulheim, Germany.*
*Received July 1, 1996; accepted October 17, 1996.*

Cancer Genet Cytogenet 97:143–154 (1997)

655 Avenue of the Americas, New York, NY 10010

0165-4608/97/$17.00
PII S0165-4608(96)00396-2

Here it is shown that all of the tumor inducing lines analyzed, reveal one common aspect: homologous polytene chromosomes are not completely paired in specific regions. A number of EMS induced alleles of tumor forming strain MBT are presented. The new class of genes disrupts the restriction of the cell cycle: tumorous masses melanize in third instar larvae. The giant chromosomes reveal incomplete somatic pairing.

All of the cells in the mutant animals carry the mutation, but not all cells are tumor cells. The development of viable larvae indicates that most cells gain a restriction point before the proliferative event is induced. Cells other than neuroblasts are restricted earlier in the developmental process. Most cells of *Drosophila* divide extensively during embryogenesis and rest without further mitosis in larval stages. Growth of larvae is because of cell growth, not cell division. Neuronal cells divide during larval stages, and thus remain proliferation potent after embryogenesis [18, 19]. In wild type *Drosophila*, only diploid neurons occur. Several glia cells show endoreplication. Polytene chromosomes are absent in brain tissue [20, 21, 22]. Proliferative mutations could induce the S-phase in proliferation competent cells. In cell cycle restricted cells this feature should be visible: polyploidization or polytenization is expected. Therefore, cells were screened for chromosome abnormalities. Here it is shown, that in MBT recombinants and in mutants of proliferative genes polytene cells appear in brain tissue. Thus, replication is allowed in differentiated cells by proliferative mutations.

## MATERIALS AND METHODS

### ARD Stain

The nonligand binding structural subunit of nicotinic acetylcholine receptor is expressed in neuropiles of the optic lobes. The expression of this subunit is a marker for appropriate differentiation of the brain tissue. ARD-like immunoreactivity in larval brains was obtained with antibody Da2-E6 [23].

### Defining Brain Tumor Formation

Parents were placed on fresh medium and shifted immediately to the restrictive temperature. The F1 generation was analyzed. Same size L3 larvae were dissected in Ringer's solution. Several different qualities of the brain can be determined: enlargement of the brain without affecting the differentiation pattern is called hyperplasia. In the case of hyperplasic brains, the cells are differentiated, i.e. small neurons are detectable by light microscopy in fresh unstained squashed preparations of brain tissue. ARD stains the neuropiles in wild type pattern. A tumor shows hyperplasia of the brain, resulting in undifferentiated tissue. No substructures are visible in MBT brains, ARD does not stain neuropiles. The cells of fresh prepared tumor tissue do not adhere. Several cell are large, neurons cannot be found in tumor tissue. Malignancy can be defined as ingrowth of the tissue into the ventral ganglion. Unstructured, malignant brain tissue is called a tumor.

### Strains

MBT, DLG-1, LGL-1, LGL-M32, FAT-GD, BRAT, and *ru st e ca* were obtained from E. Gateff, Mainz. MBT is temperature sensitive (ts) and forms, at the restrictive temperature of 29°C, malignant cells in the larval brain [6]. Recombinants of the third chromosome of MBT exhibit different phenotypes: *tld-1* carries two mutant genes (*ru st e* $tld^{MBT}$ and $yeti^{MBT}$) and induces brain hyperplasia. In *tld-2*, a malignant brain tumor is formed because of three mutant genes (*ru st e* $tld^{MBT}$, $yeti^{MBT}$, and $spätzle(spz)^{MBT}$) [11]. OregonR originates from O. Vef, Mainz. Deficiencies were obtained from the Bloomington stock center. *st e tx* was a gift from A. Preiss, Hohenheim; *st tx* was obtained by recombination with OregonR. *ri* containing flies originate from U. Thomas, Magdeburg. Chromosomes were separated in crosses with marker/balancer strains *w*/*w*;Pm/SM5;TM3/TM6B or FM7 or *w*/*w*;SM1/*Sco*;MKRS/TM2 and combined with other chromosomes.

The flies were kept and tested on a special medium (Agar 8 g/L, raisins 40 g/L, yeast 60 g/L, semolina 40 g/L, honey 30 g/L, and nipagin 0.1%) at 20°C. Markers and chromosomes have been described [24].

### Mutagenesis

The line used for mutagenesis does not induce melanomes or somatic pairing defects. *e* marked OregonR males were mutagenized with EMS as described [25]. F0 animals (* *e*/* *e*) were crossed to double balancer strain TM3/TM6B. Single F1 mutagenized TM3/* *e* animals were backcrossed to TM3/TM6B and established as * *e*/TM3 or * *e*/TM6B stocks by the elimination of one balancer chromosome.

Males of the stocks were subsequently crossed to MBT virgins at 29°C. The crosses were screened for lethal MBT/* *e* hybrids. Noncomplementation indicated an allele of MBT. From mutagenesis, screening 3,000 stocks for defects on the third chromosome, 21 mutants were obtained, providing lethality to MBT/mutant heterozygotes originating from MBT virgins at the restrictive temperature. Some of the mutants provide only incomplete temperature sensitivity to hybrids and were eliminated in the first approach, because the primary defect causing tumor formation was expected to be a lethal one. One lethal allele per 300 animals mutagenized could be obtained.

Df(3R)mbtP [6] does not complement MBT, hybrids die as pupae. Genetics has revealed, that this deficiency does not comprise the tumor suppressor locus that is responsible for 100% temperature sensitive lethality. Recombinants of MBT with this deficiency have been obtained, that eclose as adults at the restrictive temperature. Heterozygotes of MBT/Df(3R)mbtP die as pupae, whereas MBT is lethal as third instar larvae. Larvae of MBT/Df(3R)mbtP hybrids frequently show hyperplasic, but never tumorous growth of the brain tissue. Therefore, an interactive locus, called *efendi(efe)*, is hidden. To define the function of *efe*, mutants were selected according to the following scheme: EMS-treated OregonR *e* males were crossed to TM3/TM6B virgins. F1 animals balanced over TM3 were crossed to Df(3R)mbtP/TM3. The occurrence of only TM3 balanced

flies indicated noncomplementation. The *e* marked animals were crossed inter se and stocks were established.

Mutagenesis over Df(3R)mbtP revealed one lethal allele per 500 mutagenized animals. Two complementation groups were discovered. One of them showed no interactive potential with MBT and is therefore not considered here.

### Mapping of the Alleles

The phenotype, i.e. melanome formation and lethality was mapped based on 100 single animals for each combination. *mali*$^{MN}$ *e* was mapped with *ru st e ca* or *st tx, hexe*$^{77}$ *e* with *ru st e ca* or *ri e, Aus*$^{9}$ *e* with *ru st e ca, st tx* or *st e tx, merlin(mer)*$^{14}$ *e* with *ru st e ca, st tx* or *ri e, amanda(ada)*$^{19}$ *e* with *ru st e ca* or *ri e, teufel(teu)*$^{98}$ *e* with *ri e, efe*$^{89}$ *e* with *st tx, efe*$^{11}$ *e* with *ri e, efe*$^{23}$ *e* with *ri e, stern(srn)*$^{88}$ *e* with *st tx* or *ri e*, and *efe*$^{79}$ *e* with *ri e. drache(dra)*$^{47}$ *e* was not mapped.

### Recombination Frequency

The used markers *ri, st*, and *e* have been identified at 46.8 cM, 44.0 cM, and 70.7 cM, respectively. Therefore about 27 recombinants between *st* and *e* and about 24 recombinants between *e* and *ri* were expected per 100 animals tested. By several alleles of the proliferative genes, the frequency of recombination is enhanced, resulting in more recombination events than expected.

### Preparation of Salivary Gland Polytene Chromosomes

Salivary glands were prepared in 45% acetic acid from stage 3 larvae grown at 18°C unless otherwise stated. The tissue was fixed in 1 N HCl for one minute and washed with 50% lactic acid/30% acetic acid. The salivary glands were stained with 2% orceine/30% lactic acid/30% acetic acid for 10 minutes, rinsed in 45% acetic acid, and squashed.

## RESULTS

### Polytene Chromosomes of MBT Show Abnormalities

The salivary gland polytene chromosomes of *Drosophila* usually appear as a haploid set because of somatic pairing of the homologous partners. The second and third chromosomes of homozygote MBT grown at the permissive temperature specifically show pairing abnormalities. Nearly every nucleus exhibits chromosomes with unpaired regions. The banding patterns are always running in parallel. Therefore, homologous bands in the chromosomes are in the same physical order. No unusual chromosome rearrangement or deficiency, which could break the pairing process, is detectable. The unpaired regions do not show unusual irregularities in the bands and interbands. No differences in the degree of polytenization is detectable.

Pairing defects occur in animals grown at the permissive or restrictive temperatures. They most frequently affect the telomeric end of the right arm of the second chromosome; completely unpaired chromosome arms are detectable as well. They originate from regions 50 and 51 in 70% of the nuclei, region 25 is unpaired in 20% of the nuclei, and region 70 is concerned in 10% of the nuclei in animals grown at 18°C. Incomplete pairing is not observed. Whereas in hybrids from sibling species neighboring bands can be alternately paired and unpaired, MBT always shows whole regions as being unpaired. MBT/OregonR hybrid polytene chromosomes were investigated (Table 1, Fig. 1A–C). The pairing defect occurs as a dominant trait. Obviously pairing defects of the chromosomes are because of gain of function in MBT, because the MBT/OregonR hybrids show unpaired chromosomes in the same frequency and at the same sites (Fig. 1A, B) as does homozygote MBT. An additional abnormality on the right arm of the third chromosome is detectable, suggesting a chromosome rearrangement: In(3R)mali86C;87C (Fig. 1C).

### Somatic Pairing in FAT-GD, DLG-1, BRAT, LGL-M32, and LGL-1

*Drosophila* tumor-inducing lines were analyzed with respect to the pairing behavior of their polytene chromosomes. OregonR was crossed over the tumor lines. All of the F1 hybrids, that do not carry balancer chromosomes, i.e. OregonR/tumor line, show somatic pairing defects (Figure 1E–I, Table 1).

### Interrelation of the Defects of Tumor-Inducing Lines

The tumor-inducing lines were crossed over MBT. If the genes hidden would be independent, full complementation of the defects should occur, because all of the components are recessive. Table 1 shows, that none of the hybrids exhibits the expected complementation of the defects, even at the permissive temperature.

### Alleles of MBT

Genetic analysis of MBT has revealed six cooperating mutant loci, contributing to tumor formation and lethality [11]. To uncover single defects of this multigenic event, mutagenesis was performed. Seven alleles exhibiting 100% lethality as heterozygotes over MBT were chosen. MBT was crossed over the alleles at the restrictive temperature. The hybrids were screened for brain tumor induction. All of the alleles induce hyperplasia or tumor formation over MBT, i.e. more cell cycles than programmed (Table 2). This indicates, that proliferative alleles were obtained. Except allele 47, all alleles reveal 100% lethality as homozygotes at the permissive temperature, indicating temperature independence of the defects.

All new identified alleles were crossed to the tumor forming lines LGL-1, LGL-M32, BRAT, FAT-GD, and DLG-1. One of the alleles, 9, does not completely complement FAT-GD, BRAT, and DLG-1 (Table 2). Over MBT, embryonic lethality occurs. Because this allele has concerned a rather ubiquitous and lethal function over several tumor forming strains, its gene is called *Aus*. Mapping the MBT alleles revealed a hot spot at 56 cM (Table 2); however, other locations are possible as well. The chromosome inversion of MBT In(3R)mali;86C;87B was hidden by one mutation; *mali*$^{MN}$. Figure 2 shows a graph of the region 86/87. Whereas the defect of the allele MN clearly localizes to

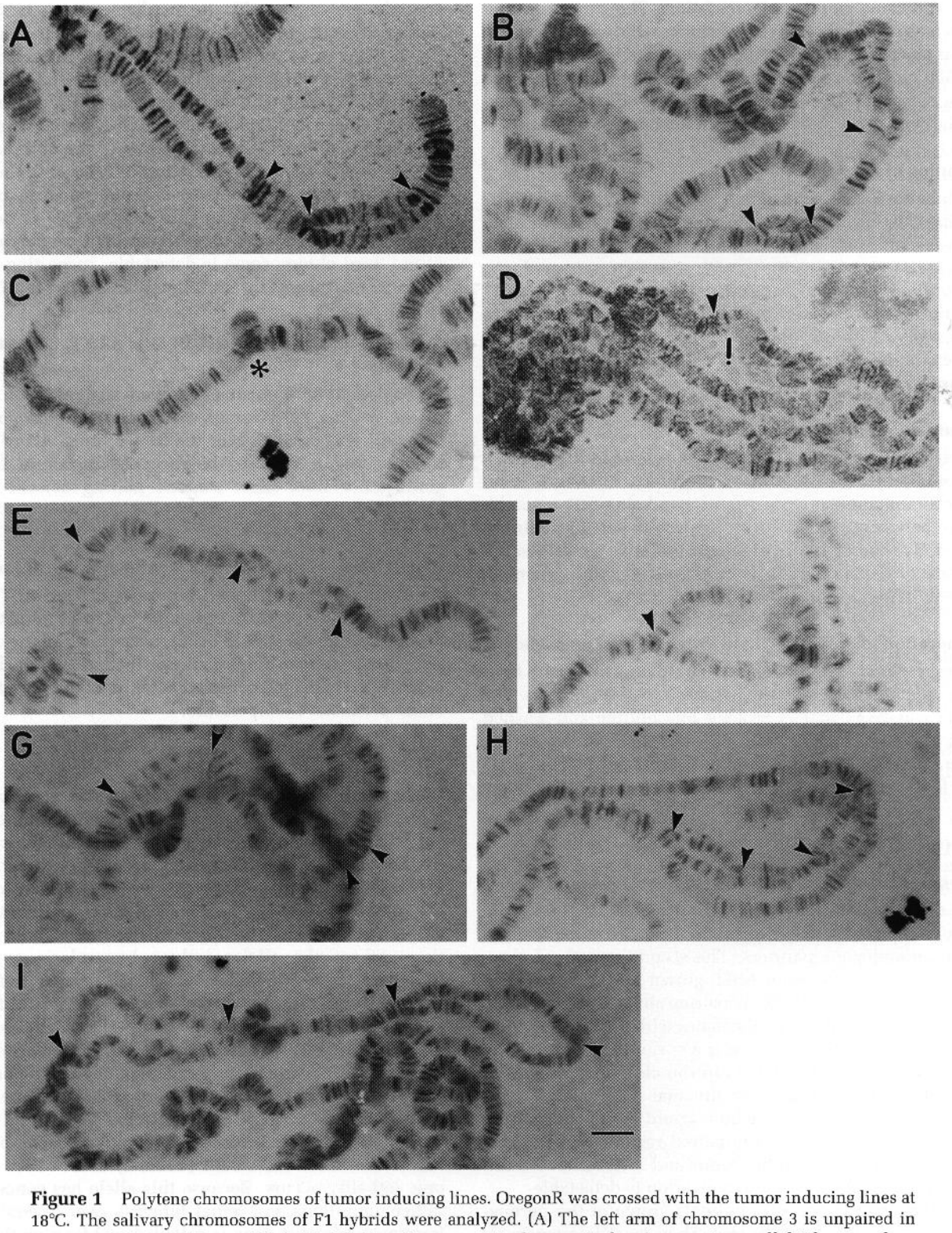


**Figure 1** Polytene chromosomes of tumor inducing lines. OregonR was crossed with the tumor inducing lines at 18°C. The salivary chromosomes of F1 hybrids were analyzed. (A) The left arm of chromosome 3 is unpaired in some nuclei in MBT/OregonR hybrids. The banding pattern in the unpaired region runs in parallel. Thus, no chromosome rearrangement or deficiency is responsible for the pairing defect. (B) The right arm of the second chromosome in MBT/OregonR hybrids shows somatic pairing defects. Here, regions 47 and 50 to 55 are unpaired. Incomplete pairing is not detected. (C) The apparent inversion (asterisk) on the right arm of the third chromosome is involved in tumor etiology, In(3R)mali86C;87C. (D) $mali^{MN}$ is the proliferative allele mapped to the inversion. The polytene chromosomes of homozygous $mali^{MN}$ always show pairing defects. In addition a phenomenon of banding abnormality is detectable: One homologue of the unpaired chromosome in region is not banded (exclamation mark) in about 10% of the unpaired regions. (E) OregonR/FAT-GD, (F) OregonR/DLG-1, (G) OregonR/BRAT,

**Table 1** Somatic pairing defect in tumor inducing lines

| | | Interrelation with MBT | | |
|---|---|---|---|---|
| | % of nuclei[a] | 18°C[b] | 29°C[b] | 18°C[c] |
| MBT | 90 (2R) | + | P/tumor | + |
| LGL-1 | 90 (2R) | P | P/tumor | E |
| LGL-M32 | 10 (nd) | P | P | E |
| DLG-1 | 90 (2R) | + | (P) | nd |
| FAT-GD | 80 (3L) | (P) | (P) | + |
| BRAT | 30 (nd) | (P) | (P) | E |
| Wildtype | 0 | + | (P) | nd |

[a]The number indicates the frequency of salivary gland nuclei exhibiting partially unpaired chromosomes. At least 100 nuclei of at least three different hybrids were analyzed. OregonR virgins were crossed with males of the tumor-inducing lines. F1 animals were analyzed. Thus, the dominant somatic pairing defect is indicated. Remarkably the first chromosome of DLG1 is not ultimately necessary for the pairing defect, because DLG1 males transmit the first chromosome as a balancer, and not the *dlg1* containing first chromosome! Genetic analysis revealed, that, as in MBT, loci on the second chromosome influence the brain phenotype [Riede unpublished results]. The chromosome arm most frequently unpaired is indicated in brackets.

[b]Virgins of the tumor inducing lines (for example LGL-1/LGL-1;LGL-2/SM5;LGL-3/LGL-3) were crossed with MBT males at the permissive (18°C) or restrictive (29°C) temperature. F1 animals containing heterozygous chromosomes, for example LGL-1/MBT-1;LGL-2/MBT-2;LGL-3/MBT-3 were analyzed. If the mutations in the lines would be independent, at the permissive and at the restrictive temperature, full complementation should occur.
+: no defects, full complementation. P: pupal lethality of the hybrids.
(P): pupal lethality is incomplete, 10% of the pupae eclose.
/tumor: brain tumor is induced in hybrids; Animals with the balancer chromosomes, for example LGL1-1/MBT-1;SM5/MBT-2; LGL1-3/MBT-3 eclose as adults.

[c]Double mutants containing homozygous chromosomes were analyzed. To obtain double mutant lines containing the third chromosome of MBT at homozygosity, and the second chromosome from LGL, for example, in two independent crosses, MBT and LGL males were crossed with *w/w*;Pm/SM5;TM3/TM6B virgins. One F1 *w*/LGL-1;Pm/LGL-2;TM6B/LGL-3 virgin was crossed with one F1 *w*/;SM5/MBT-2;TM3/MBT-3 male. F2 *w/w*;LGL-2/SM5;TM6B/MBT-3 animals are crossed inter se. *w/w*;+/SM5;MBT-3/MBT-3 or *w/w*;LGL-2/SM5;+/TM6B animals are viable. Thus, if the mutations on the second chromosome of LGL were independent of the mutations of the third chromosome of MBT, animals of the type *w/w*;LGL-2/SM5;MBT-3/MBT-3 should be viable as adults. Instead they die as embryos (E) already at the permissive temperature. Only *w/w*;LGL-2/SM5;MBT-3/TM6B adults eclose.
+: no defects;
E: embryonic lethality;
nd: not determined

87B, MBT is affected by deficiencies covering 87B and 87C. Neither allele could be identified at 92 cM, all alleles obtained complement Df(3R)mbtP. This indicates that this deficiency at 92 cM comprises an interactive locus, called *efe*.

### Alleles of *efe*

Df(3R)mbtP has been published to comprise the tumor suppressor gene of MBT [6]. To gain access to its role in interrelation, mutagenesis was performed. Five alleles of the complementation group, interacting with MBT, were chosen (Table 2). Allele 88 is lethal over all other *efe* alleles (Table 3), but could not be mapped to its position. The hidden gene is called *srn*. This indicates, that *efe* represents an interactive locus. The strong allele 88 is not localized at the site of the deficiency, but distant at a different locus. Allele 88 is interactive with at least one function comprised by Df(3R)mbtP in a quantitative manner, because the deficiency only provides one gene dosis instead of two. Thus the genes interact quantitatively.

### Complementation Pattern

Table 3 presents a complementation profile of the proliferative alleles at the permissive temperature. Most of the alleles are able to complement most other defects. One hundred percent lethality of the hybrids is rare but growth disadvantage is frequent. Several lethal combinations occur as maternal effects. Three alleles have developed a more lethal potential than the others: $Aus^{9}$, $mer^{14}$ and $srn^{88}$. All of them are localized to 56 cM on the third chromosome.

### Phenotype of Proliferative Alleles

Homozygotes of proliferative alleles reveal a striking lethal and nontemperature sensitive phenotype: proliferative cells melanize at the end of larval stage 3 and occur as melanotic tumor masses. The melanizing tumors can affect different tissues, each animal shows its individual pattern. (Table 2, examples in Fig. 3). The tumors can be small, like in $efe^{23}$. $efe^{89}$ reveals an allele with more potential to proliferate, tumor masses are large. The strong allele $Aus^{9}$, reveals dominance of melanotic tumor formation. The ability to form melanotic tumors is an indication of the proliferative potential of these gene defects. As all lethal MBT alleles reveal this phenotype, formation of melanotic tumor masses indicates involvement of this group of genes in disruption of the restriction of the cell cycle.

Lethal MBT alleles have been identified over MBT virgins. In opposite crosses, most of them revealed to have hidden maternal effects (Table 2), hybrid adults eclose. The three strong alleles, $Aus^{9}$, $mer^{14}$, and $srn^{88}$, do not exhibit maternal effects over MBT. Two of them are able to induce brain tumor formation over MBT. $Aus^{9}$ is embryonic lethal over MBT, the hybrid embryos are amorphous. No *efe* allele is lethal over MBT. However, in 20% of the larvae, hyperplasia formation of the brain can be observed.

(H) OregonR/LGL-M32, and (I) OregonR/LGL-1 hybrids show somatic pairing defects in their salivary gland chromosomes. Arrowheads show the beginning and end of the somatic pairing defect. The regions analyzed do not involve balancer chromosomes. These are frequently because of chromosome rearrangements unpaired. The balancer chromosome rearrangements would in addition be visible by differences in the banding pattern: unpaired regions would be inverted, the banding pattern would not run in parallel. In any of the cases, the chromosome bands are in the same physical order. Bar represents 10 μm.

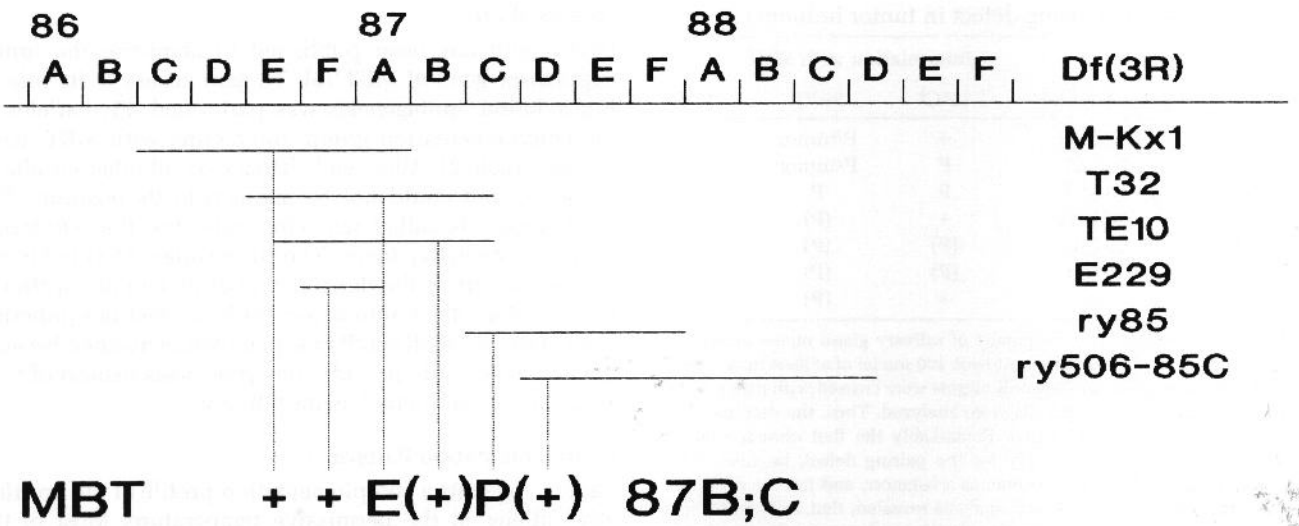


**Figure 2** Graph of regions 86–88. The deficiencies covering the region are indicated as lines. MBT or $mali^{MN}$ virgins were crossed to the deficiencies. The F1 generation was analyzed. The deficiencies M-Kx1, E229, ry85, and ry506-85C are complemented by $mali^{MN}$ (+). T32 and TE10 are not able to complement $mali^{MN}$, melanomes at the end of the larval life occur (M). Thus, the defect mapped to 55 cM lies between the breakpoints of M-Kx1 and ry85, i.e. in region 87B. MBT is fully complemented by the deficiencies M-Kx1 and E229, partial complementation occurs with TE10 and ry506-85C ((+)), several hybrids die as pupae, some adults eclose. MBT is embryonic lethal over T32 (E) and shows pupal lethality over ry85 (P). Thus, whereas the position and the phenotype of the EMS induced allele $mali^{MN}$ is clear, the situation in MBT is more complex, possibly because of interfering loci.

All mutants frequently reveal unpaired regions of the salivary gland chromosomes (Table 2, Fig. 4). Shorter or longer regions are unpaired, depending on the allele analyzed.

The phenotype of MBT is characterized by temperature sensitive formation of malignant neuroblasts in the larval brain. None of the proliferative alleles presented here, is able to induce, as a single causal component, malignant transformation of the brain tissue. No brain tumor is detectable in homozygous mutants. However, the brain of several mutants does not show the wildtype differentiation pattern of the neuroblasts (Fig. 5). ARD is a structural component of the nicotinic acetylcholine receptor in *Drosophila* and only expressed in differentiated neuropiles. In MBT, transformed neuroblasts in the brain do not express ARD, thus are not differentiated. Several proliferative alleles induce unusual overexpression of ARD in the whole brain (Table 2, Fig. 5).

### Polytene Cells in Brain Tissue of MBT Recombinants and Proliferative Mutants

Recombinants of MBT, *tld-2*, and *tld-1*, form hyperplasic or malignant tumor tissue in the larval brain. By analyzing the brain tissue in *tld-1* and *tld-2* grown at the restrictive temperatures, the enlarged brains of both strains exhibit cells, that do not adhere. These cells are heterogenous in size and appearance: some are small, some large, the nuclei appear smaller or larger (Fig. 6a). Within this hyperplastic tissue, specific cells appear: giant cells with significantly enlarged nuclei (Fig. 6b). These giant cells are not rounded like the hyperplasic cells. The giant nuclei carry polytene chromosomes (Fig. 6c). In wildtype brains, no rounded or polytene cells were observed.

Three mutant genes cooperatively induce brain tumor formation in *tld-2*. Two of them, *spz* and *tld*, encode differentiation factors, *yeti* is a proliferative gene. Thus, the polytenization of several brain cells could be because of the action of the mutant differentiation factors, or because of the defect induced by the proliferative gene. To distinguish these possibilities, the brains of $efe^{89}$, $hexe^{77}$, $mali^{MN}$, and $ada^{19}$ were screened for polytene chromosomes. In all of them polytene cells occur. Only a few nuclei exhibit giant chromosomes with a polytenization degree over 250. In about 20% of the nuclei, amplification to 50 or 100 copies of DNA occurs (Fig. 6d). More than 60% of the cells in the brain hemispheres are not polytene. About 30% of them appear in pro- or metaphase. Because the brains are not hyperplasic, this unusual high frequency of cells exhibiting pro- and metaphases does not correlate with the frequency of cell divisions.

## DISCUSSION

The *Drosophila* tumor-forming mutant lines MBT, LGL-1, LGL-M32, DLG-1, FAT-GD, BRAT, and all mutations in proliferative genes exhibit somatic pairing defects. Thus,

**Table 2** Alleles of MBT and *efe*

| | A Position | B Recombination | C Somatic Pairing | D Frequency | E Melanome Formation | F Adhesion Defect | G ARD Stain | H MBT | I Ubiquity |
|---|---|---|---|---|---|---|---|---|---|
| $Aus^{9}$ | 56cM | 1,5 | ++ | 30 | +++* | +* | | L − | +++ |
| $mali^{MN}$ | 87B | 2,0 | + | 50 | +++ | + | A | LHM | + |
| $mer^{14}$ | 56cM | 1,5 | ++ | 80 | ++ | | + | LT− | − |
| $ada^{19}$ | 88cM | 1,3 | + | 80 | ++ | | | LHM | + |
| $dra^{47}$ | n.d. | 1,0 | + | 30 | + | | | LHM | − |
| $hexe^{77}$ | 56cM | 2,0 | ++ | 30 | ++ | | + | LHM | − |
| $teu^{98}$ | 24cM | 1,5 | ++ | 70 | ++ | | | LHM | − |
| $srn^{88}$ | 56cM | 1,5 | ++ | 50 | + | +* | (+) | LT− | + |
| $efe^{11}$ | 97F | 1,0 | ++ | 20 | + | +* | | −−M | − |
| $efe^{23}$ | 97F | 1,0 | + | 50 | + | | | −HM | − |
| $efe^{79}$ | 97F | 1,2 | + | 30 | + | | A | −HM | − |
| $efe^{89}$ | 97F | 1,5 | + | 40 | +++ | + | A | −HM | + |

A: Position: The genes were mapped at 25°C with different markers. In cases, where the gene could undoubtedly be identified on deficiencies, the according cytogenetic localization is given. *efe* (92cM, 97F) does not complement Df(3R)mbtP and Df(3R)T1-P, hybrids die as pupae.

B: Frequency of recombination: By recombining the alleles with markers, a high frequency of recombination was observed. Given is the multiplicator of the expected recombination rate based on at least 100 animals in each case.

C: Somatic Pairing defect: +: indicates shorter regions usually being unpaired in OregonR/allele heterozygotes. ++: indicates longer unpaired regions or unpaired half chromosomes.

D: Frequency of pairing defect: the percentage of nuclei revealing pairing defects in OregonR/allele hybrid larvae is given. One hundred nuclei of three different animals were considered.

E: Potential to proliferate: Melanizing tumors in 100% of third instar larvae are characteristic for the mutants. +: small and few melanomes, +++: many small and large melanotic tumors (Fig. 4).

F: Cell adhesion defect: Salivary glands fall into single cells in acidic medium. This phenotype reflects a defect in cell contacts.

G: Brain type of homozygous third instar larvae. A: overexpression of ARD; +: wild type expression of ARD (Fig. 5).

H: Phenotype of MBT/allele hybrids at the restrictive temperature.

L: 100% lethality. Hybrids of strain MBT over several alleles and $srn^{88}$ die as L3 larvae, hybrids of MBT over *efe* alleles exhibit partial pupal lethality. MBT/$Aus^{9}$ animals are embryonic lethal.

H/T: Hyperplasia/Tumor formation in hybrids

M: Indicates a maternal effect: MBT virgins crossed with the alleles result in lethal hybrids; reciprocally crossed hybrids are viable. All *efe* alleles are partial lethal over MBT. This partial lethality occurs as maternal effect.

I: Ubiquity of effects: Alleles were crossed with LGL-1, LGL-M32, FAT-GD, BRAT and DLG-1 at the permissive temperature.

−: no interrelative potential was observed,
+: indicates, that lethality of hybrids occurs in one case,
+++: three combinations are lethal.
* Dominance

**Table 3** Complementation scheme of proliferative alleles

| | 9 | MN | 47 | 98 | 14 | 19 | 77 | 88 | 89 | 79 | 11 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | E | + | + | (+) | (−) | + | + | − | + | + | (+) | + |
| MN | + | L3 | (+) | + | (−) | (+) | + | (+) | + | (−) | + | + |
| 47 | − | + | + | + | + | (+) | + | − | + | + | + | + |
| 98 | + | + | (+) | L3 | nd | + | + | (+) | nd | nd | nd | + |
| 14 | (−) | (+) | + | + | L3 | (+) | + | (+) | + | (+) | (−) | + |
| 19 | (+) | + | + | nd | (−) | L3 | + | (+) | (+) | + | + | + |
| 77 | (+) | + | + | nd | (−) | + | L3 | (+) | + | + | + | + |
| 88 | (−) | (+) | + | + | + | (−) | + | L1−P | − | − | − | − |
| 89 | + | (−) | + | + | + | + | + | − | L2−3 | (+) | + | (+) |
| 79 | + | + | (+) | + | (+) | (+) | + | − | (+) | L1−3 | (+) | − |
| 11 | + | (+) | (+) | + | + | + | + | − | + | (+) | P | + |
| 23 | (+) | + | + | (+) | + | + | + | − | (+) | − | + | P |

Alleles were crossed at the permissive temperature. Homozygous animals die as embryos (E), larvae in stages 1, 2, or 3 (L1-3) or pupae (P). Heterozygous animals survive as adults (+), frequently no full complementation is observed. 20 to 50% of the hybrids die ((+)), adult hybrids require a more than four days elongated generation time. No complementation occurs in rare cases (−), incomplete lethality indicates more than 80% hybrid lethality ((−)).

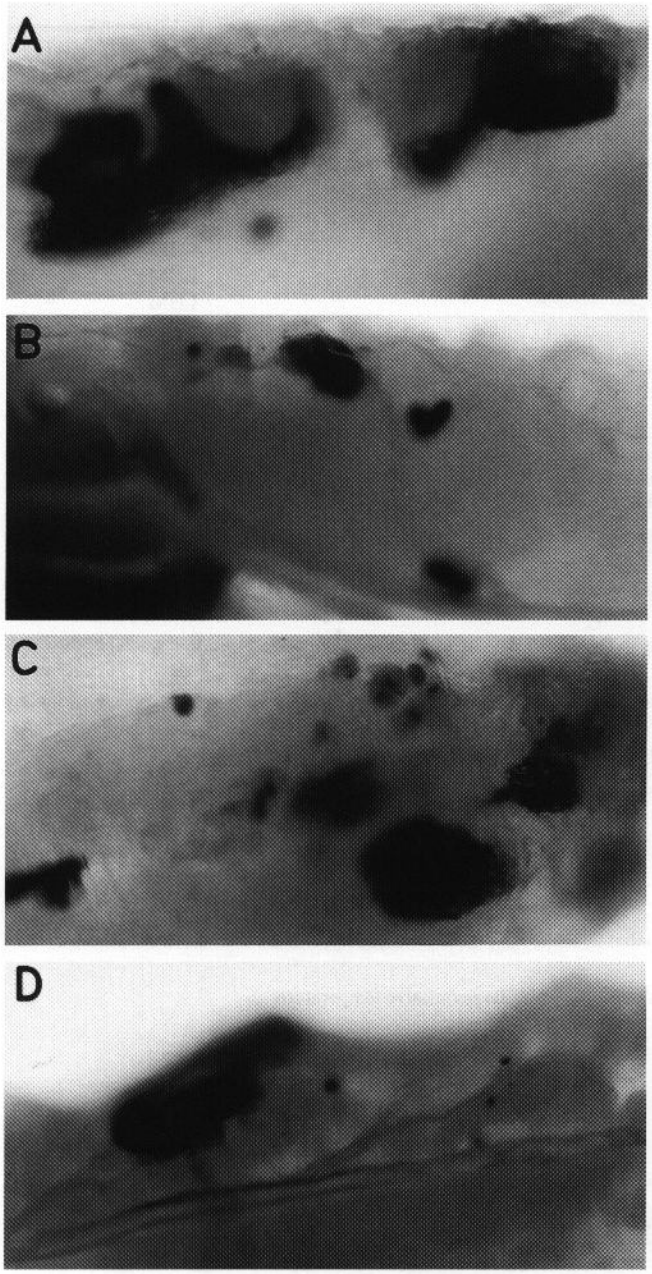


**Figure 3** Melanomes. The tumors that melanize in third instar larvae are shown at the same magnification. (A) The strong MBT allele, *Aus*$^{9}$, exhibits dominant formation of melanotic tumor masses. Here, the formation of melanomes in the abdomen of an *Aus*$^{9}$/TM6B *Tb* pupae is shown. (B) *efe*$^{89}$ shows a high proliferative potential as recessive function. Usually a few giant tumors are accompanied by several smaller ones. Part of a homozygous third instar larvae is shown. (C) Allele 77 usually shows recessive occurrence of a few small melanomes. Here a rare case (2%) of dominance in melanotic tumor formation is shown in a hexe$^{77}$/TM6B *Tb* hybrid larvae. (D) *efe*$^{23}$ exhibits low proliferative potential as homozygote. A rare case of a metastasing melanome in a third instar homozygous larvae is shown.

excessive cell proliferation in *Drosophila* is correlated with genes disrupting the pairing process. The somatic pairing defect could be a manifestation of a functional defect, a cell cycle defect, which is responsible for tumor induction. A functional information of a distinct part of the chromosomes is affected.

The somatic pairing defect in MBT is different from the pairing defect in hybrids of sibling species. In sibling species, DNA sequence heterology is because of the evolutionary separation of the species. This sequence heterology prevents the somatic pairing of chromosome bands [17]. MBT is kept as a homozygote line at 20°C. Neither the second nor the third chromosome are recombination deficient [11]. Thus, genetic exchange between the homologous chromosomes is possible. This genetic exchange can homogenize unusual DNA heterology. Therefore, the somatic pairing defect is not expected to originate from unusual DNA sequence heterology. Another aspect is affected. Usually the DNA in one band is packed by nucleosomes and histone H1. The DNA is unpacked during the replication process and repacked after the synthesis of DNA in every round, because chromosome bands are visible during the whole process of polytenization. Thus, the replication process is one probable event for the induction of the somatic pairing. Replicated homologous bands are repacked into one band instead of two. Heterologous sequences are packed in two homologous bands.

Partially homologous sequences are incompletely paired, with a visible gap in between one band [15]. Tumor inducing lines are expected to have homologous DNA in homologous bands. Therefore incomplete pairing is not observed. Either the bands pair, or the pairing process does not recognize homologous sequences, in a region specific manner. The pairing defect can be localized to hot spots. No general pairing deficiency, induced by the absence of a certain enzyme like an unwinding protein, can be defined. One possibility for creating unpaired distinct regions is uncoordinated replication. One chromosome might replicate and be repacked before the homologous partner is replicated. Alternatively, the whole region might replicate at another time, when the somatic pairing mechanism is not activated in the cell.

Proliferative alleles induce temperature independent formation of melanotic tumor masses in third instar larvae. Melanization occurs by aggregation of plasmatocytes, which encapsulate other tissues [26, 27]. Tumors indicate a cell cycle defect. In MBT the alleles are able to induce brain hyperplasia or brain tumor formation. Hyperplasic growth of tissue indicates the formation of more cells than programmed. Thus, the cell cycle is driven more frequently, leading to excessive cell proliferation. The formation of a brain tumor additionally requires the mutation of other gene defects that influence the differentiation pattern of neuroblasts. In MBT *tld* has been identified as a tumor suppressor gene; *spz* is involved in temperature sensitive lethality [11]. The mutation of differentiation genes might direct the tumor forming capability of proliferative genes in the brain. The action of oncogenes and tumor suppressor genes might mediate the cell specificity of the tumor, because of modulation of the G1 phase.

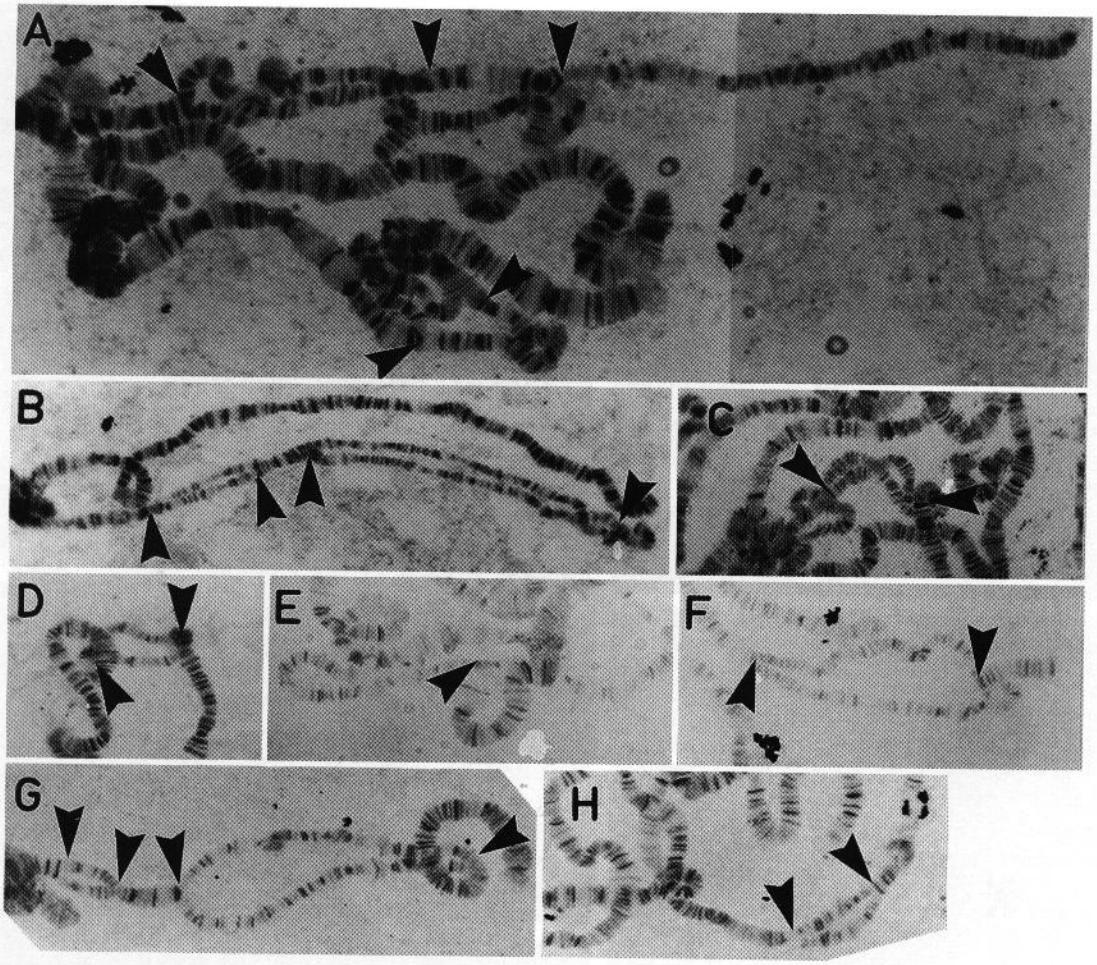


**Figure 4** Pairing defects of proliferative mutants. OregonR was crossed with the alleles at 18°C. The giant chromosomes of third instar larvae hybrids are shown at the same magnification. Arrowheads indicate beginning and end of pairing abnormalities. (A) Allele 9 induces severe pairing defects. Here a nucleus with three long unpaired regions, concerning five to ten sections each, is shown. (B) Allele 14 usually reveals several long unpaired chromosome regions, concerning five to ten sections on one chromosome half. (C) Allele 19 frequently exhibits shorter unpaired regions, concerning about three sections. (D) By allele 47 shorter regions appear unpaired. (E) Allele 77 reveals longer unpaired chromosome regions. (F) In nuclei of allele 98 longer regions are unpaired. (G) Through the strong *srn* allele 88 several regions are unpaired. (H) The pairing defect of allele 23 concerns shorter distances of about three sections.

In addition to mammalian cells, which differentiate only from the G1 phase, *Drosophila* cells are able to differentiate from G2 through endomitotic cycles and resting with polytene chromosomes. Both, the cell cycle and endomitosis, have a common interval, the replication phase, where somatic pairing is initiated [16].

Giant cells with giant chromosomes beneath the tumor cells in *tld-1* and *tld-2* brains revealed an additional cellular phenotype induced by proliferative genes: aberrant polytenization of brain cells.

Three different phenotypic manifestations of proliferative genes are presented. Dependent on the cell fate, proliferative genes (1) allow aberrant polytenization in brain cells, (2) influence a functional component of the chromosomes and induce region specific somatic pairing defects, and (3) induce a cell cycle defect.

Polytenization of brain cells indicate that proliferative genes are able to induce the S-phase and allow replication of DNA. Region specific somatic pairing defects of polytene chromosomes indicate that these genes might induce the S-phase by interference with a specific part of the DNA. Induction of tumor tissue indicates that proliferative genes possibly nullify the restriction of the cell cycle by inducing the S-phase through interference with part of the DNA.

The manifestation of a proliferative gene mutation in proliferation competent cells results in more cell cycles than programmed, a tumor appears. The somatic pairing defect points towards a replication defect. Part of the chro-

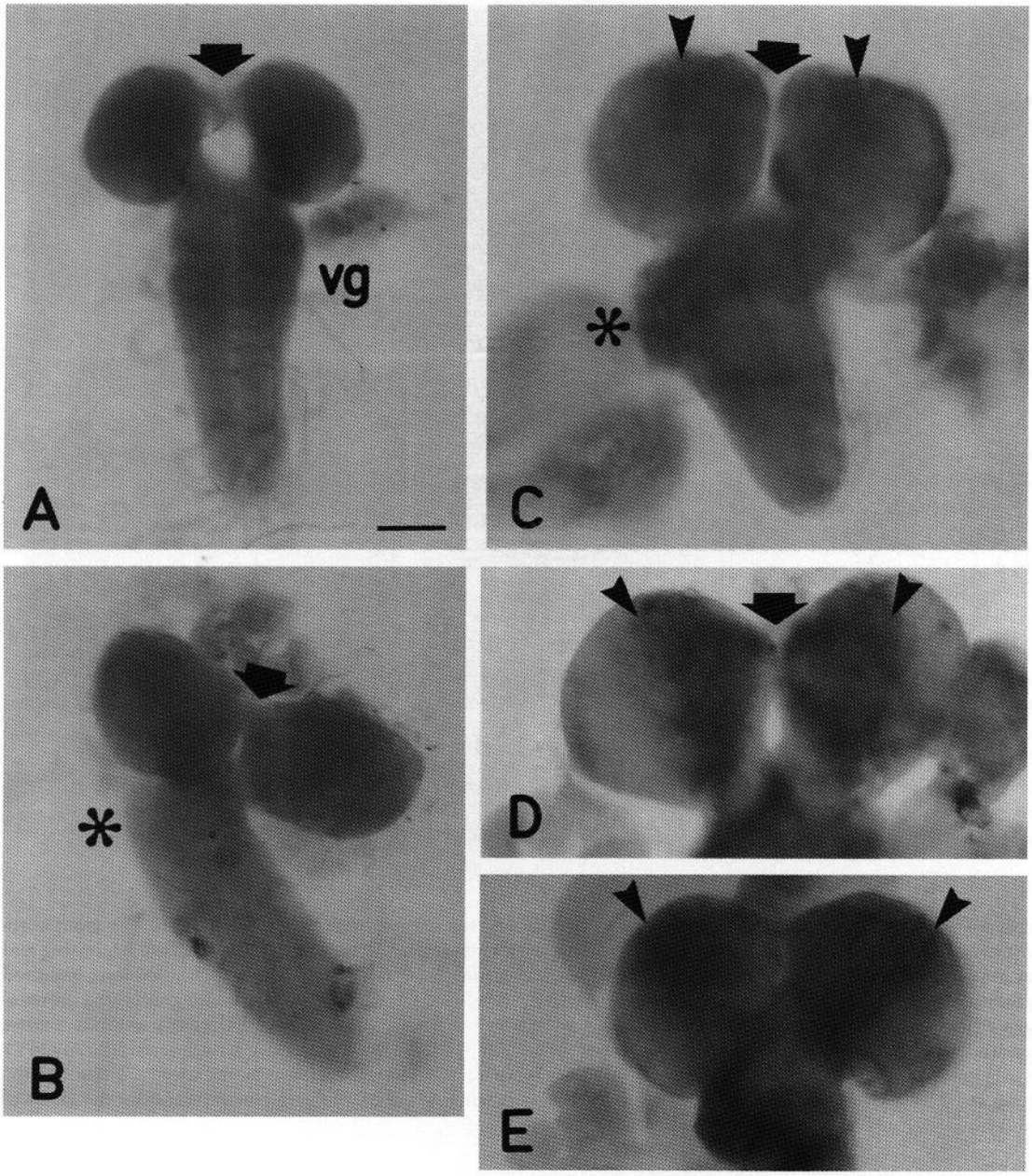


**Figure 5** Brain phenotypes. Shown are aARD stained larval brains of homozygous animals at the same magnification. Bar represents 100 μm. (A) Allele MN results in an elongated ventral ganglion (vg). The optic lobes are about half the size of wildtype lobes and not completely separated (arrow). ARD is not specifically expressed in the neuropiles. Staining occurs in the whole brain hemispheres. Exposure to dye is about five times shorter compared to wildtype stain as shown in Figures 3D and E, indicating an unusual overexpression of ARD in the whole brain. (B) Allele 89 results in a brain similar to that from allele MN, but the ventral ganglion appears with an asymmetry (asterisk). (C) Allele 88 shows brains of about wildtype size. The ventral ganglion appears to be asymmetric (asterisk). The optic lobes are separated (arrow), slight specific expression of ARD in the neuropiles is visible (arrowheads). (D) Allele 14 induces hyperplasic and not completely separated optic lobes (arrow). Expression of ARD is normal, indicating that neuropiles are differentiated (arrowhead). (E) Allele 77 results in optic lobes of normal size, neuropiles are differentiated (arrowhead).

mosome might replicate without license. This could lead to replication of the whole genome, which could induce the cell cycle in proliferation competent cells. Proliferation restricted cells form polytene chromosomes by endomitosis. The further study of the nuclear somatic pairing defect and the regulation of replication could highlight a so far undescribed principle in tumor formation.

I am grateful to I. Forner for technical assistance, to E. Gateff, and O. Vef for donating flies, and to R. Brown for reading of the

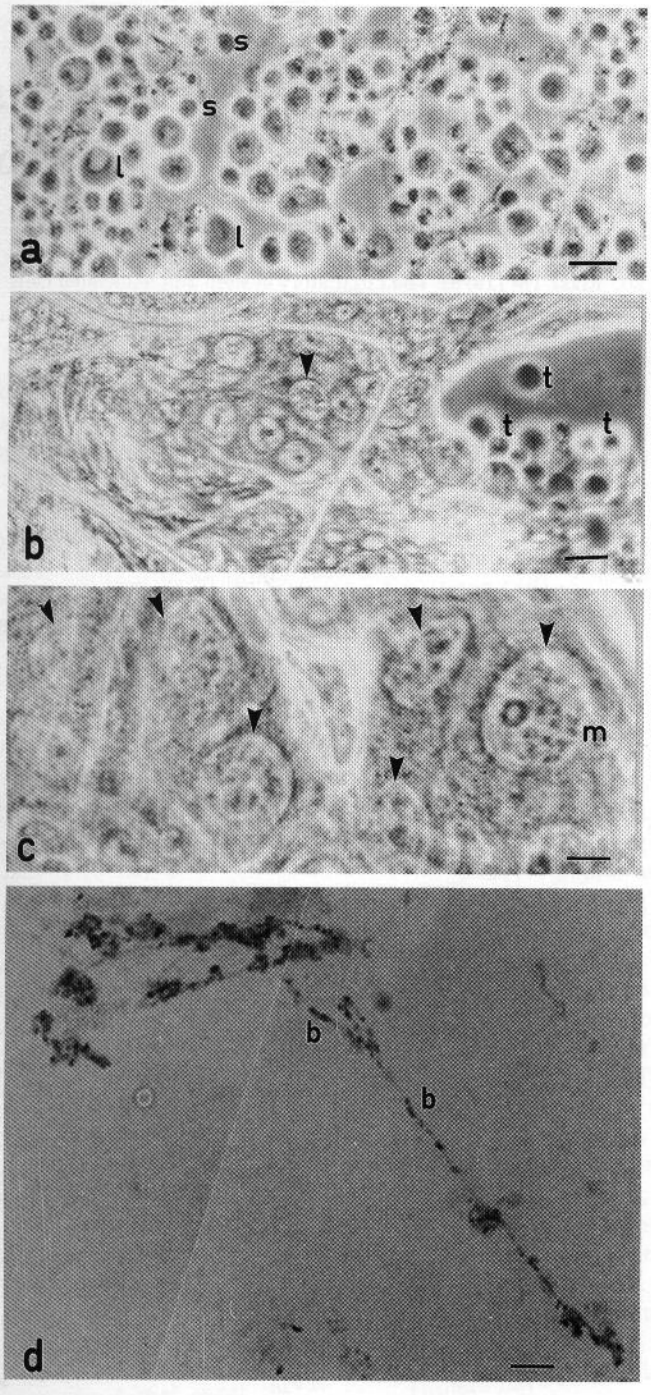


**Figure 6** Tumor formation and polytenization. (a) Hyperplasic cells in the brain of a *tld-1* L3 larvae grown at 29°C are shown. The cells do not adhere. Cell population is not homogenous, small cells (s) and large cells (l) occur. The nuclei of the overgrown cells do not appear to maintain polytene chromosomes. (b) Within the hyperplasic tissue (t) of the *tld-1* brain, a different group of cells appears. These cells adhere as tissue. Large nuclei (arrowhead) point towards polytenization of DNA within these cells. (c) Higher magnification of *tld-2* brain cells indicate, that the large nuclei (arrowheads) of the giant cells within the tumor tissue contain mäander type polytene chromosomes (m), pointing to about 50 amplified copies of the DNA [15]. (d) The chromatin of one nucleus of homozygous *efe*$^{89}$ brain cells is shown. The brain, isolated from L3 larvae with beginning melanization of tumor tissue, was fixed, stained with orceine, and squashed. Polytene chromosomes appear in about 20% of the nuclei. Here they are amplified to about 50 copies, the banding pattern of the chromosomes starts to appear (b). Bars represent 30 μm (a,b) or 10 μm (c,d).

manuscript. Advice obtained from Herbert Jäckle is gratefully acknowledged. For this study a grant of the Deutsche Forschungsgemeinschaft (DFG-Ri722/4-1) was obtained.

## REFERENCES

1. Bishop M (1982): Retroviruses and cancer genes. In: Advances in Cancer Research, vol 37. G Klein, S Weinhouse, eds. Academic Press, New York, pp 1–28.
2. Weinberg RA (1995): The retinoblastoma protein and cell cycle control. Cell 81:323–330.
3. Levine AJ, Momand J, Finlay CA (1991): The *p53* tumor suppressor gene. Nature 351:453–456.
4. Gateff E, Schneidermann HA (1969): Neoplasms in mutant and cultured wildlife tissues of *Drosophila*. Natl Cancer Inst Monogr 31:365–395.
5. Mechler BM, McGinnis W, Gehring WJ (1985): Molecular cloning of lethal(2)giant larvae, a recessive oncogene of *Drosophila melanogaster*. EMBO 4:1551–1557.
6. Gateff E, Löffler T, Wismar J (1993): A temperature-sensitive brain tumor suppressor mutation of *Drosophila melanogaster*: Developmental studies and molecular localization of the gene. Mech Develop 41:15–31.
7. Woods DF, Bryant PJ (1989): Molecular cloning of the lethal(1)discs large-1 oncogene of *Drosophila*. Develop Biol 134:222–235.
8. Woods DF, Bryant PJ (1991): The discs large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homology localized at septate junctions. Cell 66:451–464.
9. Mahoney PA, Weber U, Onofrechuk P, Biessmann H, Bryant PJ, Goodman CS (1991): The *fat* tumor suppressor gene in *Drosophila* encodes a novel member of the cadherin gene superfamily. Cell 65:853–868.
10. Gateff E (1994): Tumor suppressor and overgrowth suppressor genes of *Drosophila melanogaster*: Developmental aspects. Int J Dev Biol 38:565–590.
11. Riede I (1996): Three mutant genes cooperatively induce brain tumor formation in Malignant Brain Tumor. Cancer Genet Cytogenet 90:135–141.
12. Hashimoto C, Gerttula S, Anderson KV (1991): Plasma membrane localization of the Toll protein in the syncytial *Drosophila* embryo: Importance of transmembrane signaling for dorsal-ventral pattern formation. Development 111:1021–1028.
13. Shelton CA, Wassermann SA (1993): *Pelle* encodes a protein kinase required to establish dorsoventral polarity in the *Drosophila* embryo. Cell 72:515–525.
14. Massagué J (1990): The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641.
15. Beermann W (1962): Riesenchromosomen. Protoplasmatologia, Vol 6D, Springer Verlag, Wien.
16. Friedrich-Freksa H (1940): Bei der Chromosomenkonjugation wirksame Kräfte und ihre Bedeutung für die identische Verdopplung von Nucleoproteinen. Naturwissenschaften 38:376–380.
17. Riede I, Renz M (1983): Study on the somatic pairing of polytene chromosomes. Chromosoma 88:116–123.

18. Campos-Ortega JA, Hartenstein V (1985): The Embryonic Development of *Drosophila melanogaster*. Springer-Verlag, Berlin, Heidelberg, New York.
19. Goodman CS, Doe CQ (1993): Embryonic development of the *Drosophila* central nervous system. In: Development of *Drosophila*. CM Bate, A Martinez-Arias, eds. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 1131–1200.
20. Steinemann M (1980): Chromosomal replication in *Drosophila virilis*: diploid karyotype of brain cells. Chromosoma 78:211–223.
21. van de Flierdt K (1975): No multistrandedness in mitotic chromosomes of *Drosophila melanogaster*. Chromosoma 50:431–434.
22. Prokop A, Technau GM (1994): BrdU incorporation reveals DNA replication in non dividing glial cells in the larval abdominal CNS of *Drosophila*. Roux's Arch Dev Biol 204:54–61.
23. Schuster R, Phannavong B, Schröder C, Gundelfinger ED (1993): Immunhistochemical localization of a ligand-binding and a structural subunit of the nicotinic acetylcholine receptors in the central nervous system of *Drosophila melanogaster*. J Comp Neurol 335:149–162.
24. Lindsley DL, Zimm GG (1992): The Genome of *Drosophila melanogaster*. California Academic Press, San Diego.
25. Grigliatti T (1986): Mutagenesis. In: *Drosophila*, a Practical Approach. DB Roberts ed. IRL press Oxford, Washington DC, pp. 39–58.
26. Rizki TM (1978): The circulatory system and associated cells and tissues. In: The Genetics and Biology of *Drosophila*. Vol 2b. M Ashburner, TMF Wright, eds. Academic Press, London, New York, pp. 379–448.
27. Barigozzi C (1969): Genetic control of melanotic tumors in *Drosophila*. Natl Cancer Inst Monogr 31:277–290.