A Forward Genetic Approach to Mapping a P-Element Second Site Mutation Identifies DCP2 as a Novel Tumor Suppressor in Drosophila melanogaster

Rakesh Mishra,*1 Rohit Kunar,*1 Lolitika Mandal,† Debasmita Pankaj Alone,‡ Shanti Chandrasekharan,§ Anand Krishna Tiwari,** Madhu Gwaldas Tapadia,* Ashim Mukherjee,†† and Jagat Kumar Roy*,2

*Cytogenetics Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005, India, †Department of Biological Sciences, Indian Institute of Science Education and Research Mohali, Manauli 140306, India, ‡School of Biological Sciences, National Institute of Science Education and Research, HBNI, Jatni, Khurda 752050, India, §Division of Genetics, Indian Agricultural Research Institute, Pusa, New Delhi 110012, India, **School of Biological Sciences and Biotechnology, Indian Institute of Science Education and Research, Koba, Gandhinagar-382 007, India, and ††Department of Molecular and Human Genetics, Institute of Science, Banaras Hindu University, Varanasi 221005, India

ORCID IDs: 0000-0001-7352-8293 (R.M.); 0000-0003-1606-4992 (R.K.); 0000-0002-7711-6090 (L.M.); 0000-0003-4809-925X (D.P.A.); 0000-0002-4080-6653 (A.K.T.); 0000-0002-5050-4624 (M.G.T.); 0000-0002-6461-3274 (J.K.R.)

ABSTRACT The use of transposons to create mutations has been the cornerstone of Drosophila genetics in the past few decades. Second-site mutations caused by transpositions are often devoid of transposons and thereby affect subsequent analyses. In a P-element mutagenesis screen, a second site mutation was identified on chromosome 3, wherein the homozygous mutants exhibit classic hallmarks of tumor suppressor mutants, including brain tumor and lethality; hence the mutant line was initially named as lethal (3) tumorous brain [(3)tb]. Classical genetic approaches relying on meiotic recombination and subsequent complementation with chromosomal deletions and gene mutations mapped the mutation to CG6169, the mRNA decapping protein 2 (DCP2), on the left arm of the third chromosome (3L). Thus the mutation was renamed as DCP2l(3)tb. Fine mapping of the mutation further identified the presence of a Gypsy-LTR like sequence in the 5′UTR coding region of DCP2, along with the expansion of the adjacent upstream intergenic AT-rich sequence. The mutant phenotypes are rescued by the introduction of a functional copy of DCP2 in the mutant background, thereby establishing the causal role of the mutation and providing a genetic validation of the allelism. With the increasing repertoire of genes being associated with tumor biology, this is the first instance of mRNA decapping protein being implicated in Drosophila tumorigenesis. Our findings, therefore, imply a plausible role for the mRNA degradation pathway in tumorigenesis and identify DCP2 as a potential candidate for future explorations of cell cycle regulatory mechanisms.

KEYWORDS DCP2 tumor suppressor Drosophila genetic mapping forward genetics

With increasing interest in studies of classical tumor suppressors (Papagiannouli and Mechler 2013; Ivanov et al., 2010), the search for new candidate proteins in tumor suppression has garnered much importance (Tipping and Perrimon 2014). In Drosophila, P-element mutagenesis provides a convenient method to identify, isolate and clone tagged genes (Mechler 1994). Although identification and subsequent molecular analysis is convenient with P-element transpositions, second-site mutations devoid of any P-element insertion created during transposition, may impede further analyses (Liebl et al., 2006). A P-element, P-lac w* (Bier et al., 1989), on third chromosome at 93B position was mobilized via enhancer-trap mutagenesis to obtain regulators of hsr(93D3-4) at 93D3-4 region (Lakhotia and Tapadia 1998). In the process, one mutant line showed prolonged larval life, brain tumor in the larval brain and lethality in the late larval and pupal stages in homozygous condition. The escapee flies showed ommatidial defects and reduced survival. Molecular mapping approaches identified the P-element (P-lac w*) to reside 94 bases downstream to the TATA box of Rab11 (located at the cytogenetic position 93B12-13), which is 45 bases upstream to the first exon of the gene and is a part of the 5′UTR (Mandal and Roy, unpublished). Rab11 codes for a vesicular trafficking protein and is a component of the recycling endosomes (Novick and Zerial 1997; Satoh et al., 1997).
Introduction of a functional copy of Rab11 in the homozygous mutant background rescued the ommatidial defect, but failed to rescue the tumorous phenotype and lethality, implying the presence of a second-site mutation, besides the P-element insertion in Rab11. Allowing free recombination, the mutations, viz., the P-element insertion in Rab11 and the second-site mutation devoid of the P-element, were separated into two discrete isogenised lines (Alone and Roy, unpublished). Since the phenotypes shown by the second-site mutation phenocopies mutations in tumor suppressor genes in Drosophila (Gateff and Schneiderman 1969; Gateff 1994; Gateff E, 1978), the mutation was named as l(3)tb [lethal (3) tumorous brain] owing to its location on the third chromosome and the phenotypes manifested. Genetic and molecular analyses mapped the mutation to DCP2 on the left arm of chromosome 3 (cytogenetic position 72A1) and hence the allele was named as DCP2l(3)tb.

DCP2 codes for the mRNA decapping protein 2, which belongs to the NUDIX family of pyrophosphatases and was identified more than a decade ago through a yeast genetic screen (Dunkley and Parker 1999). Being one of the major components of the decapping complex, DCP2 is conserved in worms, flies, plants, mice, and humans (Wang et al., 2002). DCP2 is activated by DCP1 and they function together as a holoenzyme to cleave the 5′ cap structure of mRNA (LaGrandeur and Parker 1998; Coller and Parker 2004; Parker and Song 2004; She et al., 2008).

In humans, DCP2 is upregulated in lung cancer (Watson et al., 2008) and regulates histone mRNA titres (Mullen and Marzluff 2008) and regulates histone mRNA titres (Mullen and Marzluff 2008) and regulates histone mRNA titres (Mullen and Marzluff 2008). DCP2 is activated by DCP1 and they function together as a holoenzyme to cleave the 5′ cap structure of mRNA (LaGrandeur and Parker 1998; Coller and Parker 2004; Parker and Song 2004; She et al., 2008).

In Drosophila, DCP2 plays fundamental roles in the response pathways active following chronic nicotine exposure and its loss mediates locomotor hyperactivity following such exposure (Ren et al., 2012). Also, DCP2 is involved in the decapping of mRNAs involved in cell cycle progression and DNA replication and acts to restrict bunyaviral infections in cell lines and adult flies (Hopkins et al., 2013).

Despite the abundance of information from various model organisms pertaining to the roles of the mRNA decapping machinery, and/or the decay programs in cellular homeostasis, development and stress management, our understanding of the roles of the components of the mRNA decay program(s) and their interaction with the cellular signaling modules vis-à-vis gene expression programs is still in its infancy. Although mRNA decapping plays a significant role in mRNA turnover and translation, widely affecting gene expression (Mitchell and Tollervy 2001; Raghavan and Bohjianen 2004; Song et al., 2010), simultaneous links between mRNA degradation genes, retrotransposons and tumors have not been observed and/or investigated so far. Therefore, the novel allele DCP2l(3)tb reveals a new perception for functional roles of mutant lesions and the ensuing perturbations in gene regulation in tumor biology.

MATERIALS AND METHODS

Fly strains and rearing conditions
All flies were raised on standard agar-cornmeal medium at 24 ± 1°. Oregon R+ was used as the wild type control. The l(3)tb mutation (y w; +/+) was isolated in a genetic screen and the mutation was maintained with the TM6B balancer. The multiply marked “ruraca” (ru h st cu sr e ca/TM6B, Tb) and “rurPrica” (ru h st cu sr e Pr ca/TM6B, Tb) chromosomes were employed for recombination mapping (Lindsley and Zimm 1992). w; Δ-2, Sb/TM6B, Tb1, Hu, e1 (Cooley et al. 1988) and CyO, P[Tub-Phac/T2]/Wg8°;+;TM6B, Tb, Hu, e2 were used for providing transposase source for P element and piggypBac specific transposable element, respectively, in mutagenesis experiment. The y w; P[Act5C-GALA4]25f01/CyO, y w; +/+; Tub-GAL4/TM3, Sb, e, and Elav-GAL4 stocks were obtained from the Bloomington Drosophila Stock Center. The lethal insertion mutants of gene DCP2, viz., P[Bac(RB)DCP2-28854]/TM6B, Tb1 Hu, e1 (Thibault et al. 2004) and P[GTC1/DCP2-870176]/TM3, Sb, e1 (Lukacsovich et al. 2001) were obtained from Exelixis Stock Center, Harvard University and Bloomington Drosophila stock center, respectively, while the UAS-dboRNAi line used for knock-down of diablo (dibio) was procured from the Bloomington Drosophila Stock Center.

Deficiency stock Df(3L)RM96 was generated in the laboratory (for details of characterization, refer to Supplementary Table S3) using progenitor P element stocks, viz, P[RSS]5-SZ-3486, P[RSS]5-SZ-3070, P[RSS]UM-8356-3, P[RSS]UM-8241-3, P[RSS]CB-0072-3, y w P[70FLP, ry+]3Pwo/y w Y, 20°, TM2/TM6C, Sb, w1188v/y Y, 20°, TM2/TM6C, Sb obtained from Vienna Drosophila Resource Center (Golic and Golic 1996; Ryder et al. 2007). Various deficiency stocks and transposon insertion fly stocks (Supplementary Tables S1 and S2) used for complementation analysis were obtained from Bloomington Drosophila stock center and Exelixis stock center.

Analysis of lethal phase in l(3)tb homozygotes
For analysis of lethal phase and morphological anomalies associated with the homozygous l(3)tb mutation, embryos were collected at the intervals of 2h on food filled Petri dishes. Embryos from wild type flies were collected as controls. The total number of eggs in each plate was counted and the embryos were allowed to grow at 23° or 18° or 16° (±1°C). Hatching of embryos and further development of larval stages was monitored to determine any developmental delay. Mutant larvae, at different stages, were dissected and the morphology of larval structures was examined.

Identification of mutant locus
Meiotic recombination mapping of l(3)tb mutation: Genetic recombination with multiple recessive chromosome markers, ru cu ca, was performed to map mutation in y w; +/+; l(3)tb/TM6B, Tb mutant. The y w; l(3)tb/TM6B males were crossed to virgin +/+; ru Pri ca/TM6B females to recover l(3)tb without y w on X-chromosome. The F1 l(3)tb/TM6B males were crossed to virgin +/+; ru cu ca females and the F2 progeny +/+; l(3)tb/ru cu ca virgin females were selected. These
F2 virgins were then crossed to *ru* *Pri ca/*TM6B males to score the frequency of recombinants in the F3 progeny. Thereafter, all the F3 progeny males obtained, were individually scored for *ru*, *h*, *th*, *st*, *cu*, *sr*, *e* and *ca* phenotypes and then they were individually crossed with virgin *l(3)tb/*TM6B females to identify which of them had the *l(3)tb* mutation along with other scored markers.

**Complementation mapping of the *l(3)tb* mutation:** Complementation analysis of the mutation in *l(3)tb* allele was carried out in two stages. First, deficiency stocks spanning the entire chromosome 3 (Supplementary Table S1) were used to identify the mutant loci, and second, lethal *P*-insertion alleles selected from the region narrowed down through recombination and deficiency mapping (Supplementary Table S2) were harnessed to further identify the mutant gene(s) in *l(3)tb*. In either case, virgin females of *y*, *w*, *+/+*; *l(3)tb/*TM6B, *Tb* were crossed with the males of the various deficiency stocks and/or the lethal *P*-insertion alleles and the non-tubby F1 males heterozygous for *l(3)tb* and the deficiency were scored for the phenotype(s).

Reversion analysis was performed by the excision of piggyBac transposon in *DCP2*^200034^ with the help of piggyBac specific transposase source, *Cyo. P*(Tub-Phat)2/sgt^3^ (Thibault et al. 2004) or by the excision of *P*-element in *DCP2*^2001766^ strain using transposase from the ‘jumpstarter’, Δ2-3*Sh/TM6B, *Tb*, *Hu*, *e*'. Virgin flies from the ‘mutator stocks’, *viz. DCP2*^200034^ or *DCP2*^2001766^ strain were crossed to male flies from respective ‘jumpstarter stock’. F1 male flies with mosaic eye pigmentation carrying the transposase and respective transposons were selected and crossed to JSK-3 (*TM3, Sb, *e'/TM6B, *Tb*, *Hu, e'*') virgins and from the next generation (F2), rare white eyed revertant flies were selected (Figure S1).

**Fine mapping of *l(3)tb* mutation**

**Genomic DNA Isolation, PCR and Southern hybridization:** Genomic DNA for polymerase chain reaction (PCR) was isolated by homogenizing 50 male flies from each of the desired genotype or 80–100 third instar larvae from homozygous mutant *l(3)tb* (Sambrook et al. 1989). Based on the results obtained from genomic mapping, identification of the candidate region in *DCP2* was done by overlapping PCR based screening, wherein the entire genomic span of *DCP2* was amplified using 28 primer pairs from 3L:1581834.15819523 (Supplementary Tables S4, S5, S6). After identifying the candidate region, it was validated with the primer pair, Dbo_F: 5'-ACAACATCTCCTCATGAGAACC-3' and DCP2_P19_R: 5'-TGCTACCCGAACTTTTGGGATCT-3'. The primer pair Dbo_F: 5'-ATAAACAAAAAGTTATGGTACCCTGCAGG-3' and DCP2_R: 5'-AGATTTGATGTATATGGATC-3' was designed to amplify the full length gene along with flanking sequences (500 bp on either side). In either case, the thermal cycling parameters included an initial denaturation at 96° (2 min) followed by 30 cycles of 30 s at 94°, 45 s at 72°, and 15 min at 68°. Final extension was carried out at 68° for 20 min. The PCR products were electrophoresed on 0.8% agarose gel with O’GeneRuler 1 kb plus DNA ladder (Thermo Scientific, USA). An 812 bp region (3L: 15829579..15826790) spanning the candidate mutated region in *DCP2* was PCR amplified and ligated in pGEM-T vector (Promega) to generate the pGEM-T-812 clone. The ~340 bp fragment isolated during primer walking (see below) was purified and ligated in pTopo-TA-XL vector (Invitrogen, USA) to generate the pTopo-TA-XL-430 clone. Digestion, ligation and transformation were performed using standard protocols as described in Sambrook et al. 1989. Southern hybridizations were performed according to Russell and Sambrook 2001. Following electrophoresis and gel pre-treatments, DNA was transferred on to positively charged nylon membranes (Roche, Germany). Hybridizations were performed at 68° with 0.02% SDS, 5X SSC, 0.5% Blocking reagent, and 0.1% laurylsarcosine with probes generated from pGEM-T-812 and pTopo-TA-XL-430 plasmids. DIG labeling and chemiluminescent detection were performed as per the manufacturer’s instructions (Roche, Germany). Alternatively, radiolabeling of probes using α-32P dCTP (BRIT, India) and detection were performed according to Sambrook et al., 1989.

**sequencing, primer walking and CNV detection:** To confirm the fidelity of amplification automated DNA sequencing was performed (ABI – 3130, USA) as per the manufacturer’s instructions. Primer walking was initiated with the primers dbo_F and DCP2_P19_R and from the terminal part of the sequence obtained, new primers P9_W2_F: 5'-GGGACTCTGTAAAAATCCTTCGATTCT-3' and P19_W2_R: 5'-GGGGCGTATCAGTTTCATAAAGG TAC-3' were designed. Long-range PCR with P9_W2 was performed as described previously. Sequence chromatograms were assessed and analyzed with FinchTV 1.4.0, Geospiza Inc. Semi-quantitative assessment of copy number variance (CNV) of the intergenic sequence in *DCP2*^2003^ was determined through PCR analyses. A 156 bp sequence (3L: 15826497..15826652) was chosen to be amplified by CNV_F: 5'-ACAGTTGGCCCTCTGTGATAAT-3' and CNV_R: 5'-AGTGCAACGGAAAGGAATCT-3' against an internal control sequence of 153 bp, corresponding to the gene Dsor, amplified by the primer pair Dsor_F: 5'-CCACCTGGATGC ATATCTC-3' and Dsor_R: 5'-GTCCTTGAACTGGTGGGAAGA-3'. Thermal cycling parameters included an initial denaturation at 95° (5 min) followed by 28 cycles of 30 s at 94°, 30 s at 60°, and 30 s at 72°. Final extension was carried out at 72° for 10 min. The PCR products were electrophoresed on 2% agarose gel with a 100-bp DNA ladder (BR Biosciences, India).

**RNA isolation and RT-PCR**

For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from the 5 brains from wandering late third instar larvae from the genotypes mentioned in the respective cases in the results section, using TRI Reagent following the manufacturer’s protocol (Sigma-Aldrich, India). RNA pellets were dissolved in nuclelease-free water, followed by treatment with 2U of RNase-free DNAse1 (Thermo Scientific, USA) for 40 min at 37°. First strand cDNA was synthesized using 200 U of RevertAid reverse transcriptase (Thermo Scientific, USA) and 80 pmol of oligo(dT)18 primer (Thermo Scientific, USA). PCR was carried out for Cyclin A (amplon size 77 bp), Cyclin E (amplon size 163 bp) and Diablo (amplon size 179 bp) using G3PDH (amplon size 144 bp) as internal control. In either case, the thermal cycling parameters included an initial denaturation at 95° (5 min) followed by 30 cycles of 30 s at 94°, 45 s at 60°, and 1 min at 72°. Final extension was carried out at 72° for 10 min. The PCR products were electrophoresed on 1.8% agarose gel with a 100 bp DNA ladder (BR Biosciences, India). The forward and reverse primers used are listed below.

- **Forward primers –**
  - Cyclin A 5'-ACGTTTCTCCCTCCTCATGAGG-3'
  - Cyclin E 5'-GAGGGCGTCAGAAAGACTTTTCG-3'

- **Reverse primers –**
  - Dbo 5'-CAGCITTCTGATCTCATTTAGTT-3'
  - G3PDH 5'-CCACTGCGGAGGATGTCAACTA-3'
Reverse primers –
Cyclin A 5′−ACTTTGGCAGATGCGTGGAGA-3′
Cyclin E 5′−AATATTGTCAGCCAGGACCTC-3′
Dbo 5′−GGGAATTTTTGATAATTGGACC-3′
G3PDH 5′−GCTCACGGATTTGCATGCA-3′

Immunocytochemistry and Immunoblotting
The imaginal discs and/or brain ganglia were collected from wild type Oregon R+ wandering 3rd instar larvae, just before pupation (110 h AEL) and in mutant homozygous l(3)tb from day 6 and day 10/12. The tissues were processed for immunostaining/immunoblotting as described in Banerjee and Roy 2018, with the desired antibodies. Primary antibodies used in this study were - Anti-Discs large, 4F3 (1:50, Developmental Studies Hybridoma Bank, Iowa,USA), Anti-Armadillo (1:100, a kind gift by Prof LS Shashidhara, IISER Pune, India), Anti-Elav (Rat-Elav-7E8A10, 1:100, DSHB, USA), Anti-DE-Cadherin (DCAD2, 1:20, DSHB, Iowa, USA), Anti-phospho-Histone 3 (1:500, Millipore, Upstate, USA), Anti-Deadpan (1:800, a kind gift from Prof. Volker Hartenstein, University of California, USA) and Anti-Cyclin E (HE12, 1:20, DSHB, Iowa, USA). Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich, India) or Alexa Fluor 488 (1:20; Molecular Probes, USA) or Alexa Fluor 546 (1:200; Molecular Probes, USA) were used to detect the given primary antibody, while chromatin was visualized with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, 1 μg/ml Sigma-Aldrich, India). Counterstaining was performed with either DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, India) at 1 μg/ml, or phalloidin-TRITC (Sigma-Aldrich, India) at 1:200 dilutions. Tissues were mounted in DABCO (antifade agent, Sigma-Aldrich, India). The immunostained slides were observed under Zeiss LSM 510 Meta Laser Scanning Confocal microscope, analyzed with LSM softwares and assembled using Adobe Photoshop 7.0.

Statistical analysis
Sigma Plot (version 11.0) software was used for statistical analyses. All percentage data were subjected to arcsine square-root transformation. For comparison between the control and experimental samples, One-Way ANOVA was performed. Data were expressed as mean ± SE of mean (SEM) of several replicates.

Data availability
Supplemental Files are available at figshare. Fly strains are available upon request. The authors affirm that all data necessary for confirming the conclusions are present within the article, figures and tables. Supplementary Figure S6 contains necessary sequence information. Supplemental material available at figshare: https://doi.org/10.25387/g3.12568265.

RESULTS
l(3)tb homozygotes show the classic hallmarks of cancer in Drosophila including developmental delay, abnormal karyotype, larval/pupal lethality along with tumorous brain and wing imaginal disc
Developmental analysis of l(3)tb homozygotes showed that while embryos hatched normally and developed alike heterozygous siblings [l(3)tb/TM6B], the third instar larvae reached the wandering stage quite late with the larval stage extending up to 12 or 13 days (Figure 1B). Although 66.8% of the larvae survived to pupate (Table 1), they died in the pupal stage following bloating, increase in size and cessation of growth (Figure 1A). Hence, the mutation is lethal, with the lethality being pronounced in the pupal stage. Lowering the temperature to 16°C is lethal, with the lethality being pronounced in the pupal stage. In l(3)tb homozygotes showed delayed larval development with lethality at larval/pupal stage (A, B) and is not a conditional temperature sensitive allele (A, B, C). Homozygous l(3)tb progeny, at 23°C, showed lethality at larval and pupal stages and no flies eclosed as compared to wild type and heterozygous l(3)tb progeny with balancer chromosome (A). Homozygous l(3)tb progeny individuals demonstrated extended larval life up to day 12/13 where as heterozygous progeny individuals followed the normal wild type pattern of development (B). (C) and (D) show significant increase in viability of homozygous (non-tubby) l(3)tb larvae at lowered temperatures of 18°C and 16°C, respectively, though there also occurred absolute lethality at pupal stages. Each bar represents mean ± SE of three replicates of 100 larvae in each. *** indicates P < 0.005.

Figure 1  Developmental delay in l(3)tb homozygotes. Homozygous l(3)tb show delayed larval development with lethality at larval/pupal stage (A, B) and is not a conditional temperature sensitive allele (A, B, C). Homozygous l(3)tb progeny, at 23°C, showed lethality at larval and pupal stages and no flies eclosed as compared to wild type and heterozygous l(3)tb progeny with balancer chromosome (A). Homozygous l(3)tb progeny individuals demonstrated extended larval life up to day 12/13 where as heterozygous progeny individuals followed the normal wild type pattern of development (B). (C) and (D) show significant increase in viability of homozygous (non-tubby) l(3)tb larvae at lowered temperatures of 18°C and 16°C, respectively, though there also occurred absolute lethality at pupal stages. Each bar represents mean ± SE of three replicates of 100 larvae in each. *** indicates P < 0.005.
of the lobes. There was no loss of symmetry in the overgrown brain hemispheres in most of the cases, except in some cases where they were deformed and fused with the imaginal discs (Figure 2 J and K). A similar trend in morphological aberration was observed in the wing discs, which remained small initially, but later enlarged sufficiently (Figure 2L), with an abnormal protrusion in the wing pouch. Analysis of mitotically active cell population by staining for phosphorylated histone 3 (PH3), revealed an increased number of active mitoses in the homozygous mutant brains (Figure 3A-O; 3V) and wing discs (Figure 3P-V) (Day 6) in comparison to the wild type; the number increased with increase in age of the mutant. However, mitotic karyotypes of the mutant brain lacked numerical aberrations, despite showing extensive variability in condensation (Figure 2 H and I).

Table 1 Homozygous mutation in l(3)tb causes larval and pupal lethality

| Genetic cross (23°C ± 1) | Total Eggs | No. of eggs hatched | 3rd Instar larvae transferred | Pupae formed | Flies eclosed |
|--------------------------|------------|---------------------|-------------------------------|--------------|--------------|
| l(3)tb/TM6B | 1500 | 972 (O = 64.8%) | Non-Tubby- 468 (O = 48.1%) | Non-tubby 313 (O = 66.8%) | Non Tubby 0 |
| X l(3)tb/TM6B | (E = 66.7%) | (E= 50%) | Tubby 473 (O = 48.6%) | Tubby 468 (O = 98.9%) | Tubby 465 (O = 99.4%) |
| | | | (E = 50%) | (E = 100%) | (E = 100%) |

Numbers in parenthesis indicate the percentage observed (O) and expected (E) values out of the total progeny from previous stage.

Figure 2 Morphological and chromosomal alterations in l(3)tb homozygotes. Homozygous l(3)tb mutants show severe morphological alteration in delayed 3rd instar larval brain, wing and eye-antennal disc. Homozygous l(3)tb mutant 3rd instar larvae revealed tumorous brain of day 12 (C) as compared to day 6 of homozygous mutant (B) and day 5 of wild type, Oregon R+ (A). l(3)tb homozygotes exhibited highly significant differences in the overall circumference of the left and right brain lobes in the delayed stage (day 10) as compared to the respective wild type brain lobes (J). Significant differences were found in the area (μm²) of respective brain lobes of l(3)tb homozygotes and wild type (K). Dimensions of wing and eye-antennal imaginal discs of delayed 3rd instar larvae from homozygous l(3)tb mutant revealed significant increase in size (D,E,F,G). Length and breadth of wing discs from 3rd instar larva of l(3)tb mutant of day 6 were found to be smaller than the wing imaginal discs from wild type, but wing discs from extended larval period (day 10) showed significant increase in size (L). Metaphase chromosome preparation of brain cells (H) from wild type and l(3)tb homozygotes exhibited abnormal karyotypes (I) where l(3)tb homozygotes showed less condensed and extended chromosome morphology as compared to wild type, Oregon R+. *** denotes P < 0.005.
Eye-antennal discs and leg imaginal discs also show morphological and developmental anomalies in l(3)tb homozygous individuals

Global analysis of morphological aberrations in the mutant homozygotes showed that besides the tumorous brain and wing imaginal discs, eye-antennal discs and leg imaginal discs were also overgrown with a transparent appearance. Expression of Elav and Dlg in the eye-antennal discs revealed similarities to the developmental perturbations observed in the wing discs and brain (Figure 4S, V). In the early third instar mutant larvae (Day 6), all photoreceptor cells showed expression of Elav, similar to the wild type tissue (Figure 4J and N). However, during advanced stages of larval tumorigenesis (Day 10), it dwindled eventually (Figure 4R). The Elav expressing cells, posterior to the morphogenetic furrow, co-express Dlg and demonstrate the typical ommatidial

Figure 3 Tumorous tissues in l(3)tb homozygotes show enhanced mitotic potential. Enhanced mitotic potential observed in the tumourous tissues of homozygous l(3)tb as shown in larval whole brain (A), brain lobes (D, G) and wing imaginal discs (S) immunostained for PH3, a potent mitotic marker. Distribution of PH3 labeled cells counter stained with DAPI cells in wild type (A) and homozygous l(3)tb (Day 6 and Day 10) larval brain (D, G) and also in wild type brain lobes (B, C) and homozygous mutant brain lobes (E, F for day 6; H, I for day 10) indicated high mitotic index as compared to wild type. Similarly, more mitotic positive cells were seen in tumorous wing imaginal discs (day 10) of homozygous mutant l(3)tb (S) as compared to wild type, Oregon R+ (P). DIC images (C, F, I and R, U) illustrate external normal morphology in wild type and more pronounced tumorous phenotypes in homozygous l(3)tb larval brain and wing imaginal discs. Quantitative analysis showed increase in the number of mitotic positive cells in homozygous mutant larval brain lobes and wing imaginal discs as compared to wild type and the difference was highly significant (V). The images are projections of optical sections acquired by confocal microscopy. Staining was done in triplicates with 10 brains and 15 wing imaginal discs in each group. Significant difference is represented as *** P < 0.005 using one-way ANOVA.
In the mature mutant larvae, however, the eye discs demonstrate significant deviations from the usual regular arrangement of ommatidia. The leg imaginal discs, which reside close to the brain and wing imaginal discs also show enlargement in size that increases with advancement of larval stage. They show gradual disruption of normal expression of DE-cadherin and Armadillo (Figure 5), alike tumorous wing discs (see above), implying the mutation and subsequent tumor to affect developmental homeostasis in adjacent tissues as well.

**Figure 4** l(3)tb mutants show progressive loss of mature neurons along with increase in junction proteins. Confocal photomicrograph show loss of mature neurons and increase in junction protein, Dlg, in delayed (Day 10) homozygous l(3)tb. 3rd instar larval brain shows intense staining of Elav (green) in day 6 (D) of homozygous mutant later on show loss of staining in enlarged brain of day 10 (G), while the wild type brain (A) showed normal pattern of Elav staining. Dlg stained the ventral nerve chord and central brain in optic lobes of wild type (B), which is similar to that observed in day 6 of homozygous mutant brain (E) but in delayed larval brain, (day 10), the pattern was altered (H). Scale shown is 100μm. Neuronal tissue from eye imaginal discs also display loss of neurons seen through Elav staining in day 10 (R) as compared to day 6 (N) in homozygous l(3)tb mutant as well as to wild type (J). Pattern of junctional protein, Dlg, in eye imaginal discs is also altered in day 10 (S, V) as compared to day 6 (O) and wild type (K). Counter stain with DAPI shows very weak intensity in day 10 (T) reflecting disintegrating chromatin as compared to day 6 (P) and wild type (L). Scale bar represents 10 μm.
Genetic mapping through meiotic recombination and complementation mapping identify l(3)tb to be allelic to DCP2

The mutation l(3)tb, being recessive and on the third chromosome, was maintained with TM6B balancer. Analysis of meiotic recombination frequencies of an unmapped mutation with known markers is a classical technique that has been routinely employed to identify its cytogenetic position. In order to bring l(3)tb in a chromosome with such markers (ru cu ca), we allowed meiotic recombination to occur between l(3)tb and the eight recessive markers present on the “ruca” chromosome (Table 2). 113 recombinant males were observed and recombination frequencies were calculated in centiMorgan (cM). Table 3 shows the recombination frequencies of each marker (locus) with the mutation l(3)tb. Preliminary analysis suggested that l(3)tb was close to thread (th) with minimum recombination events between the two loci (2.65%). Further analysis of recombination events between h-l(3)tb [17.78%], st-l(3)tb [1.23%] and cu-l(3)tb [8.29%] (Table 4) and comparing with the positions of each of the markers, the mutation was estimated to be located left of thread (43.2 cM; band 72D1) between 41.71 cM–42.77 cM, i.e., at the cytological position 71F4–F5.

Figure 5 Tumor caused in the homozygous l(3)tb mutant completely alters the distribution patterns of DE-cadherin and Armadillo. Confocal images of 3rd instar larval wing imaginal discs immunolabeled to visualize the altered distribution pattern of cadherin-catenin complex proteins. Tumor caused in the homozygous l(3)tb mutant completely altered the distribution pattern of both, trans-membranous protein DE-cadherin (A, E, I, M, Q) and Armadillo (β-Catenin, B, F, J, N, R) adherens junction proteins. Alteration of both proteins is more pronounced in the wing imaginal discs from mutant larva during extended larval life (I, J) than in the early wing imaginal disc (E, F) as compared to distinct pattern of DE-cadherin (A) and Armadillo (B) in the wild type wing imaginal discs. Armadillo is a binding partner of trans-membranous protein DE-cadherin having roles in cell adhesion and regulate tissue organization and morphogenesis. Merged images also substantiate the altered distribution of both junctional proteins in the homozygous mutant (H, L) as compared to the wild type (D) where co-localization is indicated by yellow pattern. Higher magnification of wing imaginal disc (pouch region) demonstrate altered distribution pattern of DE-cadherin (Q) and Armadillo (R) in homozygous l(3)tb mutant as compared to wild type (N, R). Increase in cell size seen in homozygous l(3)tb mutant (Q) as compared to wild type (M). Complete loss of Armadillo staining observed in homozygous l(3)tb (R) whereas normal pattern seen in wild type wing disc (N). Chromatin size also altered in homozygous l(3)tb (S) as compared to wild type (O). Wild type shows clear co-localization of DE-cadherin and Armadillo (P), while there is complete loss of co-localization in homozygous l(3)tb wing imaginal discs (T). Scale bar represents 100 μm (A to L) and 10 μm (M to T).
Complementation analysis with molecularly defined Drosdel and Exelixis deficiency lines (N = 85), spanning the entire chromosome 3, identified four lines which failed to complement the mutation, viz., Df(3L)BSC774, Df(3L)BSC575, Df(3L)BSC845 and Df(3L)RM95, which was generated in the lab using progenitor RS stocks. Trans-heterozygotes l(3)tb/Df(3L)BSC575 were pupal lethal and the dying non-tubby larvae showed phenotypes similar to l(3)tb homozygotes, suggesting the mutation to reside between 71F1

Table 2 Rearranged genotypes of 113 males after various recombination events between all the eight visible markers of rucuca chromosome

| S.No. | Genotypes | No. of Flies | Status of l(3)tb locus |
|-------|------------|--------------|-----------------------|
| 1.    | ru h th + + + + + | 11 | l* |
| 2.    | ru h th st + + + + | 11 | l* |
| 3.    | ru h th st cu + + + | 11 | l* |
| 4.    | ru h th st cu sr + + | 3 | l* |
| 5.    | ru h th st cu sr e + | 6 | l* |
| 6.    | + + + + cu sr e ca | 1 | l |
| 7.    | + + + + sr e ca | 2 | l |
| 8.    | + + + + + + + + | 26 | l |

Table 3 Recombination frequencies (RF) between various recessive markers on rucuca chromosomes (roughoid, hairy, thread, scarlet, curled, stripe, ebony, and claret) and l(3)tb

| Sl. No. | Association of marker with l(3)tb | Flies | Recombinant (R) | Recombination frequency (RF) \( \times 100 \) |
|---------|----------------------------------|-------|----------------|-----------------|
| 1.      | ru - l                           | ru* l | ru* l* | 51 | 45.13 |
| 2.      | h - l                            | h* l | h* l* | 34 | 30.08 |
| 3.      | th - l                           | th* l | th* l* | 3 | 2.65 |
| 4.      | st - l                           | st* l | st* l* | 3 | 4.42 |
| 5.      | cu - l                           | cu* l | cu* l* | 8 | 7.07 |
| 6.      | sr - l                           | sr* l | sr* l* | 19 | 16.8 |
| 7.      | e - l                            | e* l | e* l* | 27 | 23.89 |
| 8.      | ca - l                           | ca* l | ca* l* | 49 | 43.3 |

Complementation analysis with molecularly defined Drosdel and Exelixis deficiency lines (N = 85), spanning the entire chromosome 3, identified four lines which failed to complement the mutation, viz., Df(3L)BSC774, Df(3L)BSC575, Df(3L)BSC845 and Df(3L)RM95, which was generated in the lab using progenitor RS stocks. Trans-heterozygotes l(3)tb/Df(3L)BSC575 were pupal lethal and the dying non-tubby larvae showed phenotypes similar to l(3)tb homozygotes, suggesting the mutation to reside between 71F1
and 72A1 on the left arm of chromosome 3. Further analysis using six deletion lines belonging to the above region (71F1–72A2) identified the mutation to reside between 71F4 to 71F5, which, strangely, is a gene desert region. Complementation analyses performed with lethal insertion alleles (N = 26) of genes residing proximal or distal to 71F4-F5 identified two lethal P-element insertion alleles of DCP2 (mRNA decapping protein 2; CG6169), viz., P[GT1]DCP2\(^{BG01766}\) and PBac\(^{RB}\)DCP2\(^{00034}\) which failed to complement the \(l(3)\)tb mutation (Figure 7A and C), as well as those deletions which had failed to complement \(l(3)\)tb, implying the mutation to be allelic to DCP2 (72A1).

| Table 4 Recombination events between \(h\)-l, \(st\)-l and \(cu\)-l |
|---------------------|----------------------|----------------------|----------------------|----------------------|
| S.No. | Association of marker with \(l(3)\)tb | Parentals (P) | Flies | Recombinants (R) | Recombination frequency |
|-------|-----------------------------------|------------|--------|----------------|------------------------|
|       | Genotype | No. of flies | Genotype | No. of flies | \(R\)/\(P\) x 100 |
| 1.    | \(h\)-l | \(h^+\) l | 89 | \(h^+\) l | 18 | 17.78 |
|       |          |           | \(h^+\) l |          | 40 |            |
| 2.    | \(st\)-l | \(st^+\) l | 252 | \(st^+\) l | 2 | 1.23 |
|       |          |           | \(st^+\) l |          | 6 |            |
| 3.    | \(cu\)-l | \(cu^+\) l | 269 | \(cu^+\) l | 22 | 8.29 |
|       |          |           | \(cu^+\) l |          | 50 |            |

Figure 6 Trans-heterozygotes of DCP2 mutants and \(l(3)\)tb show developmental delay and larval/pupal lethality. Viability assay performed on various hetero-allelic combinations between alleles of DCP2 and the mutation in \(l(3)\)tb. Homozygous \(l(3)\)tb exhibited larval as well as pupal lethality. 69% of homzygous larvae pupated whereas no fly eclosed from the pupae. \(l(3)\)tb trans-heterozygous with \(P[GT1]DCP2^{BG01766}\) showed only 18.4% fly eclosed (A). \(l(3)\)tb/\(PBac{RB}DCP2^{00034}\) trans-heterozygote (C) causes 100% lethality at pupal stage. Trans-allelic combination \(P[GT1]DCP2^{BG01766}/PBac{RB}DCP2^{00034}\) (E) also exhibited 100% pupal lethality. Developmental delay seen in trans-heterozygotes \(l(3)\)tb/\(P[GT1]DCP2^{BG01766}\) (B) and \(l(3)\)tb/\(PBac{RB}DCP2^{00034}\) (D) as in homozygous \(l(3)\)tb. Progeny from heterozygous for both the alleles of DCP2 gene, \(PBac{RB}DCP2^{00034}/P[GT1]DCP2^{BG01766}\) (F) also exhibited developmental delay. *** indicates \(P < 0.005\).
Trans-heterozygotes of DCP2 mutants and l(3)tb show developmental delay, tumorous larval brain with elevated neuroblast numbers. Heterozygous combination of l(3)tb with DCP2^{e00034} allele resulted in significant increase in the number of neuroblasts and mitotically active cells. Confocal projections showing immunolocalisation of Deadpan, a neuroblast marker (Green, A, F, K) and PH3 (red, B, G, L) marking the mitotic cells, show enhanced neuroblast population in homozygous mutant (F) and in heterozygous l(3)tb with DCP2^{e00034} allele (K). Similarly, increased number of mitotic cells (PH3 positive) are also observed in heterozygous l(3)tb with DCP2^{e00034} allele (L), similar to homozygous l(3)tb mutant (G). Quantitative analyses of NBs and mitotically active cells show significant differences when compared with the wild type (P). *** P < 0.005. Scale bar indicates 50 μm.

Figure 7 Trans-heterozygotes of DCP2 mutants and l(3)tb show tumorous larval brain with elevated neuroblast numbers. Heterozygous combination of l(3)tb with DCP2^{e00034} allele resulted in significant increase in the number of neuroblasts and mitotically active cells. Confocal projections showing immunolocalisation of Deadpan, a neuroblast marker (Green, A, F, K) and PH3 (red, B, G, L) marking the mitotic cells, show enhanced neuroblast population in homozygous mutant (F) and in heterozygous l(3)tb with DCP2^{e00034} allele (K). Similarly, increased number of mitotic cells (PH3 positive) are also observed in heterozygous l(3)tb with DCP2^{e00034} allele (L), similar to homozygous l(3)tb mutant (G). Quantitative analyses of NBs and mitotically active cells show significant differences when compared with the wild type (P). *** P < 0.005. Scale bar indicates 50 μm.

Trans-heterozygotes of DCP2 mutants and l(3)tb show developmental delay, tumorous larval brain with elevated neuroblast numbers, larval/pupal lethality and developmental defects in escapee flies

Trans-heterozygotes of l(3)tb with two known alleles of DCP2, viz., P(GT1)DCP2^{R101766} and PBac[RB]DCP2^{e00034}, showed developmental delay and third instar larvae persisted in the same stage till Day 10 ALH (Figure 6B and D), and showed tumorous phenotypes of brain and wing imaginal discs (Supplementary Figure S4), similar to the l(3)tb homozygotes. The expression of Deadpan (Dpn), a marker for neuroblasts, shows an increased number of neuroblasts in the larval brain of the trans-heterozygotes as well as l(3)tb homozygotes (Figure 7F, K and P). Also, the trans-heterozygous progeny showed a higher mitotic index than the wild-type progeny, similar to the l(3)tb
homozygotes (Figure 7G, L and P). While PBac(RB)DCP2\00034/l(3)tb was found to be 100% pupal lethal, P(GT1)DCP2BG01766/l(3)tb was only 81.6% lethal (Figure 6A and B), with the rest 18.4% pupae eclosing as flies. However, the escape flies showed several developmental abnormalities, viz., defects in the wing (9.5%), thorax closure (3.2%), loss of abdominal para-segments and abdominal bristles (3.2%), and presence of melanotic patches (22.2%), leg defects (41.3%) or eclosion defects (12.7%) (Supplementary Figure S2). Analysis of compound eyes in these escapees revealed complete loss of regular arrangement of ommatidia and ommatidial bristles

| Cross | l(3)tb/P(GT1)DCP2BG01766 (males) X +/+ (Virgin females) | l(3)tb/P(GT1)DCP2BG01766 (Virgin females) X +/+ (males) |
|-------|--------------------------------------------------------|--------------------------------------------------------|
| Total No. of Pair Mating | 70 | 83 |
| Fertile | 28 (40%) | 18 (21.7%) |
| Sterile | 42 (60%) | 55 (66.3%) |

**Table 5** Fertility assay of trans-heterozygotes P(GT1)DCP2BG01766/l(3)tb demonstrating male and female sterility

Figure 8  
DCP2\176b\ is an insertion allele of DCP2. Gel electrophoretogram showing the PCR analysis of the full-length DCP2 (A) and candidate region (B) in the wild type, mutant and the heterozygote. The schematic in C shows the gene arrangement along the chromosome along with the important coordinates. The primers are indicated by red arrows. Amplification of the full length gene shows that the wild type amplicon is of 8.6 kb while the mutant amplicon is sized ~17 kb (A, upper half), whereas the wild type amplicon for the candidate region is of 945 bp while the mutant amplicon is sized ~8.5 kb (B, upper half). The heterozygote harbors both the alleles (wild type and mutant) and thus shows both the amplicons. The lower half in both A and B shows the the blot of the same probed with the pGEM-T-812 probe which spans the candidate mutated region in DCP2 and is represented by the purple line. D shows the gel electrophoretogram and Southern blot of DCP2 in the wild type and mutant genome. HindIII digested genomic DNA showed banding at ~2.1 kb in the wild type genome as against ~10 kb in the mutant genome, the size difference being almost in agreement with the banding profile exemplified by BamHI digestion, with the wild type genome hybridizing at ~10.2 kb and the mutant at ~18 kb.
Abnormal external genitalia were also observed in the male escapees (data not shown). Subsequent analysis showed that the trans-heterozygous escapee flies had compromised fertility, with only 40% of the males and 21.7% of the females being fertile (Table 5).

The similarity in the pattern of development and the defects associated between the l(3)tb trans-heterozygotes and homozygotes provide strong genetic proof of allelism between l(3)tb and DCP2.

DCP2\(^{(3)tb}\) is an insertion allele of DCP2

Fine mapping, performed by overlapping PCR, identified the region (Supplementary Figure S5), amplified by the primer pair, Dbo_F and Dcp2_P19_R, to span the candidate region. The region, which is 945 bp (3L: 15826279..15827223) in the wild type and comprises of 5’UTR coding region of DCP2, the adjacent intergenic sequence and the proximal part of the neighboring gene, dbo, showed absence of amplification in the DNA of l(3)tb homozygotes, highlighting it as...
The DCP2_l(3)tb genome harbors Gypsy-LTR Like sequence in 5’ UTR coding region of DCP2 and expansion of adjacent upstream intergenic AT-rich sequence

To identify the functional genomics of mutations, it is essential to deduce the nucleotide sequence of the mutation. Thus a convergent bi-directional primer-walk was initiated with the primer pair, which identified the insertion in DCP2 in the l(3)tb genome. On sequencing, the DCP2-proximal end showed the presence of wild type sequence till 3L: 15826410 after which a 444 bp AT-rich sequence was detected (Supplementary Figure S6 B-1), which did not show any resemblance with the wild type sequence present at the region whereas the dbo-proximal end showed complete wild type sequence profile (3L: 15827143..15826738) (Supplementary Figure S6 B-2). On a homology search to identify the novel sequence obtained, the sequence showed homology with the Gypsy LTR sequence of Drosophila. On searching for DCP2 promoters in the Eukaryotic Promoter Database, SIB and aligning the sequence coordinates of the 444 bp insertion, it was found that the insertion is downstream to the transcription start site (TSS) of DCP2, which is at 3L: 15826420. Subsequently, long-range PCR was first performed with DCP2_l(3)tb and wild type genomic DNA with a new pair of primers from the distal part of the reads obtained above. Although no amplification was observed with the wild type DNA, the DCP2_l(3)tb DNA showed amplicons of sizes ~7.2 kb, ~3 kb, ~2.8 kb and ~430 bp with the 430 bp amplicon showing the highest concentration as observed from the electrophoretogram (Figure 9B). This amplification profile resembled that of tandem repeat bearing regions. To confirm the repetitive nature of the sequence, Southern hybridization was performed with the same electrophoretogram. The 430 bp amplicon was eluted from the gel, cloned in pGEM-T vector and used as a probe. The probe showed complete hybridization with all of the amplicons indicating the repetitive nature of the sequence present downstream (Figure 9B). Sequencing of the 430 bp amplicon revealed an AT-rich sequence. Homology search identified the sequence to be homologous to the distal part of the DCP2 UTR and the adjacent intergenic sequence between DCP2 and dbo, the coordinates being 3L: 15826407..15826716. After aligning the
DCP2 rescues the mutant phenotypes exhibited by \( l(3)tb \) homozygotes

\[
\begin{align*}
\text{Act5C-GAL4/CyO} & +/+ & +/+ & +/+ +/+ & \text{Tub-GAL4/TM6B} & \text{Sp/CyO} & \text{l(3)tb: UAS-DCP2/TM6B} & \text{Sp/CyO} & \\
\text{Homozygotes die as embryos} & & & & & & & & \text{and CyO TM6B homozygotes die as embryos or early larvae}
\end{align*}
\]

Homozygotes die as embryos

Table 6: Global overexpression of d(sal)–>DCP2< (italic) rescues the mutant phenotypes exhibited by \(<\text{sal}(3)tb\)<(italic) homozygotes

| Genetic Crosses | Act5C-GAL4/CyO | +/+ | +/+ | +/+ | Tub-GAL4/Sp | +/+ | l(3)tb:UAS-DCP2/Sp | +/+ | l(3)tb:UAS-DCP2/TM6B | +/+ |
|-----------------|----------------|-----|-----|-----|-------------|-----|-------------------|-----|-------------------|-----|
| Genotype        | UAS-DCP2/CyO  | X   | X   | X   | Tub-GAL4/Sp | +/+ | Sp/CyO l(3)tb: UAS-DCP2/TM6B | +/+ | Sp/CyO l(3)tb: UAS-DCP2/TM6B | +/+ |
| Eggs            | 750            | 1050| 1245| 790 | 425         | 29  | 37                | 37  | 31                | 31  |
| Unfertilized    |                |     |     |     |             |     |                    |     |                    |     |
| Eggs            |                |     |     |     |             |     |                    |     |                    |     |
| Fertilized      |                |     |     |     |             |     |                    |     |                    |     |
| Eggs            |                |     |     |     |             |     |                    |     |                    |     |
| Dead Embryos    |                |     |     |     |             |     |                    |     |                    |     |
| Dead Embryos    |                |     |     |     |             |     |                    |     |                    |     |
| Dead 1st instar |                |     |     |     |             |     |                    |     |                    |     |
| Dead 2nd instar |                |     |     |     |             |     |                    |     |                    |     |
| Dead 3rd instar |                |     |     |     |             |     |                    |     |                    |     |
| Dead Pupae      |                |     |     |     |             |     |                    |     |                    |     |
| Dead Pupae      |                |     |     |     |             |     |                    |     |                    |     |
| Eclosion        |                |     |     |     |             |     |                    |     |                    |     |
| Eclosion        |                |     |     |     |             |     |                    |     |                    |     |

DCP2\(^{3(3)tb}\) is a DCP2 hypomorph along with low expression of the neighboring gene, dbo

Following the identification of the genomic architecture of the allele, it was imperative to determine the expression potential of the allele. Semi-quantitative RT-PCR analyses confirmed the hypomorphic nature of the allele wherein the homozygous \( l(3)tb \) mutant showed extremely low levels of expression of DCP2 (Figure 9F). Since the intergenic region between DCP2 and dbo is upstream to either gene and bears an expansion, dbo transcript titre were also examined wherein they showed extremely lowered expression (Figure 9F). At present, it is doubtful whether the lowered dbo level is a cause or an effect of the mutation since dbo (Smac/Diablo/Henji) is a pro-apoptotic molecule which inhibits the inhibitor of apoptotic proteins (IAP), and its lowered levels, therefore, serve as a prognostic marker of tumor progression in human carcinomas (Martinez-Ruiz et al., 2008). Again, dbo expresses strongly in the neuronal tissues and localizes at the synapse (Wang et al., 2016) where its perturbation causes alteration in neuromuscular function. Despite being a multifaceted molecule, ubiquitous knock-down of dbo does not result in developmental abnormalities, viz., developmental delay, lethality or tumorous phenotypes in the brain, and/or wing imaginal discs (Supplementary Figure S7).

The tumor caused by DCP2 is hyperplastic with elevated Cyclin A and E

Since the mutation showed all the hallmarks of classical tumor suppressors (Merz et al., 1990), we sought to characterize the perturbations in cellular physiology caused in the wake of tumorigenesis. RT-PCR analyses depicted elevated levels of Cyclin E (G1/S phase cyclin) and A (G2/M phase cyclin), which are indicative of increased cell proliferation and rapid cell cycles (Figure 10C). Immunolocalization studies also confirmed the elevated expression of Cyclin E as well (Figure 10A and B). On observing closely, the regular arrangement of cells in the brain hemisphere and optic lobes in the wild type was severely disrupted in the mutant along with excessive growth and increased number of mitotic nuclei. The enlarged brain lobes, increased number of mitotic nuclei and disruption of the regular arrangement of cells in the mutant concomitant with elevated expression of cyclins A and E validated the tumourous nature of the mutant.
When the tumorous brains (Figure 10D) and wing discs (Figure 10E) were examined for the expression of the polarity marker Discs large (Dlg), both tissues did not show appreciable loss of polarity. On a closer look, the wing discs at 138 h AEL showed an increase in cell number concomitant with a decrease in cell size (Figure 10E-C). At the same stage, the tumorous brain shows an increased number of cells in the optic lobe (Figure 10D). Usually, neoplastic tumors are metastatic and the tumor cells lose their polarity to acquire the mesenchymal-like fate, delaminate from the matrix and migrate. In contrast, hyperplastic tumors do not show appreciable loss of polarity even in later stages of tumourigenesis, since in these tumors the cells do not delaminate but remain adhered to the original tissue matrix, concomitant with cell division (Miles et al. 2011). The expression pattern ofDlg showed retention of polarity at 138 h of development, which is an exceptionally late and delayed 3rd instar larval stage, implying that tumor is an over-proliferative and hyperplastic. Again, this is in agreement with the Cyclin E staining pattern and taken together that tumor is an over-proliferative and hyperplastic. Hence, DCP2 has an unexplored role in the development and/or cell cycle progression across phyla, which needs to be investigated. Since the physiology of an organism is tightly regulated by the optimized titres of gene expression programs, a global loss of DCP2 may lead to perturbation in mRNA titles, which may alter the cellular response to such dismal conditions and eventually lead to drastic physiological disorders such as tumourigenesis. The exact mechanism(s) by which a loss of DCP2 leads to tumourigenesis needs further analysis and identification of the novel allele, DCP2\textsuperscript{f039b}, unravels that DCP2 could be a probable candidate for future explorations of tumourigenesis.

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