Involvement of the mitochondrial protein translocator component Tim50 in growth, cell proliferation and the modulation of respiration in Drosophila

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ABSTRACT

Allelic mutants exhibiting growth defects in Drosophila were isolated. Molecular cloning identified the responsible gene as a budding yeast Tim50 ortholog, thus it was named tiny tim 50 (ttm50). The weak allele (ttm50Gp99) produced small flies due to reduced cell size and number, and growth terminated as larvae in the strong alleles (ttm50IE1 and ttm50IE2). Twin-spot analysis showed fewer cells in ttm50Gp99 clones, whereas ttm50IE1 clones did not proliferate, suggesting the gene has an essential cellular function. Tim50 is known to maintain mitochondrial membrane potential (MMP) while facilitating inner-membrane protein transport. We found that tagged Ttm50 also localized to mitochondria, and that mitochondrial morphology and MMP were affected in mutants, indicating mitochondrial dysfunction causes the developmental phenotype. Conversely, ttm50 over-expression increased MMP and apoptosis. Co-expression of p35 suppressed this apoptosis, resulting in cell over-proliferation. Interestingly, ttm50 transcription was tissue specific, corresponding to elevated MMP in
the larval midgut, which was decreased in the mutant. The correlation of $ttm50$ expression levels with differences in MMP, match its proposed role in mitochondrial permeability barrier maintenance. Thus a mitochondrial protein translocase component can play active roles in regulating metabolic levels, possibly for modulation of physiological function or growth in development.
INTRODUCTION

Sizes of organs and organisms in each species are the result of the regulation of cell size and cell number (for reviews, see Neufeld and Edgar 1998; Polymenis and Schmidt, 1999). Recent advances in the field of growth regulation have revealed the involvement of multiple signaling pathways including the insulin/TOR pathway (for a review, see Oldam and Hafen, 2003), the Myc pathway (Johnston et al., 1999; Trumpp et al., 2001; de la Cova et al., 2004) and the Ras pathway (Prober and Edgar, 2000). These findings suggest that nutrition levels are major input signals for this signaling network and that the output is the rate of protein synthesis causing changes in cell growth and proliferation. It would also be expected that intracellular energy levels affect cell growth and proliferation, although it is yet unproved whether such mechanisms are actually utilized in developmental growth regulation.
Intracellular energy levels are largely dependent on the synthesis of ATP in the mitochondria and their dysfunction causes severe developmental defects. In *Drosophila melanogaster*, mutants of nuclear coded mitochondrial proteins, including DNA polymerases and ribosomal proteins, are reported to demonstrate growth retardation, indicating that they at least have a passive role in the regulation of development (Iyengar *et al.*, 1999; Maier *et al.*, 2001; Iyengar *et al.*, 2002; Galloni, 2003; Frei *et al.*, 2005).

About 99% of mitochondrial proteins are encoded by the nuclear genome. After being synthesized by the cytoplasmic ribosomes, they are translocated into mitochondria by protein translocases (for reviews, see Rehling *et al.*, 2003; Endo *et al.*, 2003; Rehling *et al.*, 2004). In *Caenorhabditis elegans*, it has been shown that this transport of mitochondrial proteins is essential for mitochondrial biogenesis, and defects in the components of the translocases cause severe developmental aberrations (Curran *et al.*, 2004). Mitochondrial architecture consists of the outer membrane, the intermembrane space,
the inner membrane, and the matrix. Mitochondrial proteins are translocated into the intermembrane space or embedded into the outer membrane by the TOM complex. Many of the proteins translocated into the intermembrane space are further embedded in the inner membrane or translocated into the matrix by the TIM22 or TIM23 complexes. The TIM23 complex consists of Tim23, Tim17 and Tim50, and is involved in the translocation of proteins with positively charged presequences (for reviews, see Rehling et al., 2003; Endo et al., 2003; Rehling et al., 2004).

In this article, we show that Drosophila mutants of a gene similar to yeast Tim50 are defective in growth and cell cycle and exhibit reduced mitochondrial membrane potential (MMP). Overexpression of the protein induced increased MMP, apoptosis and in certain circumstances extra cell proliferation.
MATERIALS AND METHODS

Genetics: Transposon mutagenesis for X chromosomal recessive lethal mutants was performed by re-mobilizing the multiple incomplete P elements on the second chromosome of the Birmingham 2 strain with a supply of the transposase from PA2-3-32 (Robertson et al., 1988). Additional alleles were obtained by EMS mutagenesis. yellow (y) male flies were fed with 0.01M EMS and mated with C(1)DX, y w/5–Y, y′ w′ females, and the resultant male progenies were singly crossed with y ttm50^{599}/Binsc females to screen alleles.

Molecular techniques: Standard molecular techniques were employed (Sambrook et al., 1989). The DNA isolated from y ttm50^{599}/Binsc female flies was partially digested with Sau3A and the fragments were ligated with EMBL3 arms. The ttm50^{599} genomic library was screened with pπ25.1 (Rubin and Spradling, 1983) as a probe to recover genomic DNA flanking the P element inserted in ttm50^{599}. The resultant clone was then used to probe a wild-type Drosophila genomic library (Tsuda et al., 1993). Fragments derived from the wild-type
genomic clone were used as probes for cDNA library screening. A 12–24 hr embryonic cDNA library in pNB40 (Brown and Kafatos, 1988) was screened and a full length clone was obtained. The cDNA and genomic fragments were sequenced for both strands. Similarity searches were carried out using FASTA (Wisconsin GCG) and NCBI BLAST programs.

Transgene Constructs: The 3.5kb SacII/EcoRI genomic fragment for mutant rescue was cloned into the P element vector pW8 and used for transformation. The GMR element mediated expression construct was made by recloning the \textit{ttm50} containing \textit{EcoRV/EcoRI} fragment from the pNB40 cDNA clone into a pBST(SK+) vector, then again recloning the \textit{EcoRV/SmaI} fragment into the pGMR expression vector (Hay \textit{et al.}, 1994). The hemagglutinin (HA) epitope tagged Ttm50 expression construct was produced by first PCR amplifying the pNB40 cDNA with an \textit{EcoRI} site-incorporated 5' primer (GAATGTAAGGCTCTAGGACTC) and a \textit{XhoI} site-incorporated 3' primer (CTGAGCGTGCTCCACATCTGCT) and cloning it into the \textit{EcoRI/XhoI} sites of the pUAST expression vector (Brand and Perrimon, 1993) via a pGEM(TA) vector (Promega). This was cut with
Eco52I and a NotI fragment containing a triple HA epitope was inserted resulting in a C terminal tagged construct that was confirmed by sequences and used for transformation. Expression was induced using a heat-shock GAL4 transgene (Brand and Perrimon, 1993), and expression of the correctly sized tagged protein checked by western blotting.

Clonal Analysis: X-ray clones were induced by irradiation at a dose of 1.5kR using an Ohmic OM-100RE soft X-ray unit equipped with a 0.2mm aluminum filter. Flp recombinase clones were produced by recombinating mutant alleles onto X chromosomes carrying the p[2 π M]FRT construct at18A, crossing these with Ubi-GFP, p[2 π M]FRT 18A and inducing mitotic recombination with heat shock-induced flp-recombinase expression (Golic, 1991).

Histochemistry: Mitotracker Red (Molecular Probes) was used at 10-100nM, JC-1 (Molecular Probes) at 25-250nM and Acridine Orange (Wako Fine Chemicals) at 1uM was used in PBS or Grace’s medium with unfixed samples or before fixation. Concentrations were adjusted so as to obtain uniform staining in control sample tissues.
Fixation of heptane permeablized dechorionated eggs or dissected tissues were with 4% paraformaldehyde in PBS. DAPI (Wako Fine Chemicals) was used at 1 ng/ml, Rhodamine or Alexa Fluor 647-conjugated phalloidin (Molecular Probes) at 0.2 U/ml in PBS containing 0.1% Triton-X. HA tagged proteins were detected by indirect immunofluorescence using rabbit polyclonal anti-HA Y-11 serum (Santa Cruz) at 1:500 followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG serum (Molecular Probes) at 1:1000 dilutions.

*In situ* hybridization was done according to the method of Tautz and Pfeifel (1989) with standard modifications for RNA probes and pre-stain-washes in maleic acid buffer. The probes were transcribed from PCR products with T7 promoters incorporated into the primers during amplification from cDNA. Sense strand probes were used as non-specific staining controls. Bright field and fluorescent images were taken on a Nikon Eclipse E800 microscope with a DXM 1200F digital camera, SEM images on a Phillips XL 20 scanning electron microscope,
and confocal images on a Zeiss Axiovert 100M equipped with an LSM 510 module.
RESULTS

Isolation of the mutants: Among a collection of newly induced P-insertional mutations, an X-linked semi-lethal line, Gp99, was obtained. Under standard culture conditions, few mutant flies appeared. When the mutant chromosome was marked with yellow (y) and the y hemizygous male larvae were separately cultured in uncrowded conditions, 60 to 80% of the mutant larvae developed into adults but grew slowly (Figure 1D). Mutants took about three more days to develop as compared to the normal 10 days developmental time. The mutant is considerably smaller than the control (Figure 2, A and B). The weight of the mutant male flies (0.65 ± 0.06 mg, n = 483) was about 35% reduced as compared to the control y hemizygous flies (1.01 ± 0.04 mg, n = 347).

To examine whether reduced body size might be due to reduced cell size, we compared the density of wing hairs (cells) within a fixed area with an arbitrary width just behind the posterior cross vein. In the mutant, 125.5 ± 5.1 (n = 22) hairs were counted while
109.2 ± 5.1 (n = 15) hairs were counted in the control. This roughly corresponds to a 13% reduction in cell width, and partially explains the reduction of body size. The number of hairs along the posterior cross vein was also reduced in the mutant (30.0 ± 2.2 (n = 21)) as compared to the control (35.1 ± 2.6 (n = 14)), although the shape and vein pattern of the wing appeared normal. In addition, the macrochaetae (bristles) are considerably thinner and shorter than the control (Figure 2, A and B). Because they are secreted by a single polytene cell (shaft cell) that is much larger than the average diploid cell, the observed decrease in bristle size could be a result of defective cell growth or cell cycle progression.

Twin-spot analysis (mitotic clone analysis) utilizing flp recombinase showed that mutant clones induced in imaginal discs were consistently lower in cell number than their wildtype sister clones (Figure 2H), whereas wildtype control clones were the same size as their sister clones (Figure 2G). Acridine orange staining of the imaginal discs did not show increased cell death (data not shown).
Thus, we conclude that the diminuitive body size in the mutant is due to a reduction in both size and number of cells caused by a cell growth and proliferation defect.

The mutant males exhibited reduced fertility but crossings with heterozygous females produced homozygous females. These laid eggs but none of the eggs hatched. Examination of the embryos revealed a maternal effect on cleavage divisions (Figure 2, D–F) although some embryos continued development past gastrulation. Those arrested in cleavage divisions seem to be at around cycle 8 (Figure 2D), and most of the nuclei are at M phase (Figure 2E). Polyploid nuclei were observed sporadically (Figure 2F), suggesting that some nuclei failed to complete nuclear division, but continued through DNA replication only to fail again in mitosis afterwards. This could be because the G1–S check point is not in effect at this developmental stage (Sibon et al., 1997).

As cleavage divisions consist of only S and M phases, we also examined the mitotic index in the brain squashes from third instar
larvae (Table 1). In the larval brain lobes, cell cycles proceed through all four phases. If the mitotic defect were the major cause of cell cycle arrest, an increase of mitotic index would be expected. As shown in Table 1, the mitotic index was considerably reduced, suggesting that the cell cycle is affected during a phase(s) other than M phase. As aberrant mitotic figures were frequently observed in the brain squashes, mitosis is also affected. Thus, both mitosis and interphase are affected in this mutant.

Since Gp99 is a hypomorph (see below), we tried to obtain strong alleles by EMS mutagenesis. Two additional alleles, IE1 and IE2, were isolated. IE1 is thought to be null (see below), and the growth of the mutant larvae was severely affected and the larvae died as 1st instar larvae after surviving for about one week (Figure 1B). The molecular defect of IE2 was not identified, but the growth of the mutant larvae was also severely affected and died as larvae after surviving a considerable period (Figure 1C). These phenotypes resemble those of typical growth defect mutants such as peter pan (Migeon et al., 1999),
bonsai (Galloni, 2003), and the mutants in the insulin/TOR network (Chen et al., 1996; Böhni et al., 1999; Montagne et al., 1999; Weinkove et al., 1999; Oldham et al., 2000; Zhang et al., 2000; Rintelen et al., 2001; Oldham et al., 2002; Saucedo et al., 2003; Stocker et al., 2003).

Clonal analyses of IE1 produced clones of single or two cells in contrast to the control twin spots with large numbers of cells in wings (Figure 2I), indicating a loss of cell proliferation ability in the null mutant. Germ line clones of IE1 could not be obtained.

Cloning and identification of the gene: Using the P element as a probe we cloned the DNA fragment flanking the insertion site in Gp99, and the wild type locus was also cloned (see Materials and Methods). The P element is inserted in the 5' -UTR of the transcription unit, CG2713 (Figure 3A) mapping to 3B3 on the X chromosome. Excision of the P element using transposase reverted the mutant phenotypes of Gp99. Furthermore, a 3.5 kb SacII/EcoRI genomic fragment containing the intact gene but only portions of the neighboring genes (Figure 3A)
completely rescued the bristle phenotype of Gp99 and the viability of all 3 alleles showing that CG2713 is the responsible gene. A cDNA clone was isolated and sequenced. Comparison with the genomic sequence revealed the presence of four short introns and an ORF encoding a protein of 428 amino acid residues. These sequences did not differ significantly from those published by the Berkeley Drosophila Genome Project. Two more related sequences are present in the Drosophila genome (Figure 3B). A search of the Genome Databases revealed that the sequence at the carboxyl-terminal portion is highly conserved in human and nematode (Figure 3B). The sequence showed a low but significant similarity to the phosphatase for the C-terminal domain (CTD) of RNA polymerase II. Similar sequences were also found in budding and fission yeasts. The budding yeast sequence has been reported to encode Tim50, which is located in the inner membrane of mitochondria and is involved in protein translocation (Geissler et al., 2002; Yamamoto et al., 2002) while maintaining mitochondrial membrane potential (MMP) (Meinecke et al., 2006). Drosophila sequences contain a putative,
positively charged presequence at the N-terminal ends and a putative transmembrane domain, suggesting that these gene products themselves also translocate into mitochondria, and are embedded in the inner membrane. From the phenotype and sequence homology we named the genes tiny tim50 (ttm50), tiny tim2 (ttm2: CG12313), and tiny tim3 (ttm3: CG6691). The ttm50 mutant lines are indicated as ttm50<sup>0</sup>, ttm50<sup>E1</sup> and ttm50<sup>E2</sup> hereafter.

Sequencing of the PCR amplified ORF fragments from genomic DNA of ttm50<sup>E1</sup> revealed a point mutation altering guanine to adenine, which causes a change of Trp<sup>197</sup> to a TAG termination codon (Figure 3A). This causes a truncation before the conserved region, and the mutation is interpreted to be null. We could not find any non-silent alterations in the ORF of ttm50<sup>E2</sup>, and the mutation may be in the regulatory sequence.

Ttm50 is a mitochondrial protein: As Ttm50 is similar to yeast Tim50, we examined whether Ttm50 is also localized in mitochondria by expressing a HA-tagged Ttm50 protein using the GAL4/UAS system (Brand
and Perrimon, 1993). This transgene was able to rescue the \texttt{ttm50}^{HA} mutant lethality indicating it is functional. When Ttm50-HA protein was expressed using various \texttt{GAL4} drivers, the presence of Ttm50-HA was observed in a reticulated pattern in the cytoplasm in tissues such as the CNS, imaginal discs, salivary glands and fatbodies of larvae (Figure 4A). Co-staining with Mitotracker Red which stains mitochondria specifically, showed an overlapping pattern, demonstrating that Ttm50-HA is localized to mitochondria (Figure 4C) as anticipated from its sequence homology.

Northern blot and real time RT-PCR analyses demonstrated that \texttt{ttm50} is expressed throughout development (data not shown). \textit{In situ} hybridization in embryos demonstrated a large amount of mRNA in the cleavage division stage embryos (Figure 5A), suggesting a contribution by maternal mRNA. After gastrulation, \texttt{ttm50} is highly expressed in the developing midgut (Figure 5, C-H). In adult flies, significant expression of \texttt{ttm50} was detected in isolated testis as well as the remaining carcass, whereas \texttt{ttm2} and \texttt{ttm3} are expressed exclusively in
the testis when analyzed by real time RT-PCR (M. Nishimura, T. Yamamoto, S. S. and Y. N., unpublished observations).

To evaluate the effect of the ttm50 mutations on mitochondrial activity, the body wall of control and 2nd instar ttm50²² larvae were either ripped to allow the gut to spill out (Figure 6A), or the entire larvae was completely inverted, and then stained with Mitotracker Red. Mitotracker Red (chloromethyl-X-rosamine) is a lipophilic cationic dye which accumulates in mitochondria due to their negative membrane potential and is a sensitive non-toxic relative indicator of MMP although it also reflects changes in mitochondrial mass (Pendergrass et al., 2004). We found that mitochondrial activity was strongest in the gut corresponding to the transcription pattern observed in the late embryo. Gut staining in the mutant was weaker than wildtype (Figure 6B). Results were similar when the larvae were fed Mitotracker Red and the anterior midgut epithelia compared (Figure 6, C-F) as described by Galloni (2003). Since Mitotracker Red staining is
dependent on MMP, this indicates that respiratory activity is reduced in the mutant midgut.

When mitotic clones were X-ray induced in \textit{ttm50}^{E1} heterozygous embryos and observed at larval stages, polyploid clone cells identified in the salivary gland by reduced GFP fluorescence showed weakened Mitotracker Red staining compared to surrounding cells (Figure 6, G–J). The network of mitochondria appeared less developed and the staining intensity of individual components was reduced. Thus it appears that there was a decrease in overall volume of mitochondria in the cytoplasm in addition to a drop in MMP, leading to a combined decline in mitochondrial activity.

Overexpression of \textit{ttm50} can cause increased mitochondrial membrane potential, apoptosis and an increase in cell proliferation: When the Act5C–GAL4 driver was used to express \textit{ttm50} cDNA ubiquitously, some of the transgenic lines showed lethality at different stages. \textit{UAS-ttm50}^{P18} was lethal at an early larval stage, \textit{UAS-ttm50}^{P2} died as pharate adults and \textit{UAS ttm50}^{P12} was viable with no visible phenotypes,
but was still able to rescue the lethality of \textit{ttm}50^{E2}. These differences in phenotype probably reflect differences in strength of expression due to a "position effect" of the chromosomal sites in which the transgene was inserted. Thus overexpression of \textit{ttm}50 appears to be lethal and indicates that a moderated level of expression needs to be maintained for proper function and viability.

The intermediate strength \textit{UAS-ttm}50^{E2} transgene was next expressed in the eye under the control of the GMR-GAL4 driver, which expresses target sequences posterior to the morphogenetic furrow in the developing eye disc by utilizing the \textit{glass}-mediated response element (Hay \textit{et al.}, 1994). This caused a mild rough-eye phenotype indicating disruption of ommatidial cell organization (Figure 7B and data not shown). To check whether this rough-eye phenotype was caused by cell death, the deficiency \textit{DF(3R)H}99, which uncovers a set of the proapoptotic genes; \textit{reaper (rpr)}, \textit{head involution defective (hid)} and \textit{grim} was used. The gene dosage of these genes was reduced by half in flies expressing \textit{UAS-ttm}50^{E2} under the control of the GMR-GAL4. The
deficiency suppressed the rough eye phenotype suggesting that over-expression of *ttm50* was inducing apoptosis (Figure 7C). It was then found that a half reduction of *hid* gene dosage alone is sufficient for the suppression (Figure 7D), suggesting that *hid* is the main factor responsible. On the other hand, a half reduction of the gene dosage of the inhibitor of apoptosis protein gene, *DIAP1* enhanced the rough eye phenotype (Figure 7E). This was expected because *DIAP1* is negatively regulated by *hid*. Staining of the larval eye discs with acridine orange demonstrated increased cell death supporting the above results (Figure 8B).

In contrast to over-expression of *ttm50*, imaginal discs from the loss-of-function mutant, the hypomorph *ttm50^{G999}* demonstrated no evidence of increase of cell death by the TUNEL assay (data not shown). This differs from observations with human *Tim50*, whose down-regulation of expression increased the sensitivity to death stimuli in cultured cells (Guo *et al.*, 2004).
When the baculovirus P35 caspase inhibitor was co-expressed with ttm50 in the compound eyes to suppress cell death, the rough eye phenotype was almost completely suppressed (Figure 8C). At the same time the number of ommatidia was increased (798.5 ± 11.6, n=13; Figure 7F) as compared to the normal eyes (752.5 ± 23.5, n=13; Figure 7A). Expression of p35 alone did not increase the number of ommatidia (754.0 ± 10.8, n=6). The results indicate that over-expression of ttm50 induces cell proliferation when cell death is inhibited by p35 co-expression.

This was confirmed by localized over-expression in the larval wing imaginal disc using the engrailed (en) -GAL4 expression driver construct. The localized over-expression of UAS- ttm50 with p35 co-expression caused an increase in nuclear density (Figure 9A), and an increase in cell proliferation as determined by the ratio of S-phase cells detected by BrdU incorporation (Figure 9F). It also resulted in an increase in MMP as judged by staining intensity of Mitotracker Red (Figure 9H) and JC-1 (not shown). Thus raising the level of ttm50
expression causes an increase in respiratory activity and in addition stimulates cell proliferation when P35 is present to inhibit apoptosis.

The increase in Mitotracker Red staining was also observed without P35 co-expression, and was not seen with P35 expression alone (data not shown). This further suggests that controlling the selective permeability of the mitochondrial inner membrane through the expression level of ttm50 results in corresponding changes in MMP and presumably the resulting cellular energy output.
DISCUSSION

Mutants of tiny tim50 (ttm50) which encodes a Drosophila ortholog of Tim50 showed systemic growth defects, such as delayed development and reduced cell size and cell proliferation, whereas over-expression of ttm50 induced cell death, and extra cell proliferation when that cell death was artificially inhibited. These phenotypes correlated well with the presumed roles of Ttm50 in mitochondrial physiology, with the loss of Ttm50 affecting mitochondrial morphology and causing a drop in MMP, while its over-expression caused an increase in MMP. Expression of ttm50 during development was found to be tissue specifically regulated, and its expression levels matched the MMP observed in tissues, thus ttm50 may also have specific roles in tissue development and physiology.

Ttm 50 function in mitochondria and its relation to observed phenotypes: Whereas loss of ttm50 in mutants reduced MMP, its overexpression elevated MMP. The maintenance of MMP is essential for
ATP synthesis by the mitochondria, thus transport through the inner membrane is highly selective to maintain the proton gradient. The Tim23 complex serves as the channel for preproteins and the presequences in preproteins induce rapid gating of the channel by Tim50 (Meinecke et al., 2006). The function of Tim50 is to keep the channel closed in the absence of presequences, so the loss or reduction of its homolog Ttm50, would be expected to result in a “leaky” membrane with a decrease in MMP. Such a decrease was observed in mutants in this study and has been reported for Tim50 mutants in budding yeast (Meinecke et al., 2006). Extending this logic, too much Ttm50 could cause an overly tight permeability barrier with an increase in MMP as was observed in our over-expression experiments. Alternatively, reduced protein import by loss of Ttm50 could cause a general dysfunction in mitochondria leading to MMP decrease, whereas over-expression could cause increased protein import resulting in increased MMP due to a rise in overall mitochondrial metabolism.
Both M phase and interphase cell cycle defects were found in 
\textit{ttm}^{299} \textit{mutants}, although the M phase defects were prominent only during 
the cleavage stage when the G1/S phase check point is not in place. 
Malnutrition usually affects G1 phase, and mutants of the \textit{tenured} gene, 
which encodes cytochrome oxidase subunit Va, have been shown to cause 
drops in intracellular ATP levels that results in cell cycle arrest at 
the G1–S cell cycle check point. This occurs through a pathway 
involving AMP–activated protein kinase and p53 activation that causes 
elimination of Cyclin E (Mandal \textit{et al.}, 2005). Furthermore, cleavage 
stage embryos are known to be sensitive to hypoxia that induces 
declines in ATP levels and results in mitotic arrest (DiGregorio \textit{et al.}, 2001), thus a similar phenomenon may be occurring in 
\textit{ttm}^{299} \textit{mutants}.

\textbf{Induction of cell death by excess Ttm50: Over-production of}
Ttm50 induced cell death. Apoptosis is generally accompanied by 
mitochondrial permeability transition (MPT) in which the inner 
mitochondrial membrane increases in permeability, resulting in loss of
membrane potential, swelling, and eventual rupture of the outer membrane (Zoratti and Szabo, 1995). A scenario where the integration of too much Ttm50 into the inner mitochondrial membrane disrupts its integrity and induces MPT may explain the apoptosis observed. Other possibilities are that abnormally elevated MMP or abnormal protein transport caused by excess Ttm50 bring on the apoptosis observed.

When the induced apoptosis was inhibited by co-expression of p35, over-produced Ttm50 induced excessive proliferation. This could be explained by increased MMP that would result in up-regulation of ATP synthesis, and may have stimulated cellular growth and subsequently cell proliferation. It has been shown that cell cycle progression could be regulated via the mitochondria in the case of mRpl12 required for cell growth driven by Cyclin D/Cdk4 (Frei et al., 2005).

An alternative interpretation is that when cell death induced by over-expressing ttm50 is artificially prevented through p35 co-expression, it results in overgrowth through a phenomenon known as compensatory cell proliferation. Apoptotic cells induce the
proliferation of surrounding cells to compensate for their death by secreting growth-promoting signals such as Decapentaplegic (Dpp) and Wingless (Wg). Thus when apoptosis is induced but at the same time inhibited by P35 the “non-dying“ apoptotic cells can bring on excessive compensatory division (Huh et al., 2004; Ryoo et al., 2004).

Tissue specific roles of ttm50: Ttm50 is probably essential for mitochondrial function since its two homologs, ttm2 and ttm3 appear to be testes specific and probably cannot complement its function in other tissues. Thus the tissue-specific transcription seen by in-situ hybridization in embryos after gastrulation is interesting. The lethal phase for the null ttm50F1 allele was at the early larval stage, so embryonic development of tissues not transcribing ttm50 is presumably sustained by maternally provided transcripts or translation products. Accordingly, ubiquitously distributed transcripts were seen in early cleavage stage embryos. They are thought to be of maternal origin because expression of zygotic genes generally does not occur at this early stage. From gastrulation onwards in embryogenesis ttm50
transcripts are expressed strongly in the midgut, and also in the hindgut, malphigian tubules, and mesoderm. While we did not detect ttm50 transcription in the nervous system, zebrafish Tim50 mRNA expression is reported to be prominent in the brain of the embryo (Guo et al., 2004). These differences imply that the tissue-specific roles of Tim50/ttm50 differ between the two organisms, but nonetheless the control of both during development seems to be critical.

The midgut expression pattern of ttm50 is similar to that of Drosophila bonsai, which encodes the mitochondrial ribosomal protein S15 (Galloni, 2003). In bonsai mutant larvae, a gut-specific respiration defect similar to the one we report has been observed. The larval midgut is known to show high cytochrome C oxidase activity (Galloni, 2003) and in this study we find elevated MMP. So it appears that bonsai as well as ttm50 are responsible for elevated respiratory activity in this organ that could be required for its proper development or physiological function.
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protein kinase similar to MAP kinase activator acts downstream of


FIGURE LEGENDS

Figure 1. Growth defects observed in ttm50 mutant larvae. (A) A wildtype embryo at 4 days after hatching is fully grown and ready to pupate. (B) ttm50^{E1} null allele and (C) ttm50^{E2} strong allele larvae are much smaller at the same age, and later die without showing significant increase in size. (D) A ttm50^{pp9} hypomorphic allele larvae at the same age, shows smaller body size, requiring a few more days to pupate. All photos at same magnification.

Figure 2. The ttm50^{pp9} hypomorphic allele shows growth and cell cycle defects. (A) A yellow^{I} (y^{I}) adult male control fly showing normal body size and bristle morphology. (B) A y^{I} ttm50^{pp9} hemizygote male showing reduced body size and underdeveloped macrochaetae. (C) A DAPI stained wildtype cleavage stage embryo showing uniform distribution of nuclei in synchronous mitosis. (D) A DAPI stained y^{I} ttm50^{pp9} homozygous
female-derived embryo showing irregular distribution of cleavage nuclei, with some showing stronger staining intensity. (E) Higher magnification image of typical nuclei in D shows condensed chromosomes in early mitosis. (F) Higher magnification image of nuclei with stronger staining intensity in D shows condensed polyploid chromosomes which appear to have failed in nuclear division. (G) Mitotic sister (bright and dark) clones of wildtype cells are similar sized. (H) Mitotic clones of \(ttm50^{Gp99}\) homozygous cells (dark blue nuclei) have less cells than their wildtype \((ubi-GFP/ubi-GFP)\) sister clones (bright green nuclei) when induced in a heterozygous \((ttm50^{Gp99}/ubi-GFP)\) background (pale green nuclei). (I) The control sister clones only of \(ttm50^{IE1}\) null clones are observed, even after extensive growth.

Figure 3. Molecular characterization of the \(ttm50\) loci and its conservation through evolution. (A) The \(ttm50\) locus and the intron/exon organization of its transcript. The ORF is indicated by START and STOP. The \(ttm50^{Gp99}\) allele is caused by a P element-insertion in the 5' UTR.
The *ttm50* allele is caused by a nucleotide alteration of Trp<sup>197</sup> to a TAG termination codon. The extent of the 3.5 kb *SacII/EcoRI* fragment used for rescue transgene constructs is indicated. (B) Amino acid sequence comparison of genes sharing homology to *ttm50*. Amino acid residues identical to those of Dm Ttm50 are highlighted and gaps indicated by slashes. Dm Ttm50: *Drosophila melanogaster* Tiny tim50, Dm Ttm2: *Drosophila melanogaster* Tiny tim2, Dm Ttm3: *Drosophila melanogaster* Tiny tim3, HsTim50: *Homo sapiens* Tim50, Ce T21C9.1: *Caenorhabditis elegans* T21C9.1, Sc Tim50: *Saccharomyces cerevisiae* Tim50.

The single underline in *Saccharomyces cerevisiae* Tim50 indicates the mitochondria-targeting presequence and the double underline the transmembrane domain.

Figure 4. HA-tagged Ttm50 localizes to the mitochondria. (A) Highly magnified confocal image of a fat body cell. The open area in the center corresponds to the nuclei. The HA-tagged Ttm50 protein staining appears as reticulated structures in the cytoplasm. (B) Mitochondria
stained by Mitotracker Red. (C) Merged image shows a good overlap (yellow), although the distribution of intensity within the mitrochondria differs.

Figure 5. Expression of *ttm50* transcripts is developmentally regulated during embryogenesis. *In situ* hybridization with anti-sense RNA probes was used to study the tissue specificity of *ttm50* transcription at various embryonic stages. (A) Transcripts were ubiquitously distributed during the cleavage stage, and are presumably maternally derived. (B) Transcripts temporarily disappeared at the cellular blastoderm stage. (C-D) Expression reappeared in the midgut primordial and weakly in the mesoderm of gastrulas. (E-F) Additional transcripts appeared in the hindgut and malphigian tubules during germband retraction. The mesodermal expression could be seen in the differentiating skeletal and visceral muscles. (G-H) This expression pattern was retained through the duration of organogenesis. In all photos, anterior is to the left and dorsal to the top.
Figure 6. Strong alleles of *ttm50* show reduced mitochondrial activity.

(A) Bright-field image of *ttm50* (left) and wildtype (right) larvae partially dissected and incubated in PBS containing 10ng/ml Mitotracker Red. (B) Fluorescent image of same sample shows weaker respiration-dependent staining in the mutant. Staining is strongest in the wildtype gut. (C) Brightfield image of proventriculus and anterior midgut of wildtype 2nd instar larva. (D) Mitotracker Red stained fluorescent image of same sample. Bright red spots are stained yeast in the alimentary tract. (E) Brightfield image of same organs in a *ttm50* larva. (F) Mitotracker Red staining is reduced in mutant. (G) Homozygous *ttm50* mutant clone cells were induced in heterozygous embryos by X-ray irradiation and examined in 3rd instar larva. Mutant salivary gland cell (−/−) is identified by reduced GFP fluorescence. (H) Mitotracker Red staining in mutant cell is reduced both in intensity and area. (I) Alexa Fluor 647-conjugated phalloidin staining
reveals accumulation of actin at cell periphery and reduction in actin around vesicle-like structures of mutant cell. (J) Merged image.

Figure 7. Overexpression of ttm50 in the compound eye causes extra proliferation of cells. (A) Scanning electron micrograph of wild type eye. The number of ommatidia was 752.5 ± 23.5 (n=13). (B) Rough-eye phenotype caused by overexpression of ttm50 under the control of the glass mediated response element. (C) Suppression of rough-eye phenotype by a reduction by half in the gene dosage of hid grim reaper using the Df(3R)H99. (D) Suppression of rough-eye phenotype by a reduction by half in the gene dosage of hid alone. (E) Enhancement of rough-eye phenotype by a reduction by half in the gene dosage of DIAP. (F) Suppression of rough-eye phenotype by the co-expression of the caspase inhibitor p35. The number of ommatidia was increased (798.5 ± 11.6, n=13) significantly compared to wildtype. Expression of p35 alone did not affect ommatidial organization or number (754.0 ± 10.8, n=6) compared to wildtype.
Figure 8. Cell death is induced by overexpression of *ttm50*. (A) Normal levels of apoptosis detected in the eye imaginal disc by acridine orange. (B) Increased cell death induced by GMR element mediated expression of *ttm50* in the eye imaginal disc. Note the induction occurs after the passage of (to the right of) the morphogenetic furrow. (C) Almost complete suppression of apoptosis by the co-expression of *p35*. Arrowheads above each image indicate the position of the morphogenetic furrow.

Figure 9. Coexpression of UAS-*ttm50* and UAS-*p35* driven by *en*-GAL4 increases cell density (A–D) and proliferation (E–F) in the imaginal discs. (A) Wing disc nuclei stained by DAPI. (B) *en*-GAL4 driven gene expression in the posterior compartment marked by UAS-GFP. (C) Tissue morphology visualized by actin stained with Alexa Fluor 647 conjugated phalloidin shows a change in the posterior compartment. (D) Merged image. (E) *en*-GAL4 driven gene expression in the posterior compartments
of 2 imaginal discs marked by $UAS-GFP$. (F) S-phase cells visualized by Brd-U incorporation are increased in the posterior compartments. (G) Expression of UAS-GFP driven by $ptc$-GAL along the anterior posterior boundary of the wing imaginal disc pouch. (H) Increase in Mitotracker Red staining induced by over expression of UAS- $ttm50$ driven by $ptc$-GAL in same sample.
Table 1. Mitotic index in the larval brain lobe cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of mitotic cells</th>
<th>No. of cells counted</th>
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</thead>
<tbody>
<tr>
<td>Control ($y^+$)</td>
<td>1.48</td>
<td>1,356</td>
</tr>
<tr>
<td>$y\ ttm50^{p99}/Y$</td>
<td>0.68</td>
<td>2,217</td>
</tr>
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</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
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Figure 7.

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Figure 9.