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**The mitochondrial TOM complex modulates *bax*-induced apoptosis in
*Drosophila***

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List of abbreviations:

TOM: Translocase of the Outer Membrane

da: daughterless

vg: vestigial

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Abstract

Bax is a pro-apoptotic member of the Bcl-2 family proteins involved in the release of apoptogenic factors from mitochondria to the cytosol. Recently, it has been shown both in mammals and yeast that Bax insertion in the mitochondrial outer membrane involves at least two distinct mechanisms, one of which uses the TOM complex. Here, we show that in *Drosophila*, heterozygous loss of function mutations of Tom22 or Tom70, two receptors of the TOM complex, attenuates *bax*-induced phenotypes *in vivo*. These results argue that the TOM complex may be used as a mitochondrial Bax receptor in *Drosophila*.

1. Introduction

In mammals, members of the Bcl-2 family play a key role in regulating mitochondrial outer membrane permeabilization during apoptosis (for review: [1, 2]). This permeabilization authorizes the release from the intermembrane space to the cytosol of apoptogenic factors such as cytochrome c. Once in the cytosol, cytochrome c binds Apaf-1, which leads to the recruitment of procaspase-9 in the presence of dATP to form the apoptosome. Other mitochondria released factors can trigger a caspase-independent cell death. This release of apoptogenic factors is mainly controlled by proteins of the Bcl-2 family, which comprises about twenty proteins with either pro- or anti-apoptotic activities. Some of these proteins can integrate into the mitochondrial outer membrane among which the pro-apoptotic protein Bax. In most cases, Bcl-2 family proteins do not constitutively reside at this membrane but are mainly located in the cytosol before cell death induction. Apoptotic signals often target these proteins to the mitochondrial outer membrane.

The TOM (translocase of the outer membrane) complex is constituted by seven subunits and is in charge of importing nuclearly encoded mitochondrial protein precursors in the mitochondria (for review: [3, 4]). Three components of this complex, Tom70, Tom20 and Tom22 act as receptors and interact via their large cytosolic domains with mitochondrial protein precursors. Tom40, Tom5, Tom6 and Tom7 constitute the channel enabling translocation of mitochondrial preproteins. Another protein, more recently identified, Tom34, facilitates import of some proteins by interacting with their mature portion instead of binding mitochondrial localization sequences [5, 6]. It is mainly cytosolic but can be found interacting with the TOM complex. Tom34 has been proposed to be a molecular chaperone that keeps preproteins in an unfolded, import-competent state [6].

The mechanism by which proteins of the Bcl-2 family are addressed to and inserted in the outer membrane is still unclear but some recent data suggest that at least some of them could be inserted in the outer membrane like mitochondrial protein precursors, using the TOM complex. Tom20 interacts with two lysines on each side of the transmembrane domain of the carboxyterminal region of Bcl-2 [7] and Tom70 facilitates Mcl-1 import [8]. Regarding Bax, mitochondrial import has been shown to necessitate Tom22 [9], which interacts with the first helix of Bax in mammals. Furthermore, it has been shown recently that monomers, but not dimers, of Bax require the presence of both Tom22 and Tom40 to integrate into mitochondria [10]. Nonetheless, Bax mitochondrial localization can be TOM-independent in mammals, and this involves the Bax carboxyterminal region (helix $\alpha 9$). Furthermore, Bax is able to insert itself

without the TOM complex [11] in isolated yeast mitochondria, even though this insertion seems to be facilitated when a functional complex is present [12].

We have previously shown that murine *bax* is functional in *Drosophila* and induces an apoptosis associated to mitochondrial events [13, 14]. Here we show that, in *Drosophila*, Bax is found both in the cytosol and in mitochondria. In order to test whether the TOM complex is involved in Bax mitochondrial insertion in *Drosophila*, we also tested the consequence of loss of function mutations affecting TOM complex components on *bax*-induced apoptosis. Our results indicate that the TOM complex is involved in Bax pro-apoptotic abilities, indicating that Bax mitochondrial localization in *Drosophila* is important for Bax activity and showing that *Drosophila* provides a good alternative model of moderate genetic complexity to study the mechanism of action of Bcl-2 family proteins.

2. Material and methods

2.1. Fly stocks

Transgenic *Drosophila* carrying the P[UAS *bax*] construct (UAS-*bax* flies) were generated in our group [14]. These flies express a full length wild type murin Bax cDNA.

All mutants of the TOM complex were obtained from the Bloomington Stock Center. *mge^{b10}*, an EMS-induced allele null mutant, in which the Trp-94 codon (TGG) is converted to a stop codon (TGA), encodes a Mge protein truncated at the beginning of the transmembrane domain and has been described in [15]. The Tom7^{BG02496}, Tom34⁰³⁶⁹² and Tom70^{KG06223} alleles are hypomorphic. None of these four Tom mutant flies display a wing phenotype in the absence of *bax* expression.

2.2. Preparation of mitochondrial and cytosolic fractions

UAS-*bax* flies were crossed to flies expressing the GAL4 protein under the control of regulatory sequences of the *daughterless* gene (*da*-GAL4). This driver allowed us to induce ubiquitous expression of the transgene during development of the fly. 150 embryos were collected, dechorionated in undiluted bleach and rinsed off in PBS1x. Embryos were then pelleted and washed once with PBS1x. Pellets were resuspended in 150 µl of ice cold CLAMI buffer (250 mM sucrose, 70 mM KCl, 200 µg/ml digitonin), homogenized on ice using a Wheaton glass homogenizer (until the suspension became milky) and incubated at 4°C for 5 min. Samples were then centrifuged at 1000g for 5 min, at 4°C. Supernatants (cytosolic fractions) were recovered and placed at -80°C, pellets were resuspended in 50µl of IP buffer (30 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100,

0.3% NP-40, AEBSF^{SC}) and incubated for 10 min on ice. Samples were then centrifugated at 4°C for 10 min at 10,000g. Supernatants (mitochondrial fractions) were stored at -80°C.

2.3. Western-blot

Protein extracts corresponding to either 10 embryos (crude extract and cytoplasmic fractions) or 40 embryos (mitochondrial fractions) were loaded on an INVITROGEN NuPAGE™ 4-12% Bis-Tris Gels and run in MES running buffer (50mM MES, 50mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3). Electrotransfer on PVDF membrane (Immobilon™-P, MILLIPORE) was performed in transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2). Transfer efficiency was evaluated with Ponceau-S (0.3% Ponceau-S, 1% acetic acid). Membrane was then saturated in non-fat dry milk for one hour before antibody staining and probed using anti-Bax 5B7 from Santa Cruz (1:500 dilution), anti-tubulin E7 from DSHB (1:1,000 dilution), anti-cytochrome c from Pharmingen (1:500 dilution) and anti F1 ATP synthase from Mitoscience (1:2000 dilution). HRP conjugate anti-mouse or anti-rabbit are from Jackson and used at 1:10,000 dilution. Immunodetection was performed by chemoluminescence (ECL, AMERSHAM). Signals were quantified using the Bio-Rad GS-800 calibrated densitometer and the Quantity One® software.

2.4. Phenotypic suppression test and Wilcoxon test

Bax-induced apoptosis in the wing disc, driven by *vg-gal4*, results in a wing phenotype (notches) [13, 14]. However, expressivity of this phenotype displays variability. Thus, *bax*-expressing flies can be classified within three categories according to the strength of their wing phenotypes: strong, intermediate or weak [13]. Phenotypic suppression tests were performed as follow: *vg>bax* virgin flies were crossed at 18°C to either mutant flies to test or to control wild type flies. Each progeny of these crosses (expressing *bax* and heterozygous for a loss of function *Tom* gene) was classified according to the strength of its phenotype within one of the three phenotypic categories. A Wilcoxon test was then used to compare the distribution of phenotypes between the two genotypes (control vs mutant). Difference of distribution is considered significant for a value of $\alpha < 10^{-3}$. A positive W_s value indicates enhancement of the phenotype while a negative W_s shows suppression of the phenotype.

3. Results

3.1. In *Drosophila*, Bax is both cytosolic and mitochondrial

In mammals, Bax resides in the cytosol of living cells and induction of apoptosis induces its conformational change allowing its targeting to and insertion in the mitochondrial outer membrane. In order to further investigate Bax function in *Drosophila*, we performed a

subcellular fractionation to analyze Bax localization in *Drosophila* cells. Bax localization was studied in flies expressing *bax* under the control of the ubiquitous Da-Gal4 driver (figure 1). Similarly to what can be observed in mammalian cells, Bax is found both in the cytosolic and in the mitochondrial fraction (figure 1A). However, a quantification of the signal taking into account the amount of material loaded on the gel shows that 80 to 85% of Bax is found in the cytosolic fraction (figure 1B).

Release of cytochrome c during *Drosophila* cells apoptosis remains a matter of debate. In these experiments, about 15% of the cytochrome c is detected in the cytosolic fraction. *This amount is not significantly higher than the amount of F1 ATP synthase found in the cytosolic fraction in this type of experiment (figure 1B), which could suggest that the cytochrome c found in the cytosolic fraction is a contamination and thus that cytochrome c may not be released in the cytosol. However, the relatively low percentage of apoptotic cells in embryos at a given time does not allow to determine whether Bax-induced apoptosis of Drosophila cells is associated or not with a permeabilization of the mitochondrial outer membrane.*

3.2. The TOM complex receptor Maggie is necessary for *bax*-induced cell death in *Drosophila*

Given that Bax displays both cytoplasmic and mitochondrial localization, we decided to test whether the TOM complex is involved in mediating *bax*-induced cell death in *Drosophila*. Hence, we chose loss of function mutants affecting components of the TOM complex to perform phenotypic suppression tests. We used *vg-gal4* to drive murine *bax* expression in the wing imaginal discs. *bax* expression leads to apoptosis in the wing tissue that is not essential to fly survival, thus adult wings exhibit notches on their wing margins. These *bax*-expressing flies were crossed either to a control strain or to flies mutant for a TOM complex component. Progenies of these crosses were classified according to their phenotype (figure 2). Statistical analysis of the difference in distribution between the two progenies were then tested (see materials and methods and [13]).

As the component of the TOM complex described as Bax receptor in mammals is Tom22, we first tested whether a loss of function allele of its homolog in *Drosophila*, *maggie* (*mge*), could impair *bax*-induced cell death. For this purpose, we chose the *mge*^{b10} null allele that expresses a truncated form of Mge which only possesses the cytosolic portion of the protein [15]. Since *mge*^{b10} is homozygous lethal, the analysis was performed in *mge*^{b10} heterozygous flies. A shift in phenotype distribution from about 60% of strong and about 40% of intermediate phenotypes in the control cross to 30% of strong and about 70% of intermediate phenotypes was observed when using the *mge*^{b10} allele (figure 3A). This decrease in strong phenotypes percentage and the enrichment in intermediate ones reflect a global suppression of

the *bax*-induced phenotype that is highly significant according to the Wilcoxon test ($\alpha=6.15 \times 10^{-5}$). A mutation of *mge* impairs *bax* pro-apoptotic function, showing that Bax necessitates functional Mge to kill cells efficiently. We can thus hypothesize that Bax mitochondrial localization could be mediated by the TOM complex and could use Mge/Tom22 as a receptor.

3.3. The TOM complex receptor Tom70 participates in *bax* pro-apoptotic activity

To further investigate involvement of the TOM complex in *bax* killing properties in *Drosophila*, we tested whether a loss of function mutation of the TOM receptor *tom70* could impair *bax*-induced cell death. We used the *tom70*^{KG06223} allele to perform a phenotypic suppression test. In the control cross progeny, about 55% of phenotypes are classified as strong, 40% are intermediate and 5% are weak (figure 3B). In a *tom70*^{KG06223} heterozygous background, less than 40% of phenotypes are strong while the frequency of weaker phenotypes increases from 5% to 16%. This shift toward weaker phenotypes reveals a significant ($\alpha = 3.73 \times 10^{-4}$) suppression of *bax*-induced cell death even though this suppression is less efficient than what is observed with a *mge*^{b10} allele. This result suggests that Tom70 may participate in Bax mitochondrial localization.

3.4. A Tom7 mutation does not affect *bax*-induced cell death

We also tested the involvement of Tom7, a protein which does not appear to be essential for the TOM complex function as a deletion of *tom7* gene in *N. crassa* does not by itself display any phenotype [16]. We performed a phenotypic suppression test using the loss of function allele *tom7*^{BG02496}. This mutation did not modulate any significant change in *bax*-induced phenotype distribution ($\alpha = 0.028$). Indeed, we observed almost the same distribution between progenies of the control cross (which only expresses *bax*) and of the cross with the *tom7*^{BG02496} mutant allele (figure 3C). Therefore, altering *tom7* does not affect *bax*-induced cell death.

3.5. A tom34 mutation enhances *bax*-induced apoptosis

The last component of the TOM complex we have tested is Tom34. This accessory protein of the TOM complex normally resides in the cytosol. Nonetheless, Tom34 has been shown to interact with the TOM complex and facilitate translocation of some proteins through physical interaction probably acting as a molecular chaperone [5, 6]. We used the *tom34*⁰³⁶⁹² loss of function allele to perform a phenotypic suppression test of the *bax*-induced phenotype. Use of *tom34*⁰³⁶⁹² induces a significant increase ($\alpha = 4.99 \times 10^{-7}$) from 60 to 90% of strong phenotypes (compared to control cross) and a decrease from 40 to 10% of intermediate phenotypes (figure 3D). This shift in phenotype distribution reflects an enhancement of the *bax*-induced phenotype

thus showing an increase of *bax*-induced cell death. This effect on cell death could be mediated through another cell death regulator as discussed later.

4. Discussion

Recent studies on mammalian or yeast systems have shown that the TOM complex is at least to some extent involved in Bax mitochondrial localization showing that the TOM complex should regulate *bax*-killing properties. We have shown here that in *Drosophila*, Bax is located mainly in the cytosol but also in mitochondria. Therefore, Bax localization in *Drosophila* has to be regulated and the mechanisms involved in this process could be conserved during evolution. Using loss of function mutants affecting the TOM complex, we have shown that heterozygous *tom22/mge* and *tom70* mutations suppress *bax*-induced apoptosis, strongly suggesting a role of the TOM complex in Bax mitochondrial localization and pro-apoptotic activity. However, the low level of mitochondrial Bax in the whole embryo does not allow determining quantitatively if the level of mitochondrial Bax decreases. Thus we can not exclude an indirect effect of these mutations on *bax*-induced cell death. Nevertheless, heterozygous *tom22/mge* and *tom70* mutations used here do not display any other phenotype, which strongly suggests that the mitochondrial metabolism is not seriously affected in these flies. Furthermore, mutation of *tom7* does not affect Bax toxicity, which is in agreement with the observation that Tom7 is not essential for the TOM complex function. More surprisingly, heterozygous loss of function of *tom34* enhances Bax toxicity. One possibility is that Tom34 is involved in the mitochondrial targeting of an antiapoptotic component such as Buffy. This effect is therefore probably indirect.

An interesting perspective would be to study the role of the components of the TOM complex during apoptosis induced by *Drosophila* homologs of Bax. Indeed, *Drosophila* possesses two members of the Bcl-2 family: *Debcl/Drob-1* which is pro-apoptotic, and Buffy which is anti-apoptotic. However, if the mitochondrion and proteins of the Bcl-2 family seem to have an important place in the *Drosophila* apoptotic cascade, their role(s) remains elusive (for review:[17]). As it is known that mitochondrial localization of *Debcl/Drob-1* is necessary for its pro-apoptotic function [18], it would be very helpful to initiate the same kind of study on *debcl*-induced apoptosis.

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7. Figures legends

Figure 1: Bax subcellular localization.

Lysates of *Drosophila* embryos ubiquitously expressing *bax* were subjected to subcellular fractionation and analyzed by western blotting. (A) Immunoblotting was used to detect Bax, Tubulin, Cytochrome *c* and F1 ATPase. (B) Protein amounts were quantified (see materials and methods) and the average signals (+/- SEM) were corrected to match the same number of embryos. Bax is found both in the cytosolic fraction and in the mitochondrial fraction. T : total fraction, C : cytosolic fraction, M : mitochondrial fraction

Figure 2: *bax*-induced phenotypes in the wing.

Phenotypes induced by *UAS-bax* driven by *vg-GAL4* are variable and were classified into three categories (B, C and D) according to their strength. (A) wildtype fly wing. (B) Weak phenotypes are characterized by notches scattered along the margin without significant change in the wing size. (C) Intermediate phenotypes correspond to wings that are reduced in size and an important loss of the margin. (D) Individuals with a strong phenotype display partially or completely absent wings. Adapted from [13].

Figure 3: Wing phenotype distribution of *vg>bax* adult flies

Drosophila expressing *bax* under the control of a *vg-gal4* driver were crossed either to a control strain or to a mutant for a TOM complex component. Progenies of these crosses were classified according to their phenotype. Since there is no wild type fly, the phenotypes are classified within three categories (strong, intermediate or weak). Ordinate axis represents the percentage of individuals within each category. Control cross progeny is presented in pale-gray and progeny of the cross with mutant of the TOM complex in dark-gray. Genotypes of the mutant flies are: (A) *+/vg-gal4, UAS-bax ; mge^{b10}/+* (B) *P{SUPor-P}tom70^{KG06223}/vg-gal4, UAS-bax* (C) *P{GT1}tom7^{BG02496}/ vg-gal4, UAS-bax* (D) *P{PZ,ry⁺}tom34⁰³⁶⁹² ry⁵⁰⁶/vg-gal4, UAS-bax*. Genotypes of the control flies are *vg-gal4, UAS-bax/+*. Results of the Wilcoxon tests are presented under each graph.

FIGURE 1

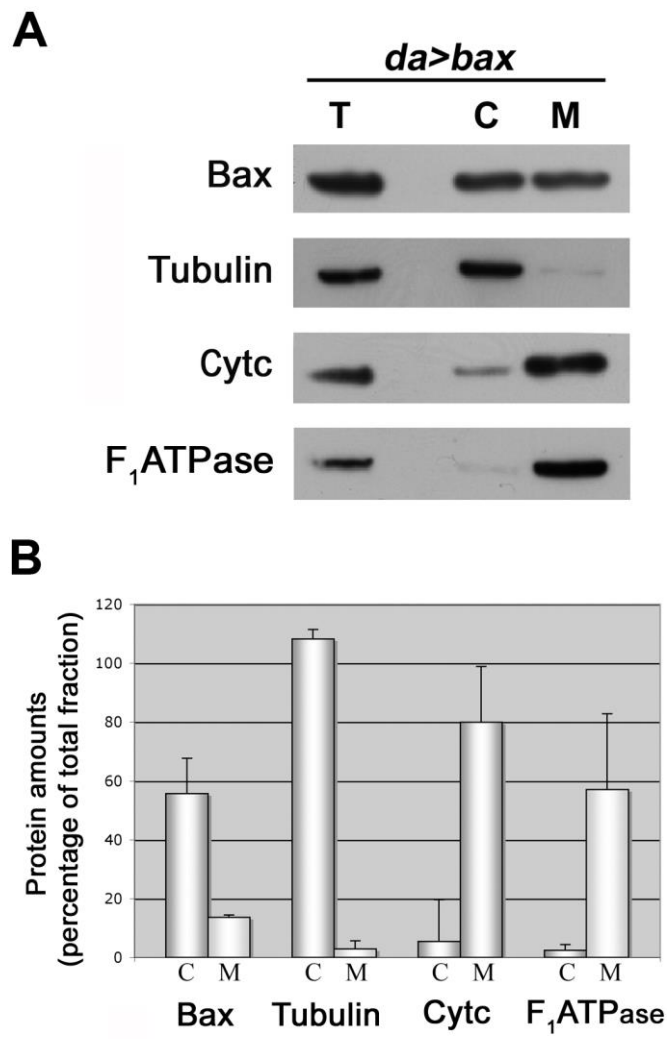


FIGURE 2

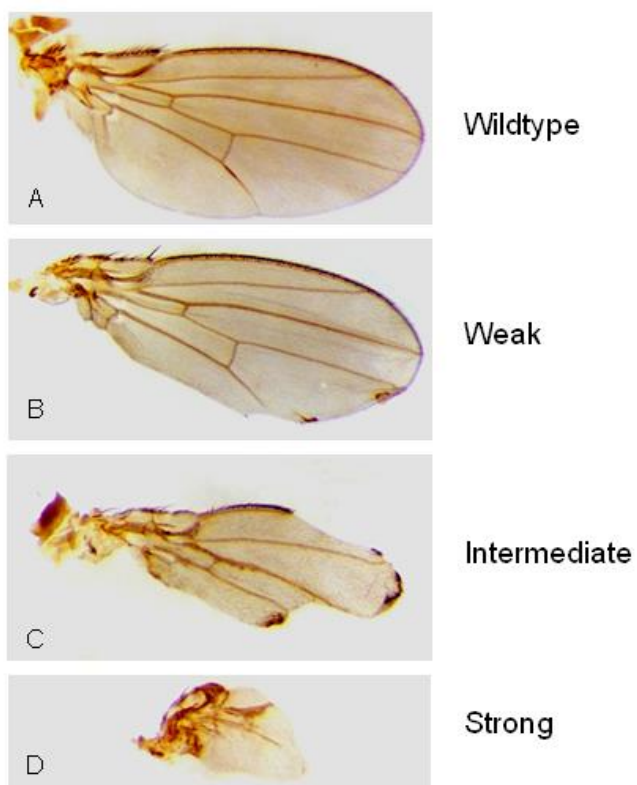


FIGURE 3

