A transgenic *Drosophila melanogaster* model to study Human T-Lymphotrophic Virus oncoprotein Tax-1-driven transformation *in vivo*

Margret Shirinian¹, Zakaria Kambris², Lama Hamadeh³,¹⁰, Caroline Grabbe⁴, Chloé Journo⁵,⁶,⁷,⁸,⁹*, Renaud Mahieux⁵,⁶,⁷,⁸,⁹* and Ali Bazarbachi³,¹⁰ #

**Running title:** HTLV-1 Tax driven transformation in *Drosophila*

¹ Department of Experimental Pathology Immunology and Microbiology Faculty of Medicine, American University of Beirut, Beirut, Lebanon

² Department of Biology, American University of Beirut, Beirut, Lebanon

³ Department of Internal Medicine, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

⁴ Department of Molecular Biology, Umea University, Building 6L, SE-901 87 Umea, Sweden

⁵ Equipe Oncogenèse Rétrovirale, Lyon, Cedex 07, France

⁶ Equipe labellisée “Ligue Nationale Contre le Cancer”, Lyon, Cedex 07, France

⁷ Centre international de recherche en infectiologie, INSERM U1111 - CNRS UMR5308, Lyon, Cedex 07, France

⁸ INSERM U1111 Ecole Normale Supérieure de Lyon, 46 allée d’Italie, 69364 Lyon, Cedex 07, France

⁹ #
Shirinian et al, 2015

Université Lyon 1, LabEx ECOFECT - Eco-evolutionary dynamics of infectious diseases, 69364 Lyon, Cedex 07, France

Department of Anatomy, Cell Biology and Physiological Sciences, American University of Beirut, Beirut, Lebanon

* Equal contribution

#Corresponding Author

Ali Bazarbachi, MD, PhD
Department of Internal Medicine, Faculty of Medicine
American University of Beirut, Medical Center
P.O.Box 113-6044 Beirut, Lebanon
E-mail: bazarbac@aub.edu.lb
ABSTRACT (75 words)

HTLV-1-induced adult T-cell leukemia/lymphoma is an aggressive malignancy. HTLV-2 is genetically related to HTLV-1 but does not cause any malignant disease. HTLV-1 Tax transactivator (Tax-1) contributes to leukemogenesis via NF-κB. We describe transgenic Drosophila models expressing Tax in the compound eye and plasmocytes. We demonstrate that Tax-1 but not Tax-2 induces ommatidia perturbation and increased plasmocyte proliferation and that the eye phenotype is dependent on Kenny (IKKγ/NEMO), thus validating this new in vivo model.
Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy secondary to HTLV-1 infection (Human T-cell lymphotropic virus type-1) (1). Although HTLV-1 and HTLV-2 share a similar genetic organization, they display major differences in their pathogenesis and disease manifestation. HTLV-1 is capable of transforming T lymphocytes in infected individuals and subsequently leads to ATL, whereas HTLV-2 has not been clearly associated with malignant diseases but only to lymphocytosis (2, 3).

Transgenic mice models over-expressing Tax-1 demonstrate its oncogenic properties (4, 5, 6). However, cellular pathways and in vivo Tax-1 partners that mediate Tax-1 induced cellular transformation are still unexplored. *Drosophila melanogaster* is a valuable model due to the availability of facile genetic screens, a nearly complete collection of mutants, RNAi lines and advanced genetic technologies in addition to the highly conserved pathways such as NF-κB. Acquisition of a “rough eye” phenotype is an accepted surrogate for cell transformation in the *Drosophila* model. Using *Drosophila* transgenic models expressing Tax proteins (Tax-1 and Tax-2), we present evidence demonstrating the ability of Tax-1 but not Tax-2 to induce transformation in *Drosophila* and show that this transformation is primarily dependent on Kenny, the *Drosophila* orthologue of IKKγ/NEMO, upstream of Relish (NF-κB) activation.

Briefly, we overexpressed Tax-1 specifically in the developing imaginal eyes using the Glass Multimer Reporter promoter (pGMR). Tax-1 was amplified from pSG5M-Tax (8) and cloned into the *Drosophila* expression vector pUAST (Genscript) with an N-terminal Myc tag. pUAST-Tax-1 plasmid was used for the generation of transgenic fly strains after injection into wild-type white-eyed (white) GMR-Gal4 BDSC (#9146, Bloomington Drosophila Stock Center, NIH P40OD018537) embryos (BestGene). Successful transgenesis was monitored through the apparition of the red eye phenotype (white+).

Analysis of the ommatidia structure by scanning electron microscopy was performed...
using a Tescan, Mira III LMU, and field emission gun SEM. A grading system was
developed depending on the severity of eye phenotype (number of ommaditial fusions
and extent of bristle organization) and statistical tests were done using one-way
ANOVA. While control flies displayed normal eyes (Figure 1A, left panel), the eyes of
adult flies expressing a single copy of Tax-1 under control of GMR-GAL4 showed a
rough eye phenotype, which appeared as a perturbation of the normal crystalline array
of the ommatidia, with fused ommatidial structures as well as lost and duplicated bristles
in some instances (compare left and middle panels in Figure 1A, i.e. GMR vs.
GMR>UAS-Tax-1 and see Figure 1B for quantification). Expression of two copies of the
Tax-1 transgene (Figure 1A, right panel, homozygous) significantly enhanced the eye
phenotype (see Figure 1B for quantification). Tax-1 expression was confirmed in whole
protein extracts by western blot using mouse monoclonal antibodies against Myc (9E10
kind gift from Prof. Bengt Hallberg) and rabbit polyclonal antibodies against β-Actin
(Sigma-Aldrich, A2066) (Figure 1C).

Since HTLV-1 specifically induces transformation of lymphocytes in humans, we next
overexpressed Tax-1 in haemocytes, i.e. Drosophila blood leukocyte-like cells. As
plasmatocytes account for 95% of the circulating haemocytes in Drosophila larvae
(reviewed in (10)). UAS-Tax-1 flies were crossed to flies expressing GAL4 under the
plasmatocyte-specific peroxidasin promoter (Pxn-GAL4) (7). Third instar larvae were
bled and haemocytes were loaded onto a Neubauer haemocytometer for counting. A
robust and statistically significant increase (P<0.001) in haemocyte numbers was
observed upon overexpression of Tax-1, as compared to control larvae (Figure 1D).
Transgene expression in haemocytes was verified by western blot (Figure 1E). This
finding demonstrates that ectopic Tax-1 expression induces an overgrowth of leukocyte-
like cells in Drosophila and further strengthens a transforming activity of Tax-1 in this
model.

To further show that Drosophila is a relevant model to genetically characterize the
mechanisms of Tax-1-induced cell transformation, we took advantage of the “rough eye”
phenotype induced by Tax-1 to screen for genes interacting with Tax-1. Previous
reports have suggested that NF-κB activation plays a key role in Tax-induced transformation (reviewed in (11)). In Drosophila, the NF-κB family member Relish is activated following IMD (Immune Deficiency) pathway activation, which is dependent on Kenny (the Drosophila orthologue of IKKγ/NEMO). In parallel, Dorsal and DIF are activated following engagement of the Toll receptor (e.g. the Toll pathway), independently of Kenny (reviewed in (12)). Together, the IMD and Toll pathways induce the expression of multiple antibacterial and antifungal peptides, including Diptericin, Drosomycin, Cecropin and Defensin. Drosophila transgenic UAS-RNAi lines (Kenny, VDRC GD1249 and KK107280; Dorsal, VDRC GD1238; and Relish, VDRC GD1199) were crossed to GMR-Gal4 to induce a knockdown of the target genes specifically in the compound eye (UAS-GAL4 system). The efficiency of inhibition induced by RNAi was validated by quantitative RT-PCR (data not shown). RNAi-mediated silencing Relish significantly reduced the "rough eye" phenotype induced by Tax-1 (P<0.001) (Figure 2A second lane and see Figure 2B for quantification), whereas reduction of Dorsal modulated the "rough eye" phenotype induced by Tax-1 to a lesser extent (P<0.01) (Figure 2A third lane and see Figure 2B for quantification). This suggests that Tax-1-driven cell transformation specifically requires Relish activation and, in extension, that it is primarily dependent on the IMD pathway. To confirm that Tax-1 activates the IMD pathway in Drosophila, we investigated whether Relish-dependent transcription was affected in Tax-1-expressing flies using diptericin expression levels as readout. Quantitative RT-PCR indeed showed that the expression of diptericin was elevated in flies expressing Tax-1, as compared to control flies (Figure 2C). Importantly, the expression of diptericin was not increased in Relish-silenced Tax-1-transgenic flies (Figure 2C), confirming that Relish is a major inducer of Diptericin downstream of Tax-1. Tax-1 binds to IKKγ/NEMO (reviewed in (11)). Since Kenny, the Drosophila orthologue of IKKγ/NEMO, is specifically involved in Relish activation, we hypothesized that the transforming activity of Tax-1 in the Drosophila compound eye could be caused by an interaction between Tax-1 and Kenny. In agreement, RNAi-mediated silencing of kenny strongly reduced the "rough eye" phenotype induced by Tax-1 expression (P<0.001).
(Figure 2A fourth lane and see Figure 2B for quantification), indicating that Kenny is required for Tax-1-driven cell transformation in Drosophila.

Given that the genetically related HTLV-2 virus does not cause cell transformation in infected individuals, we generated transgenic flies expressing HTLV-2 Tax (Tax-2), to enable comparative studies of Tax-1 and Tax-2. The 6x-His tagged UAS-Tax-2 transgene was expressed in the compound eye or in haemocytes. We first found that in contrast to Tax-1-expressing flies, the compound eyes of Tax-2 expressing flies (GMR >UAS-Tax-2) displayed ommatidia which were arranged in a normal crystalline structure (Figure 2A fifth lane and see Figure 2B for quantification). Tax-2 expression was verified by western blot using rabbit polyclonal antibodies against Tax-2 (9) (Figure 2E).

Consistent with these results, the expression of diptericin was not upregulated in response to ectopic Tax-2 expression (Figure 2C), indicating that Tax-2 is a poor activator of Relish. Thus, the inability of Tax-2 to induce cell transformation in Drosophila is correlated with an absence of Relish target gene activation. Finally, the number of haemocytes was not increased in Tax-2 expressing flies (Figure 2D and see Figure 2F for analysis of Tax-2 expression by western blot).

In this study, we have generated transgenic Drosophila expressing the HTLV-1 viral oncoprotein Tax-1 or its HTLV-2 (Tax-2) counterpart. This system is particularly appropriate since cell transformation in the developing eye results in an easily screenable “rough eye” phenotype in adult flies. This in vivo model is validated by the demonstration that the NF-κB pathway is required for Tax-1-driven cell transformation, and that Tax-2 fails to induce cell transformation, consistent with epidemiological and experimental data (3). This model will be of great importance because it will allow a rapid screening of a series of Tax-1 mutants that are impaired for NF-κB activation, CREB activation, SRF activation (13), which lack the ability to bind CBP/p300 (14), p/CAF (15), which lack nuclear localization (16), post-translational modifications (11), a PDZ-binding motif etc, as well as a series of candidate cellular genes possibly linked to Tax-1 transforming activity. Transgenic Drosophila is therefore an important new tool for deciphering how HTLV-1 Tax transforms cells in vivo.
FIGURE LEGENDS

Figure 1: Over-expression of Tax-1 induces transformation in *Drosophila*.

A. Representative scanning electron microscopy images of adult eyes of transgenic flies expressing the indicated genotypes under control of the eye-specific GMR promoter (GMR-GAL4). All flies contain one copy of the transgene, unless otherwise stated. Homozygous flies contain two copies of the transgene. GMR are control flies with no UAS transgene. B. Relative roughness was quantified based on the number of ommatidial fusions and extent of bristle organization, (*** ) represents a statistical significance of P<0.001. C. Cell lysates (150 μg) from control and UAS-Tax1 transgenic adult whole flies were subjected to electrophoresis and probed with anti-Myc or anti-actin antibodies. D. Haemocyte counts in transgenic larvae expressing transgenic Tax-1 under control of the plasmatocyte-specific peroxidasin promoter (Pxn-GAL4). (*** ) represents a statistical significance of P<0.001. E. Cell lysates (150 μg) from UAS-Tax-1 transgenic larvae were analyzed by western blot, confirming the expression of the UAS-Tax-1 transgene in larval haemocytes.

Figure 2: NF-κB pathway components Kenny and Relish are necessary for Tax-1 induced transformation in *Drosophila* and Tax-2 does not activate the Relish pathway nor cause cell transformation.

A. Relish, Dorsal and Kenny expression was inhibited by RNAi in flies overexpressing Tax-1 in the compound eye. Representative scanning microscopy micrographs of adult eyes are shown. Quantification of the relative roughness of the compound eye in each fly strain is indicated in (B): (*** ) and (**) represent a statistical significance of P<0.001 and P<0.01, respectively. C. Expression levels of diptericin, a Relish target gene encoding an antimicrobial peptide, in the indicated transgenic flies. (*** ) represents a statistical significance of P<0.001. D. Haemocyte counts in transgenic larvae expressing Tax-1 and Tax-2 under control of the plasmatocyte-specific peroxidasin promoter. (*** )
represents a statistical significance of P<0.001. E. Cell lysates (150μg) from transgenic adult whole flies were analyzed by western blot, confirming expression of Tax-2 in the transgenic flies. F. Cell lysates (150μg) of haemocytes from Tax-2 transgenic larvae were analyzed by western blot, confirming expression of the Tax-2 transgene.

ACKNOWLEDGMENTS

This study is supported by the Swedish Research Council, the American University of Beirut Medical Practice Plan, University Research Board and the Lebanese National Council for Scientific Research. MS is supported by the Lady TATA memorial trust and the Swedish Research Council. CG is supported by the Swedish Research Council. ZK is supported by the American University of Beirut University Research Board (award 102725). CJ and RM are supported by Ecole Normale Supérieure de Lyon. RM was also supported by a Contrat Hospitalier de Recherche Translationnelle. RM and CJ acknowledge the support of ARC and of La Ligue Contre le Cancer (programme équipe labellisée). Authors would like to thank CRSL (AUB Central Research Science Laboratory) for their help in scanning electron microscopy imaging.
REFERENCES:


FIGURE: 1

A

GMR (Control)  GMR>UAS-Tax-1  GMR>UAS-Tax-1 (Homozygous)

B

<table>
<thead>
<tr>
<th></th>
<th>Relative Roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMR (Control)</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>GMR&gt;UAS-Tax-1</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>GMR&gt;UAS-Tax-1 (Homozygous)</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>Anti-Myc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tax-1</td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image5" alt="Graph" /></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th></th>
<th>Haemocyte Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pxn (Control)</td>
<td><img src="image6" alt="Graph" /></td>
</tr>
<tr>
<td>Pxn&gt;UAS-Tax-1</td>
<td><img src="image7" alt="Graph" /></td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th></th>
<th>Anti-Myc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tax-1</td>
<td><img src="image8" alt="Graph" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image9" alt="Graph" /></td>
</tr>
</tbody>
</table>