Uracil-DNA in Drosophila melanogaster

Ph.D. theses

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INTRODUCTION

Textbooks say that there are two major differences between the chemical composition of DNA and RNA: i) at the sugar level (deoxyribose vs. ribose) and ii) at the base level (thymine vs. uracil). Uracil, however, frequently appears in DNA either from cytosine deamination or thymine-replacing incorporation. These appearances are usually ephemeral due to catalytic action of two key factors: dUTPase, responsible for prevention of uracil incorporation into DNA and uracil-DNA glycosylase (UNG) that excises uracil from DNA. Removal of uracil highly contributes to preserving the genetic information encoded in DNA.

The fruitfly Drosophila melanogaster represents a unique model organism for uracil-DNA targeted research. On one hand, the main and most active uracil-DNA glycosylase, UNG is not encoded within the genome therefore uracil-DNA repair is impaired in both nuclear and mitochondrial DNA. On the other hand, dUTPase, key factor in preventing appearances of uracil, was found to be present under detection limit in larval tissues, and only expressed in imaginal tissues during larval stages.

Lack of these two enzymes, dUTPase and UNG, in genetically engineered model organisms led to accumulation of uracil-substituted DNA, therefore a similar situation can be envisioned in Drosophila larvae. In this work, uracil content and cellular response to uracil-DNA were investigated in Drosophila melanogaster.

Cellular distributions of Drosophila dUTPases were also found to be different from the previously described mammalian system, as mitochondria lack dUTPase. Both of the Drosophila dUTPase isoforms, associated with monomer molecular masses of 21kDa and 23kDa, respectively, can be present either in the nucleus or in the cytoplasm in different tissues and developmental stages, although only the long (23kDa) isoform contains the complete putative NLS sequence (10PAAKKMKID18). In order to understand the distribution of the two isoforms within the cell, detailed localisation studies were initiated.
AIMS

The present study aimed to investigate aspects of uracil-DNA in *Drosophila melanogaster* and to achieve the followings:

1) Verifying the putative nuclear localisation signal, and describe the subcellular localisation of two dUTPase isoforms.
   - Identification of putative NLS segment conserved among dUTPases
   - Cloning of dUTPase-eYFP fusion protein constructs into Drosophila Schneider 2 cell line-specific inducible expression vector
   - Creating stable expression system for dUTPase-YFP constructs in Schneider 2 Drosophila cell line and follow their distribution within the cell
   - Tracking localisation of dUTPase isoforms in Drosophila embryo with special emphasis on mitosis
2) Investigate cellular response to uracil-DNA in Drosophila cell culture
   - Examining cellular response to uracil substituted plasmid DNA
   - Analysing cellular response to misregulated dUTP/dTTP ratio
3) Investigate cellular response to uracil-DNA in fruitfly
   - Examining cellular response to uracil substituted plasmid DNA in embryo
   - Creating an RNAi system for dUTPase in larvae and pupae
   - Describing phenotype of dUTPase silencing
4) Measuring uracil content of Drosophila biological samples
   - Recording changes of uracil content during development
   - Comparing uracil-DNA level in larval and imaginal tissues
   - Measuring uracil content in response to dUTPase RNAi and misregulation of dUTP/dTTP ratio
EXPERIMENTAL METHODS

Cloning of dUTPase-eYFP fusion protein constructs
pRmNDUT-eYFP (21 kDa dUTPase) and pRmDUT-eYFP (23 kDa dUTPase) vectors were constructed by cloning 21 kDa and 23 kDa dUTPase coding sequences into the Drosophila transfection vector pRm-eYFP-N-C*. These constructs produce dUTPase with a C-terminal eYFP under the control of a metallothionein promoter.

S2 cell culturing, transfection, selection
For stable transfection of S2 cells, dUTPase-eYFP expression constructs were cotransfected with pPURO plasmid in the presence of Cellfectine. Stable cell lines were selected by growing them in the presence of increased concentration of puromycin.

Localisation of dUTPase-YFP in S2 cells
In order to investigate the subcellular distribution of dUTPase isoforms, cells expressing dUTPase-eYFP were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton-X 100. DNA was stained with DAPI (Sigma) and actin was labelled with rhodamine – phalloidine.

Microinjection of S2 cell extract into Drosophila embryo, confocal microscopy
Localisation of the two dUTPase isoforms was followed separately throughout complete cell cycle in Drosophila embryos. S2 cell extract, prepared from cells expressing dUTPase-eYFP, was injected into Drosophila embryos. Rhodamine-tubulin was added to the S2 cell extract in order to follow dynamics of microtubule assembly related to nuclear divisions.

Alamar blue assay for determining the effect of 5’FU and FUdR
Cellular response given to elevated level of uracil in genomic DNA was provoked by applying 5’-fluorouracil (5’FU) and 5’-fluorodeoxyuridine (FUdR) treatment to Drosophila and human cells. Such treatment leads to perturbation of nucleotide levels and induces elevated level of dUTP incorporation. Alamar blue cell viability assay was used to follow cellular response.
U-plasmid interpretation assay in cell culture
Drosophila and human cells were transfected with exogenous uracil-DNA plasmid in order to decide whether such chemically unusual DNA may be tolerated and interpreted. Fluorescent protein encoding plasmids were amplified in *E.coli* K12 XL1Blue strain and CJ236 ung-, dut-strain producing normal plasmid and dUMP-substituted version of the construct respectively.

U-plasmid interpretation assay in Drosophila embryo
Uracil-DNA plasmid interpretation assay was carried out in Drosophila embryo similarly to the cell culture reporter assay, but here plasmids were introduced to Drosophila embryo by microinjection.

RNA interference
RNAi was induced against dUTPase by crossing UAS-IR transgenic Drosophila strains with Gal4 drivers. The Act5C-Gal4 driver causes ubiquitous expression of Gal4 while drivers GMR-Gal4 and engrailed-Gal4/CyO are imaginal disc-specific, active in eye and wing discs respectively. For UAS-IR x Act5C-Gal4 /CyO, GFP crosses, progeny were scored according to GFP marker in larvae or CyO wing marker in imago. Animals lacking GFP and CyO wing markers express the IR construct thus silence dUTPase. Reduced number of hatched non-Cyo imago as compared to the number of Cyo imago indicates the importance of silenced gene for development.

Uracil content of Drosophila biological samples
In order to quantify uracil content of DNA, a real-time quantitative PCR-based assay was applied. The technique is based on two quantitative PCR reactions, where the amount of uracil-free DNA template and the amount of all DNA templates are determined separately. Then, the amount of uracil containing template can be calculated. The frequency of uracil occurrences is estimated from the amount of uracil containing template, and the length of the measured template sequence.
RESULTS

Subcellular localisation of Drosophila dUTPases

Identification of a putative NLS segment conserved among dUTPases
The NLS sequence of Drosophila 23 kDa dUTPase (PAAKKMKID) shows homology with the NLS of human c-myc and RanBP3 and it is also well preserved in other eukaryotic dUTPase sequences. These NLS sequences belong to a rather unusual type of NLS class. Here, only 3 positively charged amino acids can be found in the middle of the NLS motif, which are flanked by neutral and acidic residues. Contrary to this, classical NLS contains at least 5 basic amino acids within a nonapeptide.

Localisation of Drosophila dUTPase isoforms in S2 cells
Investigations of S2 cells overexpressing fluorescently labelled dUTPase constructs have demonstrated that a region at the N-terminus is essential and sufficient for nuclear localisation: the 23 kDa isoform is the nuclear and the 21 kDa dUTPase isoform, lacking the NLS segment, is the cytosolic isoform. None of the constructs were detected in the mitochondria.

Localisation shifts of Drosophila dUTPase within embryos
Localisation patterns of the 23 kDa and the 21 kDa fluorescent dUTPase constructs were followed during nuclear cleavage. During interphase, the 23 kDa isoform is located within the nucleus while the 21 kDa isoform is diffusely scattered in the cytoplasm. As nuclei enter mitosis, the two dUTPase isoforms show opposite localisation shifts. Unexpectedly, the 21kDa dUTPase shows a localisation shift to the karyoplasm, meanwhile the 23kDa dUTPase diffuses from the nuclear space. Later on during metaphase and anaphase, the 21kDa dUTPase remains accumulated around the chromosomes in the nuclear space and it gets excluded from the karyoplasm only during telophase. By the end of cytokinesis, the state of interphase is restored, when 23kDa is nuclear and 21kDa dUTPase is cytoplasmic. The observed dynamic localisation character showed strict timing to the nuclear cleavage phases.
Uracil-DNA in *Drosophila melanogaster*: interpretation and developmental involvement

**Cellular response to uracil substituted plasmid DNA in cell culture**
Upon transfection of both human and Drosophila cells with normal DNA plasmid, the expression of the fluorescent protein encoded by the plasmid can be readily detected. However, upon transfection with uracil substituted plasmid (U-plasmid), only Drosophila cells were capable to express the fluorescent protein.

**Cellular response to misregulated dUTP/dTTP ratio**
Cellular responses to treatment with thymidylate synthase inhibitors (5’FU and FUdR) are different in human and Drosophila cells. Both drugs destroy human cells, while Drosophila tolerates these drugs very well, indicating that Drosophila cells might tolerate elevated level of dUTP incorporation into DNA.

**Examining cellular response to uracil-substituted plasmid DNA in embryo**
In agreement with cell culture studies, Drosophila embryo interpreted uracil-DNA, and fluorescent signal was detectable upon microinjection.

**dUTPase RNAi in *Drosophila melanogaster***
Ubiquitous silencing has reduced the protein level of dUTPase below detection limit in 3rd larvae and early pupae thus it has led to dUTPase clearance from imaginal tissues. Virtually dUTPase-free larvae did not show any adverse effects indicating that dUTPase silencing did not perturb normal life and development in the larval stages. At early pupae stage, however, dUTPase silencing led to lethality. Failure of head eversion and developmental arrest was observed.

**Uracil content of Drosophila biological samples**
Measurement of uracil content has revealed that during larval stages uracil is accumulated, and reaches its maximum level at 3rd stage larvae. In addition, uracil-DNA was found to be present in all stages of development except in embryo. Accumulation of uracil was the most pronounced in larval tissues, while imaginal discs contain much less uracil. This phenomenon was perturbed upon silencing of dUTPase in larvae which resulted in similar uracil content, as silencing increased the level of uracil in imaginal discs. Here I found clear correlation between the presence of dUTPase and uracil content of DNA (Table 1).
Cell culture experiments further supports that 5’-fluorouracil (5’FU) and 5’-fluorodeoxyuridine (FUdR) treatment truly perturbed and increased the level of genomic uracil which was well-tolerated by Drosophila Schneider 2 cells.

<table>
<thead>
<tr>
<th>Type</th>
<th>dUTPase level</th>
<th>Uracil level</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>-</td>
<td>high</td>
<td>development arrest</td>
</tr>
<tr>
<td>dUTPase RNAi</td>
<td>-</td>
<td>high</td>
<td>no observed effect</td>
</tr>
</tbody>
</table>

Table 1 Uracil content varies according to the presence/absence of dUTPase
Localisation of Drosophila dUTPases

1. PAAKKMKID sequence is the NLS of *Drosophila melanogaster* dUTPase
2. The dUTPase NLS segment is well preserved in other eukaryotic dUTPase sequences
3. Mitochondria of *Drosophila melanogaster* do not contain dUTPase at detectable level.
4. During the cell cycle of interphase, the 21kDa dUTPase isoform is present in the cytoplasm and the 23kDa isoform in the nucleus.
5. Localisation of the 21kDa dUTPase isoform is shifted into the nuclear space during mitosis.
6. Localisation of two dUTPase isoforms in Drosophila shows cell cycle dependent character and is closely timed to the nuclear cleavage phases. (Figure 1)

![Diagram](image)

Figure 1 Localisation of two Drosophila dUTPases depends on cell cycle and absence/presence of NLS. Nuclear pore complexes (NPC) are responsible for NLS–selective transport across the nuclear membrane.
Uracil-DNA in *Drosophila melanogaster*

1. *Drosophila melanogaster* tolerates and interprets uracil-DNA
2. High uracil content DNA exists in Drosophila larval tissues, due to lack of dUTPase and lack of UNG
3. Uracil level in *Drosophila melanogaster* is comparable to the uracil level previously reported in ung – /dut – *E.coli* 3000-8000 uracil/ 10⁶ bases.
4. In Drosophila, uracil-DNA is present in all stages of development except in embryo, indicating that uracil-DNA is not restricted to larval stages and larval tissues even though it is most pronounced in 3ʳᵈ larvae.
5. Lack of dUTPase does not perturb normal life and development in larval stages.
6. Due to dUTPase silencing, uracil is accumulated in imaginal disc that causes lethality at early pupal stage. Developmental arrest occurs before transition to phanerocephalic pupae. Therefore, the role of dUTPase in nucleotide metabolism is not dispensable.
7. Uracil content varies according to the presence/absence of dUTPase in different tissues. dUTPase silencing resulted in increased level of uracil in imaginal discs.
8. 5’-fluorouracil (5’FU) and 5’-fluorodeoxyuridine (FUdR) treatment truly perturbs and increases the level of uracil in Drosophila Schneider 2 cells.
Publication list

Journal articles


Submitted manuscript


Selected Presentations

Poster


Muha V, A. Békési, I. Zagyva, B. G. Vértessy "From uracil-DNA to cell death within the context of fruitfly metamorphosis" Alexander von Humboldt Workshop on Structure-Based Approaches Towards Disease Control, Mátraháza H, 2007


Oral