Determination of physiological importance of deubiquitylase DmUsp5 in *Drosophila melanogaster*

Thesis of PhD dissertation

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1. Introduction

Ubiquitin is a small regulatory protein which is used for posttranslational modification of proteins in a reversible process called ubiquitylation. An enzyme cascade catalyzes the attachment of ubiquitins to target proteins. This covalent modification targets proteins to proteasomal degradation, changes enzymatic activities, or facilitates protein-protein interactions. The importance of ubiquitylation is highlighted by the fact that many intracellular physiological mechanisms are ubiquitin-dependent. These mechanisms include regulation of transcription by histone ubiquitylation, targeted protein degradation, regulation of cell cycle and programmed cell death.

As many other processes in the cell, ubiquitylation also has its opposite reaction, called deubiquitylation. This process is mediated by specific proteases called deubiquitylating enzymes, or shortly DUBs. Certain DUBs remove ubiquitins from ubiquitylated substrates before degradation, this way they can prolong half-life of certain proteins. A group of DUBs processes free polyubiquitin chains remained following proteasomal degradation and this function is essential in ubiquitin recycling. DUBs are also indispensable for ubiquitin biosynthesis. Newly synthetized ubiquitins are fused to

ribosomal subunits or form head-to-tail polyubiquitin fusions, and DUBs are required to process these fusions into free monoubiquitins.

Although DUBs are widely characterized from biochemical aspects in yeast and human cell lines, their physiological importance especially in multicellular organisms is poorly understood. It is not totally clear, for example, how DUBs contribute to the regulation of such an ubiquitin-dependent process, like apoptosis.

2. Specific aims

The aim of our work was to identify and genetically characterize deubiquitylating enzymes in an intact multicellular model organism, *Drosophila melanogaster*. Systematic knock down of DUB genes revealed which DUBs are essential for Drosophila development. Using a specific functional screen, we identified DUBs that could be implicated in apoptosis. We would like to accomplish these aims throughout these steps:

1. Identification of Drosophila DUBs by a bioinformatic approaches.
2. Preliminary characterization of Drosophila DUB genes using transgenic RNA interference lines or mutants.
3. Performing functional screens to identify DUBs involved in apoptosis.
4. Detailed analysis of genes showing interesting apoptotic phenotype:
    - generation of allele series;
    - studying apoptosis by microscopy and immunohistochemistry;
    - studying functional homology;
    - gene expression studies;
    - characterization of free and conjugated ubiquitin pools.

3. Methods

1. Databases and prediction software applications (NCBI, EBI, UniProt, FlyBase, InterPro Scan).
2. Study of *Drosophila melanogaster* transgenic RNA interference and P element mutant lines.
3. Semiquantitative reverse transcription coupled PCR (RT-PCR) and quantitative real-time PCR (qPCR).
4. Generation of deletion mutants by imprecise P element remobilization.
5. Western blot analysis.
6. Recombinant DNA techniques.
7. Generation of transgenic overexpression lines.
8. Fundamental yeast techniques (drug resistance assay, yeast transformation).
9. Cytological studies and immunohistochemistry on larval tissues.
10. Translational inhibitor (cycloheximide) feeding experiments.

4. Results

1. We identified potential Drosophila DUB genes using a bioinformatic approaches. Known yeast and human DUB sequences were used for homology search. The identification of Drosophila orthologs was facilitated by the fact that in addition to conserved catalytic domains, many DUBs contain other characteristic functional domains as well. Based on 58 human and 21 yeast DUB sequences we identified 45 potential DUB encoding genes in Drosophila. According to our knowledge on DUB protein family, we presume this is the total number of these proteins in Drosophila.
2. The identification of Drosophila DUBs was followed by a preliminary genetical characterization of these genes. Altogether 87 transgenic RNA interference lines and 45 P element or deletion mutants were obtained to investigate their loss of function phenotypes. Lethality or sterility was observed in the mutants of 27 DUB genes indicating that these DUBs are essential for Drosophila development.
3. Functional screens were performed in order to identify DUBs which can be implicated in apoptosis. For this purpose, we systematically knocked down DUB genes in the developing Drosophila eyes. In seven cases, we found rough

and abnormally reduced eye morphology with different severities. The observed phenotypes indicated that loss of these DUBs led to apoptosis. Since not much is known about the roles of DUBs in apoptosis, we continued our work with a detailed characterization of one of DUB genes with an apoptotic phenotype.

4. This gene was the *CG12082* DUB gene. In order to study its function in details, two deletion mutant alleles were generated by imprecise P element remobilization. Both mutations eliminated the gene function and resulted in late larval lethality. Moderate expression of the wild type *CG12082* sequence rescued lethal phenotypes in both cases.
5. Sequence comparisons of yeast, Drosophila and human proteins revealed that *CG12082* encodes a protein similar to yeast Ubp14 and human USP5. In addition to sequence comparison, we performed a heterologous complementation assay to investigate functional homology. It turned out that expression of the *CG12082* gene product could rescue the canavanine sensitive phenotype of the yeast *ubp14Δ* mutant. It was previously reported that human USP5 also rescues this yeast mutant. These results suggest that the gene product of *CG12082* is a functional ortholog of the

deubiquitylating enzyme Ubp14/USP5 in Drosophila, we therefore renamed it as DmUsp5.

6. Western blot analysis showed high accumulation of free and protein-bound polyubiquitin chains in *DmUsp5* mutants. Similar changes were reported in cases of yeast and human orthologs as well. This phenotype indicates a role of DmUsp5 in ubiquitin recycling. Simultaneously with polyubiquitin accumulation, a reduction of free monoubiquitin level could be observed. The reason for this may be that a significant proportion of the ubiquitin is trapped in polyubiquitylated proteins, and the replenishment of the free monoubiquitin pool is hampered in *DmUsp5* mutants.
7. A detailed analysis of apoptosis was performed in *DmUsp5* mutants. Orcein stained microscopic preparations of larval neuroblasts revealed a high incidence of small, rounded, pyknotic cells in *DmUsp5* mutants. These cells correspond to apoptotic cells and their number increased five times in mutants compared to wild type brains. The apoptosis was also supported by acridine orange staining and anti-activated caspase-3 immunostaining of larval brains and imaginal discs. Gene expression studies revealed an increased expression of the proapoptotic *p53, rpr* and *hid* genes. Gene

products of these proapoptotic regulators interact with DIAP1. DIAP1 is an apoptosis inhibitor in Drosophila, and under normal conditions suppress apoptosis by inhibiting caspases. Upon cytotoxic stress, proapoptotic regulators are overexpressed and bind to DIAP1. DIAP1 inhibition results in caspase release and apoptosis. Overexpression of proapoptotic regulators in DmUsp5 mutants reflects the downregulation of DIAP1 apoptosis inhibitor. Although we could not detect DIAP1 level directly, we observed a partial rescue of the apoptotic phenotype in *CG12082* mutants upon transgenic overexpression of DIAP1. This indirectly suggests that DIAP1 downregulation stands behind the observed apoptosis. DIAP1 is an E3 ubiquitin-ligase and its defective function can be explained by ubiquitin shortage in *DmUsp5* mutants.

8. Yeast studies revealed a phenomenon called ubiquitin stress which is activated upon monoubiquitin depletion and it is accompanied by overexpression of a DUB called Usp14. In our studies we identified the Drosophila ortholog of this protein by sequence similarity and named it as DmUsp14. Gene expression studies demonstrated an increased expression of this DUB in *DmUsp5* mutants. Our data represent the first evidence for the presence of ubiquitin

stress in a multicellular organism and suggest the activation of this mechanism when DmUsp5 function is abolished.

9. A distinctive feature of ubiquitin stress is an increased sensitivity to different chemicals, including translational inhibitors. Cycloheximide treatments indicated that even the loss of one copy of *DmUsp5* gene dramatically sensitized animals to this compound. Altogether, our data demonstrate an indispensable role of DmUsp5 in the maintenance of ubiquitin homeostasis and demonstrated that loss of its function leads to ubiquitin shortage. Moreover, we found an increased expression of proteasome subunits in *DmUsp5* mutants reflecting on a proteasome stress response triggered by the disturbed ubiquitin homeostasis.

5. Summary

Summarizing my thesis, I described the identification of Drosophila genes encoding deubiquitylating enzymes. A preliminary characterization of these genes revealed that more than half of them are essential for Drosophila development. Loss of seven of these essential DUBs led to high incidence of apoptosis. One of them is the evolutionary conserved DmUsp5, which is implicated in ubiquitin recycling and the maintenance of free ubiquitin pool. In absence of the DmUsp5 enzyme, the ubiquitin equilibrium of the cells become disturbed and an ubiquitin stress is triggered. The free monoubiquitin level is limited even at normal cellular conditions and different ubiquitin-dependent processes are competing for that. This limited pool of free monoubiquitins links protein degradation to apoptosis and to development by making these ubiquitin-dependent processes mutually interdependent. The different pleiotropic effects observed in DmUsp5 loss-of-function mutants indicate that apoptosis induction in these animals is caused by disturbance in ubiquitin equilibrium.

6. Publications

Article related to this Ph.D. thesis:

Kovacs L, Nagy O, Pal M, Udvardy A, Popescu O, Deak P
Role of the Deubiquitylating Enzyme DmUsp5 in Coupling Ubiquitin Equilibrium to Development and Apoptosis in Drosophila melanogaster.
PLOS ONE 10:(3) Paper e0120875. (2015)
IF: 3.534
MTMT ID number: 2871663

Other article:

Lipinszki Z, Kovacs L, Deak P, Udvardy A
Ubiquitylation of Drosophila p54/Rpn10/S5a Regulates Its Interaction with the UBA-UBL Polyubiquitin Receptors
BIOCHEMISTRY 51:(12) pp. 2461-2470. (2012)
IF: 3.377
MTMT ID number: 2015348

Oral presentations related to this work:

- Hungarian Molecular Life Sciences Congress, Eger, March 27-29, 2015
- Annual Congress of Hungarian Biochemical Society, Debrecen, August 24-27, 2014.
- Conference for PhD Student in Biology from Szeged, Szeged, May 19-20, 2014.
- 23rd European Drosophila Research Conference, Barcelona, October 16-19, 2013.

- 13th FEBS Young Scientists Forum, St. Petersburg, July 3-5, 2013.
- Straub Days, Szeged, May 23-24, 2012.
- International Training Course 2nd Alumni Conference, "Multidisciplinary Approaches to Biological Problems", Szeged, September 1-3, 2011.
- 9th Hungarian Congress on Genetics and 14th Cell and Developmental Biology Days Napok, Siófok, March 25-27, 2011.

Poster presentations related to this work:

- Federation of European Biochemical Societies (FEBS) Congress 2013 “Mechanisms in Biology”, St. Petersburg, July 6-11, 2013.
- Straub Days, Szeged, May 29-30, 2013.
- Hungarian Life Sciences Congress, Siófok, April 5-7, 2013.
- FEBS3+ Meeting, "From molecules to life and back", Opatija, June 13-16, 2012.
- Annual Congress of Hungarian Biochemical Society, Pécs, August 28 - September 3, 2011.
- Ubiquitin-Like Molecules in Disease Meeting, Cambridge, June 27, 2011.
- FEBS Advanced Lecture Course, Trends in Genetics: Genomic Instability and Pathways of Response, Yerevan, February 20-26, 2011.