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Abstract of PhD thesis

Examination of ubiquitin forms and ubiquitination in *Drosophila melanogaster*

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Introduction

The posttranslational modification of proteins by ubiquitination plays an important regulatory role in most cellular processes. During ubiquitination, the covalent binding of ubiquitin moieties to target proteins occurs through an enzyme cascade containing a ubiquitin activating (E1), a ubiquitin conjugating (E2) and a ubiquitin ligase (E3) enzymes. As a result, the substrate is either mono-, multi- or polyubiquitinated, which affects its activity, stability or interactions. The ubiquitination is reversible, since the deubiquitinating enzymes (DUBs) can release conjugated ubiquitins from substrates. The released monoubiquitins are recycled in new ubiquitinating reactions. Due to the opposing effects of ubiquitination and deubiquitination, the intracellular ubiquitin content is divided into two pools, of free monoubiquitins and conjugated ubiquitins. These ubiquitin forms reach a dynamic intracellular equilibrium and its maintenance is essential for normal cell physiology. Especially the availability of free monoubiquitins is pivotal for timely ubiquitination of protein substrates. The maintenance of the free monoubiquitin pool is achieved through coordinated actions of ubiquitination and deubiquitination, ubiquitin synthesis and ubiquitin degradation.

The ubiquitin proteasome system (UPS) is tightly regulated by many genes and it appears to be evolutionarily conserved in eukaryotes. Ubiquitin recycling in the UPS greatly contributes to the maintenance of the ubiquitin equilibrium. To examine the relationship between the UPS and ubiquitin equilibrium we needed an effective method for ubiquitin quantification. Several methods were developed for ubiquitin measurements in the last 30 years, but none of them were completely suitable for simple and rapid quantification of different ubiquitin forms. Recently, Oh and his coworkers

described a new approach in mouse tissues, which fulfilled these requirements. The method is based on the densitometric analysis of Western blots, and it is suitable for simultaneous determination of total, as well as free and conjugated ubiquitins from whole protein extracts. The main feature of this assay is the utilization of endogenous DUBs present in lysates that process all forms of conjugated ubiquitins to free monoubiquitins, therefore permitting the application of only a single ubiquitin-specific antibody. In this way, Western blots of appropriate tissue lysates together with ubiquitin standards enable the quantification of the different ubiquitin fractions as a single entity by densitometric analysis.

We selected three mutants, which disrupts different steps of the system, to examine their effects on the ubiquitin equilibrium. The proteasome associated polyubiquitin receptors recognize and bind the polyubiquitinated proteasome substrates. One of these receptors, the p54/Rpn10 has an important role in the selective proteolysis of proteins. The ubiquitin signals are released from the proteasome substrates before their degradation. Rpn11 is a proteasome associated DUB, which hydrolyzes the proximal end of the polyubiquitin chains (where it is linked to its target protein), releasing whole chains to the cytoplasm. In the cytoplasm, the DUB Usp5 cleaves the unanchored polyubiquitin chains and the freed monomeric ubiquitin is recycled. Other proteasome associated DUBs, like Usp14, cleave ubiquitin from the distal tip of the polyubiquitin chains, removing and recycling them step-by-step. Shortening the conjugated polyubiquitin chains can lead to the substrates dissociation from the proteasome, thereby the suppression of protein degradation.

The ubiquitin ligases are also important players of the ubiquitin-proteasome system, since they determine the substrate specificity of

ubiquitination. Several ubiquitin ligases are present in the cell, but one of them, the APC/C outstands both in its size and in complexity. The APC/C plays key role in mitosis and the maintenance of the G1 phase by targeting the regulator proteins to proteasomal degradation. The APC/C contains at least 13 evolutionary well-conserved subunits. The smallest but still essential subunit is the Cdc26, which presumably plays role in the structural stability of the APC/C.

Aims

The aim of my thesis work was to examine the dynamic changes of free and conjugated ubiquitins in *Drosophila melanogaste*r and to characterize new *Drosophila* homologues of the small APC/C subunit, Cdc26.

- Optimization of a Western blot based ubiquitin quantification method to *Drosophila*.
- Determination of ubiquitin forms and their dynamics in all developmental stages and several tissues of *Drosophila*.
- Quantification of the effects of selected mutations to the ubiquitin equilibrium.
- Characterization of two new putative APC/C subunits in *Drosophila*

Materials and methods

The flies used in the experiments were maintained on standard *Drosophila* medium at 25 ºC. Isogenized *Drosophila melanogaster OregonR* and *DSK001* strains were used as wild type control. The RNA interference and

mutant lines were: *Usp5*^{RNSi}, *p54/Rpn10*^{RNSi}, *Usp14*$^{\Delta 32}$, *Ubi-p63E*^{EY07341}, *CG17343*^{EY19920} and *CG3457*$^{15/1}$. The UAS-Gal4 system was used to induce RNA interference.

To quantify the ubiquitin content of the samples, appropriate lysates and ubiquitin standards were separated in SDS-PAGE, followed by Western blot analysis using a single ubiquitin-specific antibody. The ubiquitin concentration was calculated from densities of the monoubiquitin bands of the samples with the help of calibration curves generated with ubiquitin standards.

Transgenic constructs carrying the human Cdc26, HA-tagged Cdc26-like and GFP tagged Cdc26 and Cdc26-like were made using the standard *Drosophila* expression system (Gatway).

Complementation tests were performed in transgenic flies overexpressing the human *Cdc26* (*HsCdc26*) in a homozygous *Cdc26* mutant background in *Drosophila*. The interaction between the hemagglutinin tagged Cdc26-like and the FLAG tagged Mákos/Cdc27 was examined in coimmunoprecipitation experiments.

Semiquantitative RT-PCR was performed to examine the expression pattern of *Cdc26* and *Cdc26-like* mRNA in *Drosophila*. To examine protein expression, transgenic constructs containing *GFP* tagged *Cdc26* and *Cdc26-like* regulated by their own promoter were analyzed with Western blot using a GFP-specific antibody.

Results

Ubiquitin pool dynamics in Drosophila developmental stages and tissues

To analyze the dynamics of different ubiquitin forms in *Drosophila*, we adapted a quick, sensitive and relatively simple technique, developed for

mouse tissues. Optimizing the method included step-by step changes in buffer composition, incubation time and temperature, to meet the requirements of *Drosophila* physiology.

After optimizing this assay to *Drosophila*, we determined the ubiquitin profile in all developmental stages and various tissues. Our result showed that ubiquitin levels and the ratio of the ubiquitin forms change dynamically in different developmental stages and also in different tissues. Developmental transitions that require complex tissue remodeling, such as larval-pupal and pupal-adult transitions, the ratio of free monoubiquitins increased substantially. High monomer ratio could also be determined in mitotically active tissues, like brain, ovary and testis.

Loss of function of the ubiquitin-proteasome system leads to quantifiable changes in the ubiquitin equilibrium

The ubiquitin-proteasome system plays important roles in ubiquitin recycling, which ensures the balanced state between free and conjugated ubiquitin forms. Mutations in genes regulating the ubiquitin-proteasome system generated measurable changes in the ubiquitin pools. The loss of function of the proteasomal ubiquitin receptor *p54/Rpn10* caused an estimated two-fold increase in the total ubiquitin concentration, which included the accumulation of both free monoubiquitins and conjugated ubiquitins. The loss of function of a DUB, Usp5, also led to a two-fold increase in the total ubiquitin level, but with different dynamics. The high ubiquitin concentration measured in the mutant samples was the consequence of the accumulation of unanchored polyubiquitin chains, while the monoubiquitin content slightly decreased.

The Usp14 proteasome associated DUB disassembles polyubiquitin chains conjugated to proteasome substrates by removing ubiquitin monomers

from their distal tip. In *Drosophila*, null mutation of the *Usp14* gene resulted in reduction of free ubiquitin concentration in the testis, while other tissues were unaffected. The severity of ubiquitin shortage was increased in *Usp14* - *Ubi-p63E* double mutants. *Ubi-p63E* is one of the five ubiquitin genes in *Drosophila* that provides newly synthesized ubiquitin predominantly for testes. This synergistic interaction of *Usp14* and *Ubi-p63E* implies that the two genes play similar roles in the maintenance of the monoubiquitin pool in testes.

Both Cdc26 paralogues are APC/C subunits in Drosophila

Our group identified two putative *Cdc26* homologues in *Drosophila*, designated as Cdc26 and Cdc26-like. One of them, *Cdc26*, proved to be essential, and its loss of function resulted in mitotic phenotypes characteristic to known essential subunits of the *Drosophila* APC/C. Overexpression of the human APC/C subunit, HsCdc26, complemented the lethal phenotype of *Cdc26*, implicating that the Cdc26 has similar function in *Drosophila*.

The *Cdc26-like* gene is not essential, but its overexpression complemented the lethal phenotype of the *Cdc26* mutations. In addition to this, coimmunoprecipitation experiments revealed physical link between Cdc26-like and known APC/C subunits.

Cdc26 and Cdc26-like show different expression patterns

The mRNA expression pattern of Cdc26 and Cdc26-like proved to be different in time and space: while *Cdc26* was expressed in all developmental stages, *Cdc26-like* mRNA was present only in adult ovaries and embryos. These results were confirmed by the Western blot analyses of GFP-tagged Cdc26 and Cdc26-like proteins from different *Drosophila* tissues, expressed by their own promoters. These results suggest, that both Cdc26 and Cdc26-like

are genuine APC/C subunits, but most probably in different stages of *Drosophila* development.

Summary of results

- A simple and reliable method for quantitative measurements of ubiquitin forms was adapted and optimized to *Drosophila*.
- Ubiquitin profile of *Drosophila* developmental stages and tissues demonstrate dynamic nature of the different ubiquitin pools.
- The loss of p54/Rpn10 function causes an increase in total ubiquitin content, which includes the accumulation of both conjugated and free monoubiquitins.
- Loss of *Usp5* results in an increase in the total ubiquitin level. This was due mostly to the accumulation of the conjugated ubiquitins, while the monoubiquitin pool slightly decreased.
- Loss of *Usp14* function results in a reduced level of free monoubiquitins in testes only that explains the male sterile phenotype of the mutants. Our data suggest that Usp14 has a role in the maintenance of the monoubiquitin pool in testes.
- Cdc26 and Cdc26-like appear to be genuine APC/C subunits, but may act in different stages of *Drosophila* development.

Publications underlying the thesis

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Nagy Á, Kovács L, Lipinszki Z, Pál M, Deák P. (2018). Developmental and tissue specific changes of ubiquitin forms in Drosophila melanogaster. *PLoS ONE*, 13(12): e0209080.

Kovács L, **Nagy Á**, Pál M, Deák P. (2019) Usp14 is required for spermatogenesis and ubiquitin stress responses in *Drosophila melanogaster*. *Journal of Cell Science* (review alatt)

Conferences

Nagy Á, Lipinszki Z, Kovács L, Pál M, Deák P: Ubiquitin pool dynamics revealed by quantifying ubiquitin forms in Drosophila UPS mutants. Hungarian Molecular Life Science, 2019.03.29-31, Eger, Hungary

Nagy Á, Kovács L, Nagy O, Pál M, Deák P: Unique subunit composition of the Drosophila APC/C. CSH Asia meeting on Ubiquitin Family, Autophagy and Diseases, 2018.04.09-13, Suzhou, China

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Nagy Á, Lipinszki Z, Kovács L, Pál M, Deák P: Developmental stage- and tissue-specific ubiquitin levels in *Drosophila melanogaster*. Hungarian Molecular Life Science, 2017.03.31-04.02, Eger, Hungary

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Nagy Á, Horváth J, Nagy O, Pál M, Kovács L, Deák P: Characterization of a new APC/C subunit in *Drosophila melanogaster*. EMBO Workshop on the Cell Cycle, 2015.09.04-07, Budapest, Hungary

Nagy Á, Horváth J, Nagy O, Pál M, Deák P: Anaphase promoting complex subunits with unusual characteristics. Hungarian Molecular Life Science, 2015.03.27-29, Eger, Hungary

Conflict of interest

I myself as corresponding author of the following publications declare that authors have no conflict of interest and Ágota Nagy Ph.D candidate had a great contribution to the published results. Results discussed in his thesis are regarded as outcomes of his own scientific work.

Nagy Á, Kovács L, Lipinszki Z, Pál M, Deák P. (2018). Developmental and tissue specific changes of ubiquitin forms in Drosophila melanogaster. *PLoS ONE*, 13(12): e0209080.

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