**Doctoral theses**

# New molecular pathways and posttranscriptional modifications in autophagy regulation

**Bánréti Ágnes Regina**

**Eötvös Loránd University**

**Faculty of Science**

**Ph.D. School of Biology**

**Molecular Cell and Neurobiology Program**

**2014**

**Leader of thePh.D. School: Prof. Erdei Anna**

**Supervisor: Prof. Sass Miklós**

Ph.D., DSc. Habil., professor

**Consulent: Dr. Yacine Graba**

Ph.D., "Research Director at CNRS, France"

## Introduction

Cellular homeostasis requires a precise balance between synthetic and catabolic processes. Autophagy (cellular "self-digestion") is one of the main catabolic mechanisms eliminating cellular organelles and proteins by the lysosomal system. To date, autophagy has been shown to play a major function in many physiological processes including response to stress factors, elimination of damaged cellular organelles and tissue remodelling during development. There are three major forms of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy. In the course of macroautophagy, portions of the cytoplasm are sequestered into specialized double-membrane-bound vesicles known as autophagosomes. Subsequent fusion of autophagosomes with lysosomes leads to hydrolytic degradation of the sequestered material. I focused on macroautophagy, hereafter referred to as autophagy. Signaling pathways that control autophagy have been originally described in yeast, and these regulatory systems have been shown to be evolutionarily conserved in worms, flies and mammals. Since autophagy has been implicated in various pathological conditions, including cancer, obesity, diabetes and neurodegenerative diseases, understanding the molecular mechanisms controlling autophagy is also important from medical aspects.

Many cellular processes and signaling pathways are dependent upon reversible phosphorylation of proteins. Several kinases are known to have essential roles in the regulation of autophagy, but the mechanisms by which phosphatases influence autophagic activity remains largely unexplored. Protein phosphatase 2A (PP2A) is a serine/threonine-specific protein phosphatase that plays multiple roles in different signaling pathways and regulates diverse cellular processes such as transcription, DNA replication, intermediary metabolism, cell proliferation, cell cycle, apoptosis and autophagy. The PP2A holoenzyme is a heterotrimeric complex, consisting of A, B and C subunits. The catalytic subunit PP2A-C binds to the carboxy-terminal of the scaffold protein PP2A-A. There are different B subunits, which determine the subcellular localization and substrate specificity of the holoenzyme. Previous studies have described that inhibition of PP2A-C by okadaic acid suppresses autophagy in hepatocytes. Furthermore, PP2A is involved in the control of TOR-dependent, stress-induced autophagy in yeast.

In the present work I show that mts (PP2A catalytic subunit) and PP2A-29B with two regulatory subunits wdb and B', compose two various heterotrimeric complexes and regulate stress-induced autophagy in two alternative ways**.**

Hox proteins regulate numerous pathways during developmental processes including cellular differentiation and apoptosis. It has been known that programmed cell death is highly active during differentiation and animal development. However, while a link between Hox proteins and apoptosis (PCD I) has been established, if Hox proteins also regulate autophagic programmed cell death (PCD II) has not been described yet. My results show that *Drosophila* Hox proteins are potent repressors of the autophagic process. In inhibiting autophagy, Hox proteins display no apparent paralog specificity and do not provide positional information. Instead they impose temporality on developmental autophagy, and act as effectors of environmental signals in starvation-induced autophagy. Further characterization establishes that temporality is controlled by Pontin, a facultative component of the Brahma chromatin remodeling complex, and that Hox proteins impact on autophagy by repressing the expression of core components of the autophagy machinery.

**Main aims of the Ph.D. study**

**A) Investigate the role of PP2A in the regulation of autophagy in *Drosophila*:**

1) Does *Drosophila* PP2A phosphatase have any role in autophagy regulation?
2) Which *Drosophila* PP2A subunits regulate autophagy?
3) How PP2A fits in the molecular regulatory network that reglates autophagy?
4) What are the potential targets of PP2A?

**B) Establishing a link between Hox proteins and autophagy:**

1) What is the temporal expression pattern of Hox gene expression in *Drosophila* larval fat body, before and after the induction of developmental autophagy?
2) What regulates the temporal expression pattern of Hox genes?
3) Do Hox genes control developmental autophagy?
4) Do environmental signals effect the temporal expression pattern of Hox genes?
5) Do Hox genes have any role in the regulation of starvation-induced autophagy?
6) How Hox genes fit in the molecular regulatory network that reglates autophagy?
7) What are the potential targets of Hox proteins?
8) Is the effect of Hox proteins on autophagy evolutionary conserved?

**Matherials and Methods**

Methods: *Drosophila* genetics, Semi-Quantitative and Quantitative Reverse Transcription PCR (RT-PCR), molecular cloning, immunohystochemistry and immunocytochemistry, fluorescent and confocal microscopy, transmission electron microscopy (TEM), vertebrate cell line transfection

Model systems: *Drosophila* L3 stage larvae, vertebrate COS-7 cells

**Results**

A) Inhibiting the PP2A catalytic subunit mts by okadaic acid treatment, RNAi or P-element insertion in the mts gene, completely blocked starvation-induced autophagy. These results demonstrate that the *Drosophila* PP2A, similarly to its mammalian ortholog, is essential to autophagy. I further proved that three other PP2A subunits, the PP2A-29B, wdb and PP2A-B' have also imortant roles in autophagy induction. Overexpression of a GFP::PP2A-B' fusion gene resulted in an intense autophagosome formation in response to starvation in the fat body cells of the larvae. GFP-labeled PP2A-B'protein was strongly localized to the membrane of the large LysoTracker-Red positive granules following starvation, by fluorescent, confocal and electron microscopy. I further showed that rapamycin treatment alters the subcellular localization of PP2A-B' and B' subunit is essential to rapamycin-induced autophagy. In contrast, silencing of wdb by RNAi did not influence rapamycin-induced autophagy. These results show that while the PP2A-B' may function upstream of the dTOR kinase, wdb acts downstream of dTOR.

I identified Srp (Serpent, the Drosophila ortholog of the yeast Gln3) as a potential target of PP2A. I showed that Srp regulates the fusion of autophagosomes and lysosomes. Furthermore, I identified three new Atg genes (Atg14, Atg17 and Atg101), which may be under the control of PP2A and Srp.

B) Investigating the spatial expression pattern of Hox proteins by immunostaing of larval fat bodies showed an unusual distribution of Ubx and AbdB that spread over the entire fat body, including the most anterior region where Dfd is expressed. These results suggest an unusual non-spatial function. Therefore, I examined the temporal dynamics of Hox gene expression by focusing on the L3 feeding (L3F) and L3 wandering (L3W) larvae which respectively provides stages of pre- and post-developmental autophagy induction. My results show that genes are downregulated at the L3F/L3W transition, at the transcriptional and

translational level. Clonal maintenance of multiple Hox gene expression in L3W animals using FRT/FLP clonal system resulted in the complete inhibition of developmental autophagy in fat body cells. These results were also confirmed by electron microscopy. Hox gene expression patterns and gain-of-function experiments support a generic temporal function for Hox proteins in the fat body. This predicts that individual removal of Hox genes should not alter Hox-controlled developmental autophagy. The Trithorax group Brahma (Brm) complex mediates epigenetic and global gene expression maintenance at Hox complexes, including at the *Drosophila* ANT-C and BX-C in embrios and other tissues. To address a likely global Hox gene regulation by the Brm complex in the fat body, I examined the impact of inactivation of different Brm complex members on the expression of Hox gene products. My results show that Hox gene expression is properly maintained and autophagy is inhibited when the constitutive Brm complex components (Brm, Osa, Moi) and the accessory component Pont are simultaneously present, as is the case in L3F fat body cells. The timing of developmental autophagy that occurs in late L3 stage is under the control of ecdysone. My results also show that ecdysone signaling controls the temporal dynamics of Pont and inhibits Hox gene expression in fat body cells. It was previously shown that starvation induces autophagy in L3F larvae, prior to the onset of developmental autophagy. Following the dynamics of Hox proteins and monitoring autophagy, I observed an anti-correlation between the time course of Hox nuclear accumulation and autophagy following starvation. These results indicate that starvation-induced autophagy is also inhibited by Hox proteins and requires a global Hox clearance. I established a link between Hox proteins and the conserved Pi3K/Akt1/dTOR pathway, the central regulator of starvation-induced autophagy. To further explore how Hox genes control autophagy, I focused on the expression of Atg genes (AuTophaGy-related genes) that encode proteins of the core autophagy molecular machinery and are essential for autophagosome formation, elongation and their fusion to lysosomes, as well as additional genes known for their involvement in autophagy. Using quantitative RT-PCR analysis, I showed that maintaining Hox gene expression in L3W stage represses a large number of genes required for proper autophagy. Finally, the potential of central and posterior mouse Hox proteins to inhibit autophagy in *Drosophila* and in vertebrate COS-7 cells indicate that regulation of autophagy is an evolutionary conserved feature of Hox proteins.

## Discussion

Autophagy is a cellular process that is conserved in all eukaryotes, occurs in a wide range of cell types and plays important functions in a variety of biological processes such as response to starvation, growth factor deprivation, aging, elimination of damaged organelles, and cellular and tissue remodeling during animal development. Induction or inhibition of autophagy involves multiple levels of regulation, including developmental signals conveyed by the steroid hormone ecdysone, and environmental signals, sensed in the case of amino acid starvation by the InR/TOR pathways. These regulatory paths do not act independently but seem rather to be interconnected as illustrated by developmentally induced ecdysone-mediated autophagy that acts by repressing the inhibitory function of the InR/Akt1/dTOR pathway.

Many cellular processes and signaling pathways are regulated through reversible protein phosphorylation. However, several kinases have essential roles in the regulation of autophagy, but the mechanisms by which these proteins influence autophagic activity remains largely unexplored. Protein phosphatase 2A (PP2A) holoenzyme is a heterotrimeric complex, consisting of A, B and C subunits. The catalytic subunit PP2A/C (microtubule star/mts) binds to the carboxy terminal part of the scaffold protein PP2A/A (PP2A-29B). In *Drosophila*, there are three different forms of B subunits (widerborst/wdb, twins/tws and PP2A/B'), which determine the subcellular localization and substrate specificity of the holoenzyme. Previous studies demonstrated that PP2A is involved in the control of TOR-dependent autophagy both in yeast and mammals. Furthermore, in *Drosophila*, wdb genetically interacts with the PI3K/ Akt1 signalling cascade, a main upstream regulatory system of dTOR. My results demonstrate that in *Drosophila*, two distinct PP2A complexes (with the participation of wdb or B' subunits) play important roles in starvation-induced autophagy in two alternative pathways. The PP2A A/wdb/C complex acts upstream of dTOR, whereas the PP2A A/B'/C complex functions as a target of dTOR and regulates the elongation of autophagosomes and their subsequent fusion with lysosomes. Furthermore, I identified three new *Drosophila* Atg orthologs (*Atg14*, *Atg17* and *Atg101*), which may represent potential targets of the PP2A A/B'/C complex during autophagy. A new member of the regulatory network of autophagy was also identified as Serpent (Srp), the *Drosophila* ortholog of Gln3. Taken together, my results provide support for a new, dual role of PP2A in starvation-induced autophagy in *Drosophila* with a number of interesting candidates being identified as PP2A-interacting partners.

Temporal regulation of autophagy is key to its function. Genetic studies in the fly model indicate that autophagy is developmentally programmed and essential for normal *Drosophila* development. Autophagy is activated in several larval tissues including fat body, salivary gland, midgut and trachea. This allows the degradation and recycling of cellular materials permitting tissue remodeling, leading to the transformation of larvae into adult flies. I established a link between Hox protein function and developmental autophagy. The results of my Ph.D work show that programmed autophagic cell death in the *Drosophila* larval fat body is inhibited by multiple Hox proteins, including Dfd, Scr, Ubx, AbdA and AbdB. This highlights the generic use of Hox proteins for controlling the timing of a developmental process, which contrasts with their widely described role in controlling the spatial specificity of developmental programs. I further identified that proper timing of Hox gene expression is under the control of the steroid hormone ecdysone thought the regulation of the Brahma chromatin remodelling complex. Besides controlling developmental autophagy, Hox proteins mediate environmental control of autophagy and they are integral components of the InR/Akt1/dTOR pathway. In regulating autophagy, Hox proteins may act through multiple paths, which include the regulation of Atg genes and other genes encode proteins that are implicated in autophagy. I show that Ubx and AbdB repress most of the known autophagy genes and have common targets like *Atg8b*. In summary, my findings broaden the framework of Hox protein function, showing that besides providing spatial information, they also coordinate temporal processes and more surprisingly act as mediators of environmental signals for regulating autophagy.

Given that the autophagic process is conserved from yeast to mammals these new autophagy regulators and molecular pathways may open new possibilities for pharmacological manipulation of autophagy.

**Publications related to the Ph.D thesis**

**Agnes Banreti**, Bruno Hudry, Andrew Saurin, Miklos Sass and Yacine Graba

Hox proteins mediate environmental control of autophagy, under review in Developmental Cell, 2013 Dec 31. pii: S1534-5807(13)00705-3. doi: 10.1016/j.devcel.2013.11.024. [Epub ahead of print]

**Agnes Banreti**, Tamas Lukacsovich, Gyorgy Csikos, Miklos Erdelyi, and Miklos Sass

PP2A regulates autophagy in two alternative ways in *Drosophila*, Autophagy. 2012 Apr;8(4):623-36.

**Other publications**

**Agnes Banreti**, Miklos Sass and Yacine Graba

The emerging role of acetylation in the regulation of autophagy, Autophagy. Autophagy. 2013 Jun 1;9(6):819-29.

**Conference presentations related to the Ph.D thesis**

**Oral communications:**

**Banreti A,** Hudry B, Saurin A, Sass M, Graba Y

Hox Proteins Regulate Programmed Autophagic Cell Death in *Drosophila*

Symposium on HOX and TALE Transcription Factors on Development and Disease, Madrid, Spain, 2012

**Banreti A,** Sass M, Graba Y

Hox Protein-Regulated Autophagy

Seminar at IBDML, Marseille, France, 2011

**Poster presentations:**

**Banreti A**, Hudry B, Sass M, Graba Y.

Hox proteins mediate autophagic programmed cell death in *Drosophila*

Autophagy in health and disease-EMBO meeting, Ma'ale Hachamisha, Israel, 2011

**Banreti A**, Hudry B, Sass M, Graba Y.

Hox proteins mediate autophagic programmed cell death in *Drosophila*

Hox and TALE Transcription Factors in Development and Disease, Carry le Rouet, France, 2011

**Banreti A**, Csikos G, Lukacsovich T, Sass M.

The *Drosophila* Target of Rapamycin (dTOR) and Protein Phosphatase 2A (PP2A) in autophagy regulation.

Hungarian Biochemical Association Assembly, Budapest, Hungary, 2009

**Banreti A**, Csikos G, Sass M

The role of Protein Phosphatase 2A in the regulation of autophagy.

8th Hungarian Genetic Congress and 15th Cell and Developmental Days, Nyiregyhaza, Hungary, 2009

**Banreti A**, Csikos G, Sass M

The role of PP2A (Protein Phosphatase 2A) in the regulation of autophagy.

2nd International Conference on Molecular Perspectives on Protein-Protein Interactions, Dubrovnik, Croatia, 2008

**Banreti A**, Csikos G, Sass M

The role of Protein Phosphatase 2A in the regulation of autophagy - The Janus-faced phosphatase.

38th Membrane Transport Conference, Sumeg, Hungary, 2008

**Scientific Grants and Awards that supported the Ph.D. work**

**2012** STSM COST grant to young investigators for conference participation

**2011** "The best EMBO poster of the conference" award at the EMBO meetings-Autophagy in health and disease, Ma'ale Hachamisha, Israel

**2011** Richter Gedeon Predoctoral fellowship

**2011** STSM COST Short Term Scientific Support

**2011** Bourse de l'Espace Campus France Hongrie, Ambassade de France en Hongrie

**2010** Special Scientific Support from Eotvos Lorand University

**2010** STSM COST Short Term Scientific Support

**2010** Erasmus fellowship for Ph.D. students

**2008** Special Scientific Support from Eotvos Lorand University