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UBIQUITIN AND ITS ROLE IN PROTEOLISIS: THE 2004 NOBEL PRIZE IN CHEMISTRY

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Our story shows that the ubiquitin system could not have been discovered without biochemical approaches. So my advice to young investigators in biomedical sciences is: if you have a problem that cannot be solved by molecular genetics alone, do not be afraid to use biochemistry, do not hesitate to enter the cold room, and do not be wary of approaching the FPLC machine!

Avram Hershko

The achievement is not the Nobel, the achievement is the science. Prizes and recognition are not targets that one should aim for. Breakthrough achievements that expand our knowledge of the world and benefit mankind are.

Aaron Ciechanover

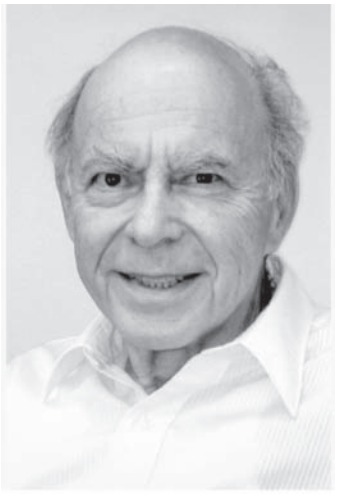
In the early 1980-s, Aaron Ciechanover, Avram Hershko, and Irwin Rose discovered one of the most important cyclic cellular processes - a regulated ATP-dependent protein degradation, for which they were awarded the 2004 Nobel Prize in Chemistry. These scientists proved the existence of a non-lysosomal proteolysis pathway and completely changed the perception of intracellular protein degradation mechanisms. They demonstrated pre-labelling of a doomed protein in a cell with a biochemical marker called ubiquitin. Polyubiquitylation of a protein as a signal for its proteolysis was a new mechanism discovered as a result of collaborative efforts of three scientists on isolation of enzymes involved in this sequential process, clarification of the biochemical stages, and substantiating the energy dependence mechanism. The article contains biographical data of the Nobel laureates, the methods applied, and the history of the research resulted in the discovery of the phenomenon of proteasomal degradation of ubiquitin-mediated proteins.

K e y w o r d s: A. Ciechanover, A. Hershko, I. Rose, ubiquitin, regulated protein degradation, PROTAC.

From 1950-s till 1980-s, the scientific studies were characterized by intensive development of molecular biology and a revolutionary breakthrough in elucidating the DNA structure, the mechanisms of transmission of genetic information from DNA to RNA, and protein synthesis. However, the question of how proteins degrade remained beyond the researchers view. Early on, it was thought that only dietary proteins broke down to provide energy, while the body's structural proteins remained relatively stable and underwent only minor "wear and tear". After the discovery of lysosomes in the 1950-s, it became clear that these organelles, equipped with proteolytic enzymes, active at acidic pH, were capable of breaking down proteins. However, some data accumulated over time contradicted the ideas about the role of lysosomal activity in protein degradation. Thus, the half-life of different

Aaron Ciechanover (1947)

Avram Hershko (1937)



Irwin Rose (1926–2015)

proteins varied by several orders of magnitude (from minutes to many days), and inhibitors of lysosomal proteinases suppressed the cleavage of extracellular proteins that arrived through the receptor endocytosis pathway, but had no effect on the cleavage of proteins with a short half-life, as well as of abnormal/mutated proteins. Moreover, unlike the exergonic process of protein cleavage with lysosomal proteases, intracellular proteolysis required the metabolic energy, which was quite paradoxical in terms of thermodynamics principles.

The data obtained showed the existence of a non-lysosomal mechanism of protein degradation, however, to confirm its existence, a cell-free system that would have copied protein degradation by a specific energy-dependent mechanism at neutral pH was lacking. Such a cell-free proteolytic system was first produced from reticulocytes in 1977. Reticulocytes are terminally differentiated blood cells, which lose lysosomes and other structural elements at final stages of maturation in the bone marrow before entering the bloodstream, yet retain the ability to break down intracellular proteins. Aaron Ciechanover (Technion, Israel Institute of Technology, Haifa, Israel), Avram Hershko (Technion, Israel Institute of Technology, Haifa, Israel), and Irwin Rose (University of California, Irvine, USA) used this same cell-free system for their research of protein degradation. These scientists revolutionized the concept of protein degradation mechanisms. They discovered that doomed cellular protein is pre-labeled with a biochemical marker called ubiquitin. In 2004, the three scientists were awarded the Nobel Prize in Chemistry for the discovery of ubiquitin-mediated protein degradation. Usually, the Nobel Committee adheres to strict scientific terminology in its communications, but this time, explaining the essence of the award-winning discovery, it resorted to the figurative expression “a kiss of death”, thus highlighting the role of ubiquitin in protein degradation. The Nobel Prize was shared equally by the laureates.

A brief summary of the award winners

Avram Hershko was born on December 31, 1937, in the small Hungarian town of Karcag, near Budapest. His father, Moshe Hershko, was a schoolteacher at a Jewish primary school, and his mother, Margit (Shoshana) Hershko, taught the children piano lessons. Abraham has adored the pre-war period of his childhood and considered it a happy time when he lived in a beautiful house surrounded by a garden, with loving parents and his older brother Haim. This paradise was lost with the outbreak of World War II. In 1942, after Hungary became a satellite of the Nazi Germany, Avram’s father was enlisted to the army and later sent to the Russian front, where he was taken prisoner. In 1944, Avram, together with his mother, older brother and other Jewish families, were sent to the Auschwitz concentration camp, where, as it turned out later, many of Hershko’s relatives died. By a happy coincidence, the train carrying Avram arrived in Austria, where Jews were sent to involuntary labor. In 1946, after returning his farther from a POW camp, the family moved to Budapest, and three years later immigrated to Israel.

In Jerusalem, his father worked as a school teacher, thus making possible for Abraham and his brother to study at the expensive private school.

Avram was a good student, easily catching mathematics, physics, literature, history and even the Talmud. Upon graduating from the school, he decided to study medicine not least because of his brother Heim had already been a medical student (he could inherit all his books for free).

In 1956, Avram began studying at the Hadassah School of Medicine with the Hebrew University of Jerusalem. A part of the course was taught by Jacob Mager, a prominent biochemist and an individual of encyclopedic knowledge. Avram was so impressed with the depth of Mager's knowledge of biochemistry that managed to get invited to work at his laboratory, where he started working in 1960. At his relatively small laboratory, Mager simultaneously worked on several biomedical research projects and managed to excite interest of his students in various branches of biochemistry. By the end of the year, Avram already knew that he would work rather in scientific research than in clinical practice.

In 1965, he graduated from the medical school, earned the master's degree, served in the military in 1965–1967, and then returned to Mager's laboratory for another two years to complete his PhD (1967–1969). The experience gained from collaboration with this scientist not only deepened Avram's knowledge of enzymology, but also significantly influenced his progress as a scientist. Jacob Mager was a very pernickety experimenter, using all possible positive and negative controls for every experiment, repeating every significant new result several times to prove its validity.

In 1968, A. Hershko met Gordon Tomkins from the University of California in San Francisco, who was giving a course of lectures in Israel. In 1969-1971, at the invitation and under supervision of Tomkins, Avram worked as a postdoc at the laboratory of the UCSF Department of Biochemistry and Biophysics. G. Tomkins was very different from J. Mager, as he was sociable, lively, did not care much about experimental details, but constantly burst out with great ideas and was an efficient stimulator for many researchers. G. Tomkins explored the problem of tyrosine aminotransferase (TAT) synthesis activation in hepatoma cells with hormones. Hershko recalled: "When I arrived at the lab and saw many PhD students working on various aspects of TAT synthesis, I thought it was too crowdy and asked Gordon for another project. He suggested to study the TAT degradation as a process on which the level of this enzyme also depends. This is how I learned about the breakdown of proteins and caught interest in this problem, which I have been working on ever since" [1]. The scientist found that TAT degradation could be completely stopped by ATP synthesis inhibitors. This fact proved the fact that highly selective degradation of a specific enzyme required energy, which is unlikely to be provided by lysosomes, and that a new, yet unknown, proteolytic system existed within cells [2].

At the end of 1971, A. Hershko returned from San Francisco to Israel, took a position of the medical faculty dean offered to him, and took a charge of the biochemistry department of the newly opened Technion University in Haifa. Although the Medical Faculty was a rookie at the time and, as everyone hoped, temporarily (in fact until construction of a new building in 1987) occupied a two-story building of an old monastery, it was a venue of a significant part of research related to the discovery of ubiquitin role in protein degradation. For several years, Avram and his dedicated research team were attempting to create a cell-free system that would reproduce in vitro energy-dependent protein degradation, using various sources such as liver homogenate, cultured cell extracts, and even bacteria. Despite many unsuccessful attempts and persistent pleading by colleagues from other laboratories to quit this hopeless idea, Avram Hershko remained very stubborn and convinced of a viability to learn how proteins decomposed only with the help of a cell-free system that might be subject to biochemical analysis. In the end, the researchers used a reticulocyte-based soluble ATP-dependent proteolytic cell-free system (discussed above), first produced and characterized in 1977 by A. Goldberg from Harvard Medical School, as a biochemical fractionation tool [3].

The experimental technique used by Hershko`s team was quite simple – all started from marking reticulocyte proteins with a radioactive label, then obtaining a lysate, its incubation in the presence of ATP, and differentiating the breakdown of proteins after precipitation with trichloroacetic acid by volume of the radioactive label in supernatant liquid compared to the one initially found in precipitated proteins. This model proved the responsibility of a soluble energy-dependent proteolytic system for protein degradation in reticulocytes, yet the mechanism of the process remained completely unknown. A. Hershko was confident that the clue was in using the methods of classical biochemistry, namely fractionation and separation of active components, purifica-

tion of each of them, and restoration of a complete system from isolated components.

Having started a reticulocyte lysate fractionation, Hershko took advantage of the experience gained from collaboration with Mager in purification of erythrocytes from hemoglobin using the method of anion exchange chromatography. The volume of hemoglobin in reticulocytes, as well as in erythrocytes, makes approximately 80% of the total protein content, and therefore the primary task of the researcher was to get rid of its significant portion. To do this, the lysate was separated into two fractions on the basis of DEAE cellulose, which bond most proteins, except hemoglobin - the first one containing non-adsorbed proteins (fraction I), and the second one - bound proteins eluted from the column (fraction II). And here the researchers came across the first unexpected discovery. None of the two fractions demonstrated any proteolytic activity, however, being combined, they resumed the activity. The discovery became the grounds for a conclusion that the subject protease was not a classical single enzyme, but the one consisted of at least two components. To isolate and characterize the active component, it was decided to use fraction I, which did not contain many secondary proteins, except for hemoglobin.

In 1977, at a meeting of scientists at the Fogarty Center of the National Institutes of Health (NIH), USA, the scientist met Irwin Rose, a famous specialist in enzymology at that time. During the conversation, Rose let know that he was interested in protein degradation, which quite surprised Avram, because he had not come across any of Irvin's articles on the subject. It turned out that Rose tried somehow to comprehend the energy dependence of protein degradation, but did not make significant progress experimentally, and therefore did not have relevant publications. A. Hershko believed it was a lucky encounter, because after six years of work at the Technion, he had become convinced that the circle of scientists interested in the proteolysis was very narrow, so an internship at the laboratory of the Fox Chase Cancer Center (Philadelphia, USA) offered by Rose was very much to the point. The collaboration with E. Rose played an important role in his further research.

In 1978, Aaron Ciechanover joined the Hershko's team in Haifa. In 1972–1973, he worked on his master's paper under the supervision of Avram Hershko, and after medical qualification and retirement from military service in 1976, he returned to the laboratory to continue his scientific research and to earn PhD degree. He was given a task to isolate an active component from fraction I of reticulocyte lysate.

Highly appreciating A. Ciechanover's contribution to elucidating the proteolysis mechanisms, A. Hershko made a special mention of his managerial abilities: "After telling Ernie Rose how small the Israeli research grants were, Ernie suggested that I should apply for a foreign research grant from the NIH to support my work in Israel. I was inclined to do a couple of more experiments instead of writing a grant application, but Aaron pushed me into a chair and commanded: "now write the NIH grant application!" I wrote it and got the grant, the first of five consecutive grant periods supported by the NIH. It saved the situation in the Haifa lab at a very critical time. I am very grateful to the NIH for supporting my work and also to Aaron for forcing me to write the initial grant application" [1].

The scientist continued the work that led to the discovery of three enzymes responsible for linking the ubiquitin label to a substrate protein and to the development of a step-by-step scheme of the process in close cooperation with E. Rose and A. Ciechanover.

In the 1990s, A. Hershko worked with the Marine Biological Laboratory (MBL) in Woods Hole (USA), where he studied the mechanisms of cyclin degradation in a non-cellular system based on Spisula solidissim fertilized eggs [4].

The scientist kept up his scientific and teaching activities at the medical faculty of the Israel Technion University of Technology, where he is currently an honored professor.

Irwin Rose was born in 1926 in Brooklyn, New York. His mother, Ella Greenwald, was born in America to a family of immigrants from Hungary. His father, Harry Rose, born in Odessa region, was the owner of a floor covering shop. Because Irwin's brother suffered from rheumatism, the family moved from New York to Spokane (Washington), a place with a drier climate. When Irwin was 13, he happened to work at a local hospital in the summer, where he helped care for psychiatric patients. The experience gained greatly influenced the young man and made him ponder on solving medical problems. However, no one in the family had been taking any part in scientific research and the young man had no one to consult with, so he chose his future profession on his own and entered the medical faculty of

Washington State College. He studied there for only a year, as his studies were interrupted by service with the navy during World War II. Upon decommissioning, he entered the University of Chicago, where he earned a bachelor's degree in 1948, and defended his PhD thesis in 1952. The purpose of his research was to determine whether the DNA content in tissues of rats fed with B12 diet increased. This project was doomed to fail because the genetic role of DNA had been discovered a short while ago, yet Rose managed to show that the DNA content in liver cells did not depend on the diet [5].

A new project was needed and the young scientist devoted it to the study of biosynthesis reactions of DNA components. For this purpose he used C-14 radioisotope, which had become available only recently, and showed that the deoxycytidine isotope originated from cytidine, its ribonucleoside precursor [6].

In 1955, E. Rose received an invitation to become a biochemistry teacher at the Yale University Medical School. Here he met Mel Simpson, who had previously shown in his experiments on rat liver slices that ATP decrease lead to a lower rate of amino acid release from proteins. This observation indicated an ATP need for breaking down proteins and required further research, but Simpson's mission at Yale University was to find a system for studying *in vitro* synthesis of proteins rather than their degradation, which was considered irrelevant at the time. Since then Rose started planning experiments to understand the energy dependence of protein breakdown and kept an eye on scientific reports on progress in researching the problem.

In 1963, Rose and his research group moved to the Fox Chase Cancer Center in Philadelphia. Here, the scientist, using Ehrlich's ascites cells and other cellular preparations, began the search for a cell-free system that would show the dependence of protein degradation on ATP, but the attempts were unsuccessful. As it was shown later, the inability of cell extracts to provide ATP-dependent cleavage of proteins was explained by the presence of a lysosomal trypsin-like protease that destroyed ubiquitin [7].

The main Rose's task at that time was to explore the mechanisms of enzymatic reactions and to find an answer to the question of how enzymes "reboot" after each catalytic act, how to detect and characterize mysterious intermediate products during the enzymes' work. Erwin Rose's contribution to finding the answers was one of his greatest achievements. For example, to identify intermediate reaction products, he developed a method of positional isotope exchange (PIX), which is now used to study complex ATP-dependent reactions and consists in labeling ATP with O18 and monitoring the movement of labeled oxygen to a certain position within the initial material. In 1972 Rose et. al. developed the Pulse/Chase method consisting in short-term labeling of a protein with subsequent fixation of the label disappearance rate over a certain time, which allowed determining the binding constants and dissociation rates of the enzyme-substrate complex. These methods helped unveil a rather complex tangle of enzymatic reactions of the ubiquitin-dependent proteolytic cycle [8].

In the summer 1978, Avram Hershko and his student Aaron Ciechanover, who had already fractionated reticulocyte extract and discovered the two-component proteolysis system, arrived in Rose's laboratory at the Fox Chase Cancer Center. Since Avram Hershko asked to share Rose's lab opportunities for collaboration, he and his team have been welcomed to Fox Chase for sabbaticals and summers during 22 years of research into the mechanism of intracellular proteolysis.

Irwin Rose was distinct in his exceptional modesty, and getting his consent for co-author of a publication always was a significant challenge for colleagues, despite his great contribution to collaborative research. He always downplayed his role in the development of the ubiquitin mechanism, for which he was awarded the Nobel Prize, and the word "ubiquitin" was omitted in his autobiography wrote in 2004.

Irwin Rose worked at the Fox Chase Cancer Center until his retirement in 1995. The scientist died at the age of 88 on June 2, 2015 in Deerfield, Massachusetts.

Aaron Ciechanover was born on October 1, 1947 in Haifa, Israel (a month before Israel was recognized as an independent state by the UN) to a family of Polish immigrants who moved from Poland to Palestine before World War II and followed Jewish religious traditions. His father was a lawyer, his mother was a housewife and English teacher. From a young age, Aaron adored biology, learned extracting chlorophyll from leaves with ethanol, and conducted his first experiments on cellular osmosis, microscoping the process using the instrument gifted to him by his older brother. While studying at high school (1953–1965), he focused on studying biology, but at

that time biology was rather a descriptive than an experimental science, and the concept of DNA as a carrier of genetic information entered school textbooks only at his graduation from the school. Therefore, the young man became interested in physics, chemistry and mathematics, which seemed more grounded to him. In thinking about his future specialty, medicine seemed to him a balance of physics, chemistry, fundamental biology, physiology and pathology.

After graduating from the school, Aaron, like every graduate in Israel, had to serve in the army. However, the Army encouraged some high school graduates to postpone service in order to pursue a university education first, particularly in military-related fields such as medicine. Having lost his parents at an early age and having no financial support, the young man decided to master a profession to earn a living and in 1965 entered the medical school of the Hebrew University in Jerusalem. At the end of his studies, when he began examining patients, he had doubts about whether he had made the right choice and whether he wanted to be a practicing physician. The reason was dissatisfaction for medicine turned out to be no less descriptive than biology, and lacking scientific explanation of the mechanisms of many diseases. In 1969, Aaron decided to deepen his experience in fundamental research and devoted his first experimental work on the study of the phosphatidic acid phosphatase activity as a key enzyme in the synthesis of triglycerides in pathological liver conditions of an animal model. When he graduated from the medical school in 1972, he already firmly knew that his vocation was biochemistry.

To get his medical license, Aaron had to complete a year of internship. Colleagues recommended him a young, talented biochemist, Dr. Avram Hershko, who had just finished his doctoral studies under the supervision of G. Tomkins at the University of California in San Francisco and had been appointed dean of the medical faculty of the newly established Technion University of Haifa. Ciechanover wrote to Avram about his intention to move to Haifa for a year to complete his master's thesis under Avram's supervision. A. Hershko agreed to accept him as a master's student, and in October 1972, their collaboration began. In 1972-1973, Ciechanover worked at the laboratory mainly in the evenings, nights and weekends, because he had to combine the research of phospholipid metabolism with clinical practice. His medical degree did not replace his service in the army, and during 1973–1976, Ciechanover served as a medical officer on a warship, periodically combining his service with lecturing biochemistry to medical university students.

Upon discharge from the military service, Ciechanover finally decided to make his career in scientific research. In 1977 he returned as a doctoral student to A. Hershko's Technion laboratory, whose team focused on the study of ATP-dependent intracellular proteolysis at that time. The first task for him from Avram Hershko, who had left for an internship to E.Rose's laboratory, was to purify the active component from fraction I obtained during the reticulocyte lysate fractionation. But all Aaron's attempts to do this were unsuccessful, until the laboratory staff fell over the "crazy" idea of heating a fraction to assess thermal stability of the components. And here the scientists got a second surprise - it turned out that this idea was indeed the case, as after 5-10 min of heating at 90°C, hemoglobin in the fraction precipitated, and the activity remained in the soluble supernatant. Technion protein chemists doubted the component could be a protein, but it appeared to be sensitive to trypsin and precipitated by ammonium sulfate, and further characterization confirmed that it was indeed a protein with a molecular weight of 8,500 Da. This is how the first ATP-dependent Proteolysis Factor 1, named APF-1, was discovered. These intriguing results were published in BBResCom by Ciechanover and Hershko in the article that became the first ever reference of the discovery of a multicomponent ubiquitin-dependent proteolytic system [9].

Ahead, Aaron Ciechanover had exciting five-year doctoral studies in collaboration with A. Hershko and E. Rose, which ended with decoding of the ubiquitin system. In 1982, Ciechanover received a doctoral degree in biology, and in 1982–1984 he had a postdoc course at the Department of Biology, Massachusetts Institute of Technology (Cambridge, USA).

After 1986, Ciechanover worked at the Biochemistry Department of the Technion University. Currently, he is a professor emeritus at the Cancer Research and Vascular Biology Center, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, (Haifa, Israel) [10].

It's worthy to note that Aaron Ciechanover is an extremely friendly and attractive person. He has given lectures at the invitation of scientists from different countries of the world and is always happy

to share both his personal scientific achievements and the achievements of medical and biological sciences as a whole. The scientist visited Ukraine several times at the invitation of various scientific societies. In 2008, at the invitation of the Ukrainian Biochemical Society, he gave a lecture in Kyiv at the O. V. Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine.

He left his impressions in the guestbook, in which he praised the contribution of the Palladin Institute of Biochemistry and the Memorial Museum, placing the emphasis on the importance of preserving the history of science and its creators.

Let's dwell in more detail on the research by these prominent scientists

So, in the 1970s, A. Hershko and A. Ciechanover discovered two previously unknown phenomena. The first one suggested that ATP-dependent intracellular proteolysis system consisted of two components contained separately in fractions I and II of reticulocyte lysate, and the second one consisted in identification of the active component of fraction I, which appeared to be a small thermostable protein APF-1. Further progress was made at the A. Hershko's laboratory in Haifa in winter of 1978–1979, when APF-1 was purified to homogeneity and labeled with radioactive iodine. When the labeled protein was incubated with fraction II and ATP and the mixture subjected to gel filtration chromatography, significant ATP-dependent binding of APF-1 to high molecular weight material was observed [11].

At first, scientists suggested that APF-1 might be an activator or proteinase subunit. However, further analysis of the reaction products using SDS-polyacrylamide gel electrophoresis showed that APF-1 bound to a very large number of endogenous proteins. Since fraction II consisted not only of enzymes, but also endogenous substrates of the proteolytic system, the scientists grew suspicious that APF-1 bound not to the enzymes at all, but to protein substrates. Indeed, it has been demonstrated that some substrate proteins of the ATP-dependent proteolytic system, such as lysozyme, could bind to APF-1.

A breakthrough was made when A. Hershko and A. Ciechanover, working at the Fox Chase laboratory headed by E. Rose, focused on elucidating the sequence of work of the proteolytic system. A series of experiments, planned on the grounds of Rose's deep knowledge of protein chemistry and enzymology, showed that APF-1 bound covalently to its substrate through a bond demonstrating all properties of a peptide bond. Most surprising and unique was the fact that individual APF-1 units formed a chain attached to a protein intended for cleavage. The reaction proved to be reversible, as APF-1 could detach from the substrate and be reused, perhaps not by a reverse of the conjugation reaction, but apparently with the involvement of a certain proteinase [12]. This same time scientists assumed that covalent binding of numerous of APF-1 units to the substrate was necessary for its recognition and degradation by a protease not yet identified at the time (it was later found to be 26S proteasome complex), which exerted its action only on proteins labeled that way. The proposed model, published in 1980 in PNAS, first described the general mechanism of ATP-dependent proteolysis [12].

Academician Serhiy Komisarenko, the Director of the Palladin Institute of Biochemistry, showing Aaron Ciechanover, Nobel Laureate in Chemistry, round the exhibition of the O. V. Palladin Memorial Museum, August 11, 2008

This model survived the test of time, albeit needed clarification and detailed elaboration. For example, in the beginning it was doubted whether one protein could be covalently modified by another one.

The attention of researchers was drawn to a conjugate of two proteins characterized by I. Goldknopf in 1977- a small 8.6 KDa protein known as ubiquitin, and histone H2A, connected by isopeptide bond between C-terminal Gly^{76} residue within the ubiquitin component, and Ɛ-NH2 Lys^{119} group within the histone one [13].

The fact of identity of APF-1 and ubiquitin was proved by postdoctoral researchers K. Wilkins and A. Haas of Rose's laboratory. The scientists found

that both ^{125}I-ubiquitin and ^{125}I-APF-1 formed electrophoretically identical conjugates in interaction with endogenous reticulocyte proteins. Comparison of the results of isoelectric focusing and of the amino acid ubiquitin sequencing with the same results obtained by A. Ciechanover in the study of APF-1 at A. Hershko's laboratory also showed the complete coincidence. The results of the two studies were simultaneously published in 1980 in J. Biol. Chem [14, 15].

The isopeptide nature of the bond between APF-1/ubiquitin and a target substrate became possible to prove through the convergence of the two research approaches, the study of histones and proteolysis. It should be noted that while modification of a protein destined for proteolysis consists in binding to a poly-ubiquitin chain (polyubiquitination), a histone molecule binds ubiquitin only once (monoubiquitination), which does not lead to its destabilization. The role of such histone modification remained unclear for quite a long time, and only now its importance, as one of the epigenetic transcription control mechanisms, has become widely known.

As for ubiquitin, it is a conservative, small heat-resisting protein of 76 amino acid residues, discovered by H. Goldstein in 1974 in the study of thymus hormones. Its function was unknown, but its universal prevalence in all organisms gave grounds to its name (lat. ubique means "everywhere") [16].

A. Ciechanover wrote in his biography: "While in retrospect the name ubiquitin is a misnomer as it is restric ed to eukaryotes and is not ubiquitous as was previously thought, from histori al reasons it has still remained the name of the protein. Accordingly, and in order to avoid confusion, we suggest that names of other novel enzymes and components of the ubiquitin system, but of other systems as well, should remain as were first coine by their discoverers. Thus, in a relatively short period of time, ubiquitin was converted from a ubiquitous thymopoietic hormone to a eukaryotic proteolytic marker".

Fig. 1. Schematic representation of the ubiquitin molecule [17]

Conjugation of ubiquitin and protein, demonstrated by the researchers, as a signal for proteolysis, was a new mechanism, the discovery of which required isolation of participating enzymes and understanding of the reasons for energy dependence of the reaction, which, according to the perceptions of the time, should have been the exergonic one. The future Nobel laureates took a total of 10 years (1980–1990) for elucidation of these questions using completely justified biochemical methods of component fractionation and system reproduction. It was found that ubiquitin was bound to a protein not by one enzyme, but through the sequential action of three enzymes. All three enzymes were isolated from the crude extract of reticulocytes and characterized using Sepharose-bound affinity chromatography with covalently bound ubiquitin as a "bait" [18, 19]. The sequence of action and the final stage of ubiquitin-dependent proteolysis are presented in the scheme proposed by A. Ciechanover (Fig. 2) [20].

Comprehension of the first reaction gave an answer to the question of energy dependence. The enzyme that catalyzed the reaction covalently bound ubiquitin on the column in the presence of ATP, and it could be eluted with AMP and pyrophosphate, or with a highly concentrated thiol compound. Judging from the fact, it was concluded that the first enzyme (E1) was responsible for ATP-dependent activation of the carboxy-terminal glycine residue within ubiquitin by forming ubiquitin adenylate, and transfer of activated ubiquitin to the cysteine residue sulfhydryl group in its active center along with the formation of a thioester bond (Fig. 2, stage 1).

$$E1 + ATP + Ub \leftrightarrow E1.AMP\text{-}Ub + PPi$$
$$E1.AMP\text{-}Ub \leftrightarrow E1\text{-}S\text{-}Ub + AMP$$

Enzyme 1 was named ubiquitin-activating, and given a systematic name - ubiquitin: [E1] ligase (forming AMP). Of all three enzymes, only E1 directly interacted with ubiquitin as a substrate. It

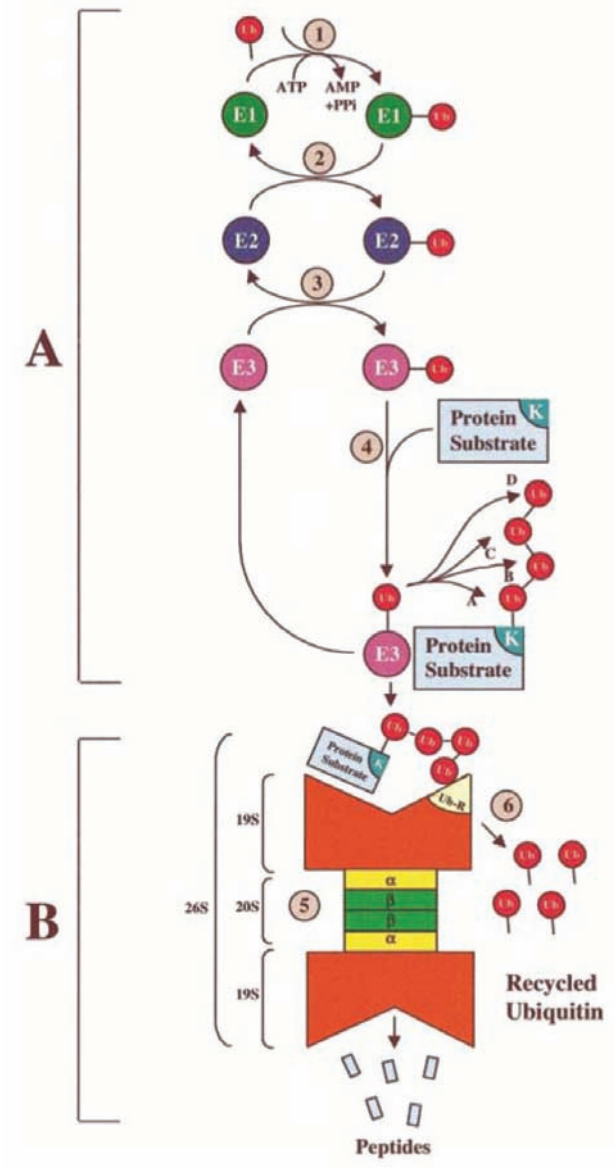


*Fig. 2. The ubiquitin–proteasome pathway. **(A)** Conjugation of ubiquitin to the target molecule. **(B)** Degradation of the tagged substrate by the 26S proteasome. **(1)** Activation of ubiquitin by E1. **(2)** Transfer of activated ubiquitin from E1 to a member of the E2 family. **(3)** Transfer of activated ubiquitin from E2 to a substratespecific E3. **(4)** Formation of a substrate–E3 complex and biosynthesis of a substrate-anchored polyubiquitin chain. **(5)** Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex. **(6)** Recycling of ubiquitin via the action of isopeptidases [20]*

was also established that ATP preceded ubiquitin in binding to E1. The affinity of E1 for ATP (~40 μM) is higher than cellular ATP level. Therefore, the enzyme easily acquires the E1-ATP configuration, capable of "catching" free ubiquitin even at low concentrations (K_m = 0.58 μM) [21].

Two subsequent enzymes are responsible for the sequential transfer of activated ubiquitin units to substrate with the formation of a polyubiquitin chain. At stage 2 (Fig. 2), E2 transfers activated ubiquitin of a transacylation reaction to the sulfhydryl group of the cysteine residue in the active center with the formation of a thioester bond. E2 is called ubiquitin carrier enzyme, or ubiquitin-conjugating enzyme, the systematic name - S-ubiquitinyl-[E1]-L-cysteine:[E2] ubiquitin-yl-transferase.

The discovery of the next E3 enzyme gave the answer to the question of selectivity of ubiquitin-mediated degradation of proteins and their different half-lives [22].

The accepted name of the E3 enzyme is ubiquitin ligase, although this name is not absolutely correct. The systematic name of the enzyme is [E2]-S-ubiquitinyl-L-cysteine: [acceptor protein]-L-lysine ubiquitintransferase (forms isopeptide bond, RING type). E3 contains both a domain that interacts with E2 and a domain that specifically recognizes a target protein. There are a number of unique E3 enzymes that differ in substrate specificity and exist in the form of a monomer or a multimeric complex. Their substrate can be specific proteins involved in controlling various cellular processes, such as signal transduction, transcription, DNA replication, and protein kinase activity regulation.

After the specific ubiquitin ligase E3 has bound the target protein and E2 (Fig. 2, step 3), the activated ubiquitin is transferred from E2 to the substrate with the formation of an isopeptide bond between the C-terminal glycine residue consisting of ubiquitin and Ɛ - lysine residue amino group of the substrate (Fig. 2, step 4). Repeating the cycle and sequential conjugation of the lysine residue within ubiquitin bound to the substrate with the C-terminal glycine residue of the new ubiquitin molecule results in the formation of a polyubiquitin chain directing the protein for degradation by 26S proteasome [4, 5]. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63) capable forming bonds required for its polymerization into a chain, but only

the polyubiquitin chain formed with the involvement of Lys^{48} serves as a proteolytic signal and is recognized by the proteasome.

Within a short period of time, the hypothesis of ubiquitin labeling of proteins received a comprehensive support. The only missing link was protease identification (downstream protease), which, according to the proposed model, should have specifically recognized ubiquitinated substrates. A. Hershko's observation that ATP energy was needed not only for ubiquitin activation, but also for degradation of ubiquitin-protein conjugate [23] was a certain clue, but the enzyme complex responsible for cleavage of labeled ubiquitin substrate was discovered and characterized by other researchers .

First, a protease with an unusually large molecular weight (~2.5 mDa), which was inactive on unmodified lysozyme, but cleaved ubiquitinated lysozyme in an ATP-dependent manner, was purified. This protease, later named a 26S proteasome, met all the criteria to be a specific proteolytic "tool" of the ubiquitin system [24].

Only in the early 1990s, a convincing evidence that the active 26S proteasome complex was formed by ATP-dependent assembly of two separate units - 20S core catalytic and 19S regulatory (one or two) - was obtained [25].

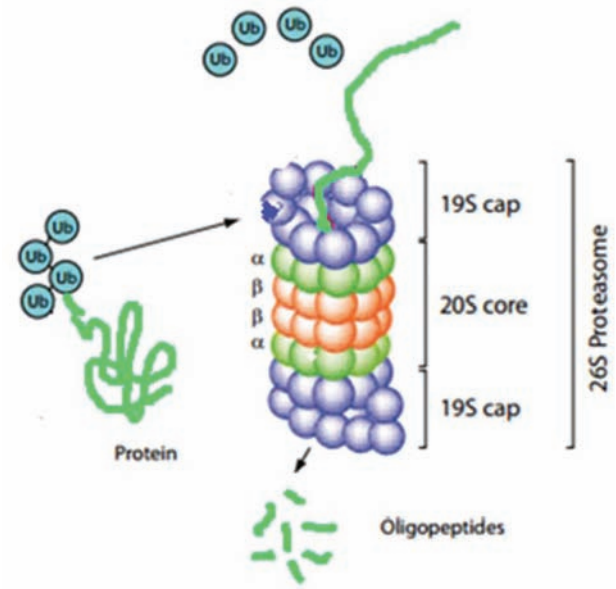


Fig. 3. Schematic representation of the structure and function of 26S proteasome [26]

The core 20S particle is a barrel-shaped structure of four stacked rings, of which the central two are formed by β-, and the two outermost - by α-subunits (Fig. 3). The catalytic centers of the proteosome are located on the β-subunits, while α-subunits are responsible for joining the regulatory 19S particle, consisting of 17 different subunits, to 20S complex. In addition, the N-terminal regions of α-subunit close the entrance to the proteasome cavity, preventing uncontrolled proteolysis. The regulatory 19S particle is responsible for several important functions. It recognizes ubiquitinated proteins, as its specific subunits are able to bind polyubiquitin chains with high affinity. In addition, the regulatory particle ensures unfolding of the polypeptide chain, which requires the ATP hydrolysis energy. Therefore, the base of the 19S particle contains six different ATPase subunits. ATP binding to these subunits is necessary not only for ATP hydrolysis, but also for opening the entrance to the catalytic chamber, translocation of the polypeptide chain, and proteolysis. The 26S proteosome action is resulted in cleavage of substrate into short peptides and release under the action of ubiquitin C-terminal hydrolases or ubiquitin isopeptidases, which can be reused by the ubiquitin system.

Given numerous cellular substrate proteins that can be potential targets for ubiquitin labeling and proteolysis, and many cellular processes in which such proteins are involved, it is not surprising that ubiquitin system disturbances are considered part of pathogenesis of many inherited and acquired human diseases. Pathological conditions caused by abnormal ubiquitin-mediated proteolysis can be attributed to two groups: 1) those resulting from abnormal or accelerated degradation of target protein, and 2) those resulting from malfunction or mutation of a specific enzyme of the ubiquitin system or target substrate, which may lead to the stabilization and accumulation of certain proteins [27, 28].

Here are some examples.

Cystic fibrosis (CF) is a multisystem disorder characterized by chronic obstruction of the respiratory and indigestion tracts due to pancreatic dysfunction. The pathology is caused by a mutation of the gene encoding the transmembrane protein (CFTR), which is a chloride channel localized on the plasma membrane of epithelial cells. The mutation causes a protein to fold in such a way that the protein fails to reach the cell surface, but is instead retained in the endoplasmic reticulum, and then polyubiquitinated and cleaved by the proteasome, thus resulting in a membrane ion channel deficiency.

Changes in the course of ubiquitination reactions can be directly related to the etiology of

many malignant neoplasms. Specific cancer types can originate from stabilization of oncoproteins or destabilization of tumor suppressor genes. The link between carcinogenesis and the ubiquitin system has been convincingly demonstrated in the case of human papillomavirus-induced cervical cancer. It has been proven that the concentration of p53 tumor suppressor protein in cervical tumors is extremely low. Detailed studies both *in vitro* and *in vivo* showed the reason being the ability of one of the viral oncoproteins to simultaneously bind to both ubiquitin ligase and p53 protein in cervical cells, forming a triple complex. Spatial convergence of ubiquitin ligase and p53 as a target substrate triggers the mechanism of proteolytic cleavage. Removal of the p53 tumor suppressor by viral oncoprotein is an important mechanism used by the virus for malignant transformation of cells.

Accumulation of ubiquitin-conjugated proteins in inclusion bodies of brain cells has been reported in many neurodegenerative pathologies, such as Alzheimer's and Parkinson's diseases. If it was previously admitted that inclusion bodies are formed due to the inherent trend of abnormal proteins to bind to each other and aggregate. But later it was found that these conditions lead to a disturbance of ubiquitin-dependent proteolysis in neuronal proteins. For example, such disorders could be caused by a decrease in the activity of ubiquitin ligase due to a mutation of the enzyme, or by the inability of the ubiquitin and proteasome mechanisms to label and remove proteins damaged by oxidative stress or other factors.

Illustrative is the history of application of thalidomide - a drug widely used in 1950s by pregnant women to relieve their symptoms but discontinued later because it caused teratogenic deformities in children born. Less well known has been the resurgence in its use as a therapy to treat hematologic malignancy. The property of thalidomide to inhibit angiogenesis inspired the suggestion that it might be useful in attempt to control drug-resistant myeloma. Indeed medical trials have confirmed that thalidomide is active in patients with multiple myeloma, but antiangiogenesis was not the mechanism of action that explained its clinical effect. The breakthrough emerged in 2010 when thalidomide was found to cause the loss of two transcription factors named Ikaros and Aiolos, which are regulators of B and T cell development [29]. It was shown that thalidomide binds to the protein cereblon (CRBN), which activates the enzymatic activity of the CRBN E3 ubiquitin ligase complex. resulting in the rapid ubiquitination of Ikaros and Aiolos, targeting them for degradation in proteasome. This alters the function of T cells and B cells with a toxic outcome for multiple myeloma cells. It was concluded that enhancing the ubiquitination and degradation of specific target proteins may represent a new class of therapeutics for manipulating proteins that were previously viewed as undruggable [30].

To date, a significant progress has been made in the practical development of a method of targeted ubiquitination and destruction of proteins threatening the normal cell functioning. In particular PROTAC (PROteolysis TArgeting Chimeras) technology is now widely used for the design of new therapies. Since the first PROTAC was introduced in 2001[31] biopharmaceutical companies are actively working to advance the development of PROTACs. PROTACs are heterobifunctional molecules composed of two ligands: protein of interest (POI) and the E3 ubiquitin ligase, linked together by a linker of variable length and nature. The POI ligand will mobilize the desired biological target. The ligase ligand role is to recognize and recruit an E3 ubiquitin ligase. Once PROTAC forms the ternary complex with a POI and E3 ubiquitin ligase the E3 ubiquitin ligase will transfer the ubiquitin (via an E2 ligase) to the protein of interest and the protein will be sent to the proteasome for its proteolysis. In recent years, many proteins have been successfully degraded by PROTACs, including nuclear receptors, protein kinases, neurodegenerative disease-related proteins and anti-apoptotic proteins. The existence of more than 600 E3 ubiquitin ligases in mammalian cells highlights their importance in terms of specificity in the regulation of protein homeostasis. Only a few E3 ubiquitin ligases were targeted, suggesting that a large pool of E3 ligases is potentially available for targeted protein degradation. The discovery of novel E3 ligases with novel binding ligand could lead to the design of new PROTACs for diseases that are still incurable today [31, 32].

In conclusion, it is appropriate to quote Aaron Ciechanover, a well-known supporter of Ukrainian biochemists, who emphasized in his welcoming speech to the participants of the 12th Ukrainian Biochemical Congress (Ternopil, 2019): "…it can be argued that all modern developments are based on basic scientific research. As an example, I want to mention our own research. We were interested in

how proteins degrade in the human body. But we did not think then about diseases and medicines. We have only identified a niche in biology that had remained unexplored: how proteins degrade in a specific way; how a cell identifies a protein that is no longer needed either because it has completed its function or denatured or mutated, retaining all other proteins in the cell at the same time. That means we were interested in a specific degradation, and we discovered the ubiquitin system. And only 28 years later, people discovered that deviations in this system lead to disease".

УБІКВІТИН ТА ЙОГО РОЛЬ У ПРОТЕОЛІЗІ: НОБЕЛІВСЬКА ПРЕМІЯ З ХІМІЇ У 2004 РОЦІ

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Аарон Чехановер, Аврам Гершко та Ірвін Роуз на початку 1980-х років відкрили один з найважливіших циклічних процесів у клітині – регульовану АТР-залежну деградацію протеїнів, за що було нагороджено Нобелівською премією з хімії у 2004 році. Вчені довели існування нелізосомного шляху протеолізу і повністю змінили уявлення про механізми деградації протеїнів усередині клітини. Вони продемонстрували, що, обираючи протеїн, який підлягає знищенню, клітина попередньо позначає його біохімічним маркером під назвою убіквітин. Поліубквітування протеїну як сигнал для його протеолізу було новим механізмом, розкриття якого уможливила спільна робота трьох учених із виділення ензимів–учасників цього послідовного процесу, з'ясування його біохімічних етапів та причин енергозалежності. У статті наведено біографічні дані Нобелівських лауреатів, описано застосовані методи та історію відкриття феномену опосередкованої убіквітином протеосомної деградації протеїнів.

Ключові слова: А. Чехановер, А. Гершко, І. Роуз, убіквітин, регульована деградація протеїнів, PROTAC.

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