Protein Breakdown

Intracellular Protein Degradation: From a Vague Idea, through the Lysosome and the Ubiquitin–Proteasome System, and onto Human Diseases and Drug Targeting (Nobel Lecture)**

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Between the 1950s and 1980s, scientists were focusing mostly on how the genetic code is transcribed to RNA and translated to proteins, but how proteins are degraded has remained a neglected research area. With the discovery of the lysosome by Christian de Duve it was assumed that cellular proteins are degraded within this organelle. Yet, several independent lines of experimental evidence strongly suggested that intracellular proteolysis is largely non-lysosomal, but the mechanisms involved remained obscure. The discovery of the ubiquitinproteasome system resolved the enigma. We now recognize that degradation of intracellular proteins is involved in regulation of a broad array of cellular processes, such as the cell cycle and division, regulation of transcription factors, and assurance of the cellular quality control. Not surprisingly, aberrations in the system have been implicated in the pathogenesis of human disease, such as malignancies and neurodegenerative disorders, which led subsequently to an increasing effort to develop mechanism-based drugs.

Keywords:

lysosomes · Nobel Lecture · proteasomes · protein breakdown · ubiquitin

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1. Biographical Notes

The Formative Years—Childhood in the newly Born State of Israel

I was born in Haifa, a port city in the northern part of Israel, in October 1947, one month before Israel was recognized by the United Nations as an independent state. It took several additional months to establish the necessary institutions and for the British to leave, and on May 15th 1948, David Ben-Gurion, the founding father of the modern Jewish state and its first Prime Minister made Israel a fact and declared its establishment as a democratic state and a home for every Jew in the world. The neighboring, but even more distant Arab countries, along with powerful Arab parties from within did not accept the UN resolution and deliberately decided to alter it by force. A bloody and costly war erupted. It lasted a year, and more than 1% of the population of the newly born and defenseless state sacrificed their lives on its defense. I assume that the first two years of my life (1947–1949) were extremely difficult for my parents, Bluma (née Lubashevsky) and Yitzhak, who immigrated from Poland with their families as adolescents in the mid-1920s. Why did their families leave Poland—their "homeland"—their homes, working places, property, relatives, and friends, and decide to make their new home in a place with a vague, if any, clear

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future that was part of the British Empire? They were idealists who enthusiastically followed the call of the Zionist movement that was established at the turn of the century by Benjamin Ze'ev Herzel (the seer of the Jewish State) to settle the land and make it—after two thousand years in the Diaspora, since the destruction of the second temple in Jerusalem—a home for the Jews. Following the Jewish Congress in Basel (Switzerland) in 1896, Herzel declared: "*In Basel I founded the Jewish State*". At that time Israel was part of the Ottoman Empire and became in 1917 part of the British Empire. My parents came from religious families, and the move, I believe, also had religious roots: Jews, throughout their lives in the Diaspora, have not stopped dreaming of having their own country, a dream that was driven by a biblical decree and prophecy:

"Thus saith the Lord GOD: Behold, I will take the children of Israel from among the nations, whither they are gone, and will gather them on every side, and bring them into their own land" (Ezekiel 37:21); "And they shall dwell in the land that I have given unto Jacob my servant, wherein your fathers dwelt; and they shall dwell therein, they, and their children, and their children's children, for ever" (Ezekiel 37:25); "And I will rejoice in Jerusalem, and joy in my people; and the voice of weeping shall be no more heard in her, nor the voice of crying" (Isaiah 65:19); "And they shall build houses, and inhabit them; and they shall plant vineyards, and eat the fruit of them" (Isaiah 65:21).

The question of timing was an important one, as despite centuries of continuous persecution and discrimination in Europe, the initial idea to establish a Jewish State had been the dream of a few. Only small groups of Jews settled in Israel during the 18th, 19th, and the beginning of the 20th century. It was only towards the end of the 19th century, with the ideas of

Herzel and the moves that led to the Balfour declaration (the British Minister of Foreign Affairs who declared in 1917 the recognition of the need for a Jewish homeland) that an active Zionist movement and Institutions were established, resulting in the translation of the dream into reality. Yet, it took an enormous amount of courage and daring by these European Jews to materialize this dream and try to establish, with almost no resources or support, a homeland in a place they had dreamt of for two thousand years, but that was not theirs at the time. The process was clearly accelerated by the heavy clouds that then covered the skies of Europe and that ended with the Holocaust. Many members of my parents' families immigrated to Israel before the Holocaust, but those who remained in Poland perished at the hands of the murderous Germans and their loyal Polish collaborators. The conversion of this movement into a State at that particular time (1947-1948) was no doubt the direct historical result of the holocaust, and symbolized the rise of the Jewish Nation from ash.

My father was a clerk in a law firm (later, in parallel with my brother, he studied law and became a lawyer), and my mother was a housewife and English teacher. My brother, Joseph (Yossi), who is 14 years older then me, was already on his national military compulsory service when I was 4 years old, the age from which I remember myself. I grew up in Haifa and enjoyed the wonderful beaches and Mount Carmel that rolls into the Mediterranean Sea. From my early days at home I remember a strong encouragement to study. My father worked hard to make sure we obtained the best possible education, and at the same time he was a member in the "Haganah" (defense), one of the prestate military organizations that fought the British for an independent Jewish State. Working in a law firm in the Arab section of the city, he risked his life daily going to work during the prewar hostilities and then the war time. My brother Joseph told me that the family waited daily on the balcony to see him return home peacefully. At home he used every free minute to delve into classic literature, Jewish religious law (Mishnah and Talmud), and modern law books. An important part of the education at home involved Judaism and Zionism. On the Jewish side, we obtained a liberal modern orthodox education. We attended services in the synagogue every Saturday and during holidays, and celebrated all Jewish holidays. Needless to say that my mother kept a Kosher kitchen.

It was extremely important for my parents to educate us as a new breed of proud Israeli Jews in their own independent country. I inherited from my father his love of Jewish studies and cultural life. To this very day, along with several physicians and scientist colleagues, I take regular periodical lessons taught by a Rabbinical scholar on how the Jewish law views moral and ethical problems related to modern medicine and science. Jewish cantorial music reflecting the prayers of Jews along many centuries has become my favorite music, and I avidly search for it in flea markets, used records stores, and auctions all over. Different Judaica artifacts also decorate my study.

In parallel, my parents made sure we should also receive an excellent general education. My father spoke several

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languages fluently, Hebrew, Polish, Arabic, French, English, German, and Yiddish, and wanted me to acquire his strong love for books: while our home was not a rich one, we had a huge library. My parents also loved classical music, so we had a great collection of 78 rpm, and later 33 rpm records. I remember that Bizet's Carmen occupied more than 20 RCA (His Master Voice) 78 rpm bakelite records.

The apparently peaceful life of our family in Israel (although under the British Crown) during the years of the Holocaust in Europe was overshadowed by the murder of family members and of many families of friends and relatives that did not escape Europe in time. For my parents, the establishment of the State of Israel as an independent and sovereign Jewish State was a direct historical result of the Holocaust in Europe and a clear statement of "*Never Massadah shall fall again*!" (Massadah was one of the last strongholds of Jews during the Roman Empire. It fell into Roman hands after all its defenders committed suicide.) They left us with the idea that the Jewish State will not only protect us as free people, but will allow us to develop our own unique culture in a more general national context rather than as minorities scattered in different countries in the Diaspora.

Falling in Love with Biology

From early days I remember my strong inclination towards biology, though it has taken different directions at different times. I remember collecting flowers on Mount Carmel and drying them in the heavy Babylonian Talmud of my brother. I will never forget his rage on discovering my love of nature hidden among the pages of the old Jewish tracts. Then came the turtles and the lizards, extracting chlorophyll from leaves with alcohol, and the first microscope my brother bought me from his trip to England when I was 11 years old. With this microscope I discovered cells (in the thin onion epithelium) and did my first experiment in osmosis, when I followed the alteration in the volume of the cells after immersing the epithelium in salt solutions of different strengths. With friends we tried to launch a self-propelled rocket. The flower collection kept growing, now in special dedicated albums, and with it, a small collection of skeletons of different animals: fish, frog, toad, snake, turtle, and even some human bones I received from an older friend who was a medical student.

After several years of amateurish flirting with biology, I decided to formalize my knowledge and love of biology, and to major in Biology in high-school. While my years in elementary (1953–1959) and junior-high (1959–1962) school were mostly uneventful and passed without any thoughts on my future, the last two years in "Hugim" (Circles) high-school in Haifa (1963–1965) were not. I had wonderful and inspiring teachers in biology (Naomi Nof), chemistry (Na'ama Greenspon), and physics and mathematics (Harry Amitay). Biology at that time was largely a descriptive discipline: while we studied the mechanism of conversion of glucose into H_2O and CO_2 and production of energy in yeast and mammals (and the opposite process of photosynthesis in plants), and became

acquainted with simple graphic descriptions of mitotic and meiotic cell divisions, most of our studies were devoted to detailed descriptions of the flora and fauna in our region, to comparative zoology (I remember well the efforts invested in memorizing the twelve differences between the frog and the toad, or between the circulatory systems and skeletal structure of the cat and dog), and to basic descriptive human anatomy and physiology (for example, how the human skeleton enables posture to be maintained). Pathogenetic mechanisms of diseases had not been mentioned, and the structure of DNA and the genetic code had entered our textbooks only towards the end of our high-school studies, in 1964/65. On the other hand, chemistry and physics appeared to me, maybe naively, to be strong mechanistic disciplines built on solid mathematical foundations. As a result, I had a deep feeling that the future somehow resided in biology, in deciphering basic mechanisms, as so little was then known. Yet, the complexity of biological and pathological processes looked to me enormous, almost beyond our ability to grasp, and I was intimidated: while I was clearly attracted to the secrets of biology, I was afraid to get lost. Importantly, I had nobody around, close enough, to consult, to clarify my thoughts. While deliberating between the largely unknown of biology and what I naively thought were the already wellfounded physics and chemistry, medicine emerged as a compromise.

While it suffered from an even higher level of complexity compared to biology, it enjoyed some other advantages, such as the fascinating ability to cure or at least to provide some temporary solution to diseases. For me, this choice offered also a practical solution as in these years I lost both of my parents: my mother died in 1958 and my father in 1964. Their death meant that I needed to become independent as soon as I could. After the death of my mother, I was left with my father who took wonderful care of me. When my father died several years later, my late aunt Miriam (Wishniak; my mother's sister), with the support of my brother, took me to her home in Haifa, enabling me to seamlessly complete my high-school studies, in the same class and along with my friends, without interruption. The other option was to move to Tel Aviv, to my brother's home, but this would have been much more complicated. Their help was a true miracle, as thinking of it retrospectively, being left alone, without parents, at the age of sixteen, the distance to youth delinquency was shorter than the one to the high-school class. Yet, with the help of these wonderful family members, I managed to continue.

How my Love of Biology Evolved to Become a Career

Towards graduation from high-school I had to make a decision: The regular track would have taken me, like most Israelis, to national compulsory service in the Israeli Defense Forces (IDF), a duty we were all eager to fulfill. In addition to the regular service, the army encourages certain high-school graduates to postpone their service and first obtain a university education, particularly in areas that are relevant

to the military, such as medicine and different disciplines in engineering and sciences. Lacking any economic support, I thought it would be better to acquire a practical profession as soon as I could. As I mentioned, it was also a compromise between the complexity and mysteries of biological mechanisms to what I thought were the already exhausted physics and chemistry. What also attracted me to medicine was my impression that diseases could be cured: as children, we may have been influenced by short, self-limiting diseases that affected us, like influenza and measles, and were not directly aware of the major killers that left physicians and scientists alike helpless (much like these days), such as malignancies, vascular diseases, and neurodegenerative disorders. I had not appreciated at the time how far more descriptive medicine is, much more than biology. Practically, and no less important (which helped me solve my dilemma), was the fact that biology was not an option in the military-supported service postponement program.

So, after a fierce competition I was accepted at the only medical school in Israel at that time, that of the Hebrew University and "Hadassah" in Jerusalem (1965). The first four years (1965-1969) were exciting. We studied basic and clinical sciences, and I began to seriously entertain the idea of broadening my knowledge base in biochemistry or pharmacology. Towards the end of the fourth year, once we started to see patients, I started to have serious doubts whether I had made the right choice and truly wanted to become a practicing physician. The imbalance between phenomenology and pathogenetic mechanisms on one hand, and the lack of mechanism-based treatment for most of the major killers on the other hand, made me seriously think that I was on the wrong trail. I felt restless and started to realize how little we knew, how descriptive is our understanding of disease mechanisms and pathology, and as a consequence how most treatments are symptomatic in nature rather then causative. The statement "with God's help" I heard so frequently from patients that were praying for a cure and health received a real meaning. I had a feeling clinical medicine was going to bore me, and decided to take one year off in order to "taste" true and "wet" basic research.

The Faculty of Medicine had a special, one-year program for the few who elected to broaden their knowledge in basic research, and I decided to major in biochemistry. I had to convince my brother that this was the right thing to do, as I needed his help to further postpone my military service by one year. This was not easy, as he too had a "dream"-to see me independent with a profession from which I could make my living, and which in the traditional Jewish spirit was nothing else but practical medicine. Following our parents' death, he felt he was responsible for my future and well-being, and wanted to see me independent as soon as he could. I nevertheless managed to convince him, and during that year (1969–1970), under the guidance of first-rate biochemists, Jacob Bar-Tana and Benjamin Shapira, I investigated mechanisms of CCl₄-induced fatty liver in a rat model, and discovered that it may be caused, at least partially, by an increased activity of phosphatidic acid phosphatase, a key enzyme involved in di- and triglyceride biosynthesis. Completing this research year (and obtaining an MSc degree), I knew I had found a new love—biochemistry. Jacob and Benjamin walked me through the exciting maze of biochemical pathways, and I was mystified. Yet, the consummation was still far away. Being loyal to the promise I made to my brother, and also to my commitment to the Israeli army, I completed the clinical years (1970–1972) and graduated Medical School.

To obtain my medical license, I still had to complete one additional year of rotating internship. At that time colleagues told me that a young talented biochemist, Dr. Avram Hershko, had just finished his postdoctoral training with Gordon Tomkins at the University of California in san Francisco (UCSF) and was recruited by the Dean and founder of the newly established Faculty of Medicine at the Technion in Haifa, the late Professor David Ehrlich, to establish a unit of Biochemistry. I wrote to Avram, with the intention to relocate to Haifa, to carry out my rotating internship there, and to use this year to carry out my MD thesis research project under his supervision. This was a small thesis I had to submit to the Medical School in partial fulfillment of the requirements for graduation. Typically for this thesis, most medical students evaluate statistically on-going treatments/ procedures, but I decided to return to the laboratory and touch on yet another research project. He agreed to accept me as an MD student, and in October 1972 we started our more than three decades voyage.

Avram was still not certain about his own main research direction, and we discussed two possibilities for my MD thesis. One was obviously to further dissect the tyrosine aminotransferase (TAT) ATP-dependent proteolytic pathway. Avram started his own trip into the world of intracellular proteolysis with Gordon and discovered that the degradation of the gluconeogenetic enzymes in cells requires energy. This was a corroboration of an earlier finding of Simpson who demonstrated in the early 1950s that the degradation of the entire population of cellular proteins in liver slices requires energy, but the mechanism(s) of this thermodynamically paradoxical requirement had remained elusive.

The other possibility was to study the mechanisms involved in the cell's "pleiotropic response"—the immediate response of serum-starved, G0-synchronized cells to the addition of serum. During his postdoctoral studies with Gordon, Avram found that among the many stimulated processes are rapid uptake of nucleotides, amino acids, and phosphate. As during my studies on fatty liver I acquired experience working with lipids, and since Avram felt the elucidation of the TAT proteolytic mechanism may be a too difficult undertaking for a short MD thesis research project, we decided to add one additional layer to the study on the "pleiotropic response" and to analyze the effect of serum on the synthesis of phospholipids.

We assumed that following serum addition, cell membranes undergo major changes that will be reflected in phospholipid metabolism. Indeed, a few minutes after serum addition we were able to detect a dramatic increase in the turnover of the phosphoinositol moiety on the diglycerol skeleton. A review of the literature revealed a similar effect of different target cells in response to a broad array of stimuli, including parasympathetic secretory cells responding to acetylcholine and thyroid gland cells to their cognate hormones, thyrotropin (TSH). The year (1972/73) I spent in the laboratory (it was not a real year but rather moonlighting, as a significant part of the time I was busy in the hospital rotating among the different clinical departments completing my duties as an intern towards graduation; I worked in the laboratory in my free evenings, nights, weekends, and holidays) finally convinced me to pursue a career in Biochemistry. But I still had three years of military service ahead of me (1973–1976).

Military Service and Professional Career—Have they Collided with One Another?

Following graduation, it was time to repay my national debt and serve in the IDF. I served for three years (1973–1976) and did it gladly. Serving in the army has always been regarded as an integral and important part of Israeli life and an entry card to its society, giving one the feeling of sharing—every one takes part in protecting this land and its inhabitants. In addition, the service itself was extremely interesting, technically, but also socially and historically. Technically, since I served in interesting units and socially, since the military service is a wonderful humane experience, the best melting pot one can go through, generating true friendships during hard times, friendships that are therefore deep, true, and lasting.

Historically, it spanned an interesting period. Initially I served in the navy, as a physician in the missile boats fleet. The year was 1973, immediately after the October Day of Atonement (Yom Kippur) war, and Israel faced a problem of protecting its southern gates, the Red Sea and the narrow Tiran (Sharm-a-Sheikh) strait that led to the port of Eilat. These were threatened by the Arab countries that neighbored the Red Sea, mostly Saudi Arabia and Egypt but also Yemen and Somalia, and Israel had to stretch its marine arm. To do so, it was necessary to transfer missile boats from the main naval bases in the Mediterranean to the Red Sea. At that time Israel did not have diplomatic relationship with Egypt, and the Suez Canal was closed by ships sunk by the Egyptians during the June 1967 Six Day War, so the decision was made to bring the boats from Haifa to Eilat, sailing through the Mediterranean Sea, and around the West and then East coasts of Africa. I was the physician on the "Reshef", one of the two boats (modern Israeli missile boats that were built in the Haifa naval shipyard) selected for the mission. One can imagine that for small missile boats, such a long (several weeks) voyage, a large part of it in the open oceans, is rather complicated, and for many reasons also risky. Beyond fueling and provision of supplies and spare parts to the crews and boats, one has to think of sailing in waterways surrounded by hostile countries, many miles away from home and a long flight distance for the Israeli Air Force. Another problem was obviously medical, how one treats emergencies, from possible gunshot wounds through "simple" daily problems like appendicitis, in a small ship, far from any medical facility and with limited diagnostic and treatment capabilities. I was particularly concerned, as I was a young physician with almost no clinical experience. I assume this would have been a challenge for more experienced physicians as well. Luckily, the voyage was smooth.

The remaining part of my three years service was also interesting. I spent that time in the Research and Development unit of the Medical Corps, developing a broad array of sophisticated devices for the soldier in the battlefield. Because of the broad range of experiences, the military service has been my ever best school for real "life sciences". During all these years (1973-1976) I maintained tight connections with Avram and fulfilled my duties as an "external" department member: during vacations from the military and along with other members of the department, which grew meanwhile, I taught continuously the course in Clinical Biochemistry to third year medical students. I should mention in particular Michael (Mickey) Fry, with whom I have remained a good friend to the present day, and my good friend and colleague Erela Gorin, who died untimely in the early 1990s.

In 1975, during the military service, I married Menucha, a physician and a graduate of Tel Aviv University School of Medicine. Menucha was a resident in internal medicine in Tel Aviv Municipal Hospital, and we built our first home in this city. Marrying Manucha brought my wanderings to an end and I felt I had again a family and a home. During all the years since the death of my father (1963–1975) I did not have a really stable home, and I wandered between the homes of my brother and my aunt in Haifa. They were truly wonderful, but I needed a base, and Menucha, with her quiet approach and warm acceptance, along with our beautiful apartment, provided me with this so much needed shelter.

Discovery of the Ubiquitin System—Graduate Studies

Towards the end of the military service, I had to make what I assume has been the most important decision in my career: to start a residency in clinical medicine, in surgery, which was my favorite choice, or to enroll into graduate school and start a career in scientific research. It was clear to me that I was heading to graduate school. My disillusionment with clinical medicine that diseases can be cured based on the understanding of their pathogenetic mechanisms, along with a magical and enchanting attraction to biochemistry, made the decision easier. I received strong support and encouragement from my wife Menucha, who started to realize she was married to a graduate student with no clear future rather than to a physician with a bright career and broad financial horizons that she thought she had married.

So in November of 1976, after my discharge from the national service and a two months driving trip across the USA, I started my graduate studies with Avram Hershko. Since I had worked with him and known him for several years now, I thought he would be an excellent mentor. At that time his group focused mostly on studying intracellular proteolysis,

and I learnt from him that he had given up on trying to identify the mediator(s) and mechanism(s) involved in the serum-induced "pleiotropic response". The choice of Avram was to work on the degradation of abnormal hemoglobin in reticulocytes, a terminally differentiating red blood cell. The reason for the selection of the reticulocyte as a model system was that we were looking for a non-lysosomal (and energyrequiring) proteolytic system, as from many studies it had become clear that regulated proteolysis of intracellular proteins is non-lysosomal, and the reticulocyte no longer contains lysosomes which are removed during the final stages of its maturation before its release into the circulation. From he work of others, it was clear that the reticulocyte contained such a proteolytic system.

Interestingly, in the summer of 1978, during a Gordon Conference on Lysosomes, I met Dr. Alex Novikoff from Yeshiva University School of Medicine in New York. Alex, along with Dr. Christian de Duve, was one of the pioneers of the lysosome research field. When I told him we were working on the reticulocyte because this cell does not have lysosomes, he angrily dismissed this argument, telling me that he characterized, though morphologically, acid-phosphatasepositive organelles in reticulocytes. He even gave me the relevant paper he published on the subject, though it was not clear that these are proteolytically functional organelles.

Another reason for the choice of the reticulocyte as a model for studying intracellular proteolysis was that in its final stages of maturation in the bone marrow and prior to entering the peripheral circulation, a massive proteolytic burst destroys most of its machineries, making it clear that the cell is equipped with an efficient proteolytic system. Earlier studies by Rabinovitz and Fisher demonstrated that the reticulocyte degrades abnormal, amino acid analogue containing hemoglobin, yet the mechanisms had remained elusive. We assumed that it was probably the same mechanism that was also involved in the natural maturation process and also in the removal of "naturally occurring" mutant abnormal hemoglobins that are synthesized in different hemoglobinopathies, such as thalassemias and sickle-cell anemia. Thus, this important piece of information-the existence of a nonlysosomal proteolytic system-made the choice of the reticulocyte an obvious one.

It was still necessary to demonstrate that the process required energy, and indeed, following an initial characterization of energy-requiring degradation of abnormal hemoglobin in the intact cell (which was published in 1978 in the proceedings of a proteolysis meeting held in Buffalo, NY), we felt the time was ripe to break the cell open and isolate and characterize the non-lysosomal and ATP-dependent proteolytic enzyme(s). Shortly before, in 1977, Dr. Alfred Goldberg and his postdoctoral fellow Dr. Joseph Etlinger at Harvard Medical School characterized, for the first time, a cell-free proteolytic system from reticulocyte, which was exactly the point where we wanted to start our own march, so we basically adopted their system.

I will not describe here the detailed history of the discovery of the ubiquitin system, but rather highlight two important points along the five years of my exciting graduate

studies (1976–1981) with Avram and Irwin A. (Ernie) Rose that led to the discovery of the system. The more detailed history can be found in several review articles written on the system at that time (see, for example, A. Hershko, A. Cienchanover, *Annu. Rev. Biochem.* **1982**, *51*, 335–364) and later, and in the accompanying Nobel Lecture.

The first point relates to the multiplicity of enzymatic components in the system: our first aim along the purification process of the ATP-dependent "protease" was to remove hemoglobin, the major protein in the crude extract. Towards that end, we resolved the extract on an anion-exchange resin, where we encountered the first exciting finding. The proteolytic activity could not be found neither in the non-adsorbed material, which we denoted fraction I, nor in the material eluted with a high salt concentration, denoted fraction II. Rather, we recovered the activity following reconstitution of the two fractions. We learnt two important lessons from this experiment which was published in 1978 in Biochem. Biophys. Res Commun. (in my opinion the first paper in the long historical trail of the ubiquitin proteolytic system) and which I regard as one of two or three key publications in the field. We learnt two lessons from this experiment: 1) The first lesson was that the protease we were after was not a "classical" single enzyme that degrades its substrate, but had at least two components. This was already a digression from the paradigm in the field at that time that proteolytic substrates, almost without exception, could be cleaved at least partially by single proteases with limited, yet defined specificities. Here we needed two components for proteolysis to occur. Now we know that the number of components of the ubiquitin system exceeds one thousand, but the first hint was already there; once one is left without a paradigm, all possibilities are open. 2) The second lesson was a methodological one. Each time we lost an activity during purification of any of the components we were characterizing, we returned to the chromatographic column fractions and tried to reconstitute it by complementation: "classical" biochemistry at its best was on our side.

Standing at a crossroads, we (luckily but thoughtfully) decided to start first with purification and characterization of the active component in fraction I. We decided so, because fraction I was the hemoglobin-containing fraction that did not adsorb onto the resin, and therefore we thought that it should not contain too many additional proteins. In the summer of 1977, ten months after I started my studies, Avram departed to a sabbatical with Ernie at the Fox Chase Cancer Center in Philadelphia, USA, and left me with the task of purifying the active component from fraction I. After many unsuccessful trials (along with another graduate student of Avram, Yaacov Hod), my colleague Mickey Fry, who was appointed as my substitute thesis advisor for this year (1977/78), came up with the "crazy" idea to heat fraction I and see if the active component was heat-stable, and indeed it was. He did so as all our attempts to resolve the activity from hemoglobindespite the large difference in the molecular mass between the active protein (ca. 10 kDa) and hemoglobin (65 kDa)failed. Following 5-10 min at 90°C, the hemoglobin in crude fraction I was "cooked" and precipitated like mud, and the activity remained soluble in the supernatant. It was hard to believe it was a protein, but Mickey remembered several other heat-stable proteins. Immediately after, we showed directly that the activity in fraction I was also a protein: it was sensitive to trypsin and precipitable with ammonium sulfate. Further characterization revealed that the protein had a molecular mass of about 8500 Da, and we called it ATP-dependent proteolysis factor-1 (APF-1). All along the way I corresponded with Avram, sent him the data, and during his sabbatical we wrote the paper for *Biochemical and Biophysical Research Communications*.

The second key finding was also discovered in Haifa during the winter of 1978/79. We purified APF-1 to homogeneity and labeled it with radioactive iodine. When the radiolabeled protein was incubated in crude reticulocyte fraction II in the presence of ATP, we observed a dramatic increase in its molecular weight: it now migrated as a sharp peak in the void volume of the gel-filtration chromatographic column. For several months we tried to elucidate the mechanism that underlies this change, hypothesizing, for example, that APF-1 could be an activator of a protease that must generate a binary complex with the enzyme in order to stimulate it, but to no avail. An important breakthrough occurred during our 1979 summer stay of several months in the laboratory of Ernie. Through a series of extremely elegant, yet simple, experiments, in which we used the broad knowledge of Ernie in protein chemistry and enzymology, we found that APF-1 is covalently attached to the substrate through a bond that had all the characteristics of a peptide bond. Furthermore, we found that multiple moieties of APF-1 are attached to each substrate molecule, and that the reaction is reversible: APF-1 can dissociate from the substrate, though not by reversal of the conjugation reaction. Accordingly, we hypothesized that covalent attachment of multiple moieties of APF-1 to the target substrate is necessary to render it susceptible to degradation by a downstream protease that recognizes only tagged proteins, followed by the release of free and reusable APF-1.

The APF-1 cycle demonstrated unequivocally the existence of three, entirely novel activities: 1) APF-1-conjugating enzyme(s), 2) a protease that recognizes specifically the tagged substrates and degrades them, and 3) APF-1-recycling enzymes. All the enzymes involved were identified later by us (the three conjugating enzymes, E1, E2, and E3) or by others (the conjugates degrading protease known as the 26S proteasome complex, and the ubiquitin-recycling enzymes, the isopeptidases). The findings describing the covalent tagging of the target substrate by APF-1 as a degradation signal as well as its release, along with the first model of the newly discovered proteolytic system, were published in 1980 in two papers that appeared in the *Proceedings of the National Academy of Sciences.*

Another important development also occurred during our stay in Ernie's laboratory, and I am not sure whether it was shear luck or serendipity, probably both. We were not aware of any other precedent of a modification of a protein by another protein. The neighboring laboratories of Martin Nemer, Alfred Zweidler, and Leonard Cohen studied dynamics of variants of different histones during sea urchin development. They drew our attention to a protein called A24 (uH2A) which was discovered earlier by Ira Goldknopf and Harris Busch, and that was a covalent conjugate between two proteins: a small, approximately 8.5 kDa protein called ubiquitin and histone 2A (H2A). Goldknopf and Busch, and in parallel Margaret Dayhoff, identified the nature of the bond between the two protein moieties in the conjugate. They found that the ubiquitin–histone bond was an isopeptide/ bifurcated bond between the C-terminal Gly⁷⁶ residue in the ubiquitin moiety, and the ε -NH₂ group of Lys¹¹⁹ in the histone moiety of the conjugate. The role of this conjugate was not clear at the time, though its level was found to be dynamic and change during differentiation, when the histone moiety is subjected to ubiquitination and deubiquitination.

This information on the ubiquitin-histone adduct and the similarity we found between APF-1 and ubiquitin in general characteristics, molecular mass, and amino acid composition, led Keith Wilkinson and his colleagues Arthur (Art) Hass from the laboratory of Ernie, along with Michael Urban from Zweidler's laboratory, to carry out a series of direct experiments, which showed unequivocally that APF-1 is indeed ubiquitin. Our study on the characterization of APF-1 and its possible similarity to ubiquitin, and Wilkinson's study (along with Urban and Haas) on the identification of APF-1 as ubiquitin, led to the convergence of two fields, that of histone research and of proteolysis. More importantly, they suggested that the bond between ubiquitin and the target proteolytic substrate maybe identical to that between ubiquitin and histone, which we demonstrated later to be true. The elucidation of the nature and structure of the bond clearly paved the road to the later identification of the conjugating enzymes and their mode of action. The two studies on APF-1, ours and that of Wilkinson and co-workers, were published in tandem in the Journal of Biological Chemistry.

As for ubiquitin, the protein was identified in the 1970s by Gideon Goldstein (in the Memorial Sloan-Kettering Cancer Center in New York City) as a small, 76-residue thymic polypeptide hormone that stimulates T-cell differentiation by activation of adenylate cyclase. Additional studies by Gideon Goldstein had suggested that it was universally distributed in both prokaryotes and eukaryotes, thus giving rise to its name (coined by Gideon Goldstein). Later studies by Allan Goldstein showed that the thymopoietic activity was due to an endotoxin contamination in the protein preparation, and not to ubiquitin. By using functional assays, it was found in my laboratory (and I believe in others as well) that ubiquitin was limited to eukaryotes, and its apparent presence in bacteria was due to contamination of the bacterial extract with the yeast extract in which the bacteria were grown: growing the bacteria in a synthetic medium resulted in the "disappearance" of ubiquitin from the preparation. The later unraveling of the bacterial genome demonstrated unequivocally that the ubiquitin tagging system does not exist in prokaryotes, though there is some similarity between the proteasome and certain bacterial proteolytic complexes. Thus, in a relatively short period of time, ubiquitin was converted from a ubiquitous thymopoietic hormone to a eukaryotic proteolytic marker. While the term ubiquitin is not justified anymore, as it is clearly not ubiquitous, we stopped using the term APF-1 and adopted the term ubiquitin as the modifying protein in the newly discovered proteolytic system. At times habits and tradition are stronger from the scientific validity and/or from logic in nomenclature. Accordingly, we adopted a general policy to use in our terminology the name that was first coined by the discoverer of any novel protein.

From that point on, the road was relatively short to the identification and characterization of the conjugation mechanism and the three enzymes involved in this process. En route to the unraveling of the conjugation mechanism, we followed partially the footsteps of Dr. Fritz Lipmann, the great biochemist from Rockefeller University (who was awarded the 1953 Nobel Prize in Physiology or Medicine for the discovery of coenzyme A). Lipmann continued to contribute to our understanding of basic biochemical processes. Among his many discoveries was the mechanism of non-ribosomal (and hence nongenetically encoded) peptidebond formation that occurs during the biosynthesis of bacterial oligopeptides such as gramicidin S. We learnt that the principles of basic biochemical reactions, such as generation of high-energy intermediates involved in peptide-bond formation, were preserved along evolution regardless of whether the bond is encoded genetically or not, or whether it links two amino acids or two proteins. Initially, we identified the general mechanism of activation of ubiquitin in a crude extract. Later, using "covalent" affinity chromatography over immobilized ubiquitin and a stepwise elution (that was based on the general activation mechanism we deciphered earlier), we purified the three conjugating enzymes that act successively in a cascadelike mechanism, and catalyze this unique process: 1) the ubiquitin-activating enzyme E1, the first enzyme in the ubiquitin system cascade, 2) the ubiquitin carrier protein E2, to which the activated ubiquitin is transferred from E1, and 3) the ubiquitin protein ligase E3, the last and critical component in the three-step conjugation mechanism that specifically recognizes the target substrate and conjugates it with ubiquitin. The binding of E1 and E2 was mediated by the activation mechanism. The E3 was also adsorbed onto the resin, although by a mechanism distinct from that of E1 and E2.

Later studies by Avram in the late 1980s revealed that the E3 adsorbed by the column was $E3\alpha$ that recognizes substrates through their N-terminal residue. At this point, however, we were extremely lucky, when unknowingly we used as model substrates commercial proteins such as BSA, lysozyme, and RNase A that were all recognized by this ligase and through a similar targeting motif: their N-terminal residue. Had we used other substrates, such as globin, the model substrate we used in our initial experiments, the E3 α adsorbed to the column would have escaped our attention, as E3 enzymes do not typically adsorb to ubiquitin. In parallel and independently, I also used this enzyme in the late 1980s in order to characterize a distinct subset of proteins recognized by this signal (see below). Last, and most importantly, using antibodies that we raised against ubiquitin with the help of Arthur Haas, we found that the ubiquitin system is involved in degradation of abnormal, short-lived proteins in hepatoma cells, thus demonstrating that the system was not limited to the terminally differentiating reticulocyte, but was probably distributed more "universally" in nucleated mammalian cells, playing a role in maintaining the cell's quality control.

During my graduate studies at Avram's laboratory, I collaborated with Hannah Heller, an extremely talented and knowledgeable research associate (who also joined us for some of our summer stays in the laboratory of Ernie in Philadelphia) and with Yaacov Hod who was also a graduate student with Avram at that time. Other colleagues in the laboratory provided me with a lot of help during this period, including Dvorah Ganoth, Sarah Elias, and Esther Eythan who were research associates with Avram, and Clara Segal and Bruria Rosenberg, two dedicated technicians.

The Interaction with Irwin Rose

As noted, I spent an important part of my graduate studies in Ernie's laboratory. Avram spent a sabbatical in his laboratory in 1977/78, and I joined him for the first time for several months in the summer of 1978, after I completed the initial characterization of APF-1 in Haifa. I returned to Ernie's laboratory during the summers of 1979, 1980, and 1981. As noted, during our summer stay in 1979, we resolved the problem of the nature of the high-molecular-mass "compound" generated when APF-1 was incubated with fraction II in the presence of ATP. The change in the molecular mass of APF-1 was discovered several months earlier in Haifa, however, we were not able to unravel the nature of the "compound"; this had to await the knowledge and wisdom of Ernie. In a breakthrough discovery, we found that the target substrate is covalently modified by multiple moieties of APF-1, a modification that renders it susceptible to degradation. This was a novel type of posttranslational modification and clearly a new biological paradigm, that required—as I feel today in retrospect—a different type of knowledge and experimental approach. This would not have been possible without Ernie's advice that was based on his immense knowledge in enzymology and protein chemistry, accompanied by his unbiased way of original thinking and approach to problem resolving. This discovery, along with the discovery that APF-1 is ubiquitin in 1980, made Ernie, his fellows (in particular Keith Wilkinson and Arthur Haas), and laboratory crucial players in the historical trail of the discovery of the ubiquitin system. Interestingly, Ernie also studied proteolysis before Avram joined him first, but had never published in the field before.

Postgraduate Training at MIT and How I Continued My Studies on the Ubiquitin System Independently

The five years in graduate school had a significant impact on my future career, not only because I played an active part in such an important discovery, but maybe more importantly, because I learnt several basic and important principles of how to approach a scientific problem. From my mentors, first and

foremost Avram, but also Ernie, I learnt two important principles: first, to select an important biological problem (but in order to avoid fierce competition and to be original to ascertain it is not in the mainstream), and second, to make sure there are appropriate research tools to approach it experimentally.

From Avram I also learnt to become a book rather then a short stories writer: I learnt not to be opportunistic but rather to adhere to a project, to dig deeply into a problem, to resolve it mechanistically, to untangle complex mazes—peeling them like an onion, and not to be tempted to be dragged after fashions. I learnt to pay attention to small details, to carefully examine hints, as the important findings were not always obvious from the beginning. I learnt to be stubborn, to fight difficulties uphill, and most importantly to be critical: I believe I developed good senses that enable me to distinguish false from truth, and artifacts from meaningful findings.

Interestingly, I learnt all these principles not in frontal lessons or formal presentations, but as an apprentice, following my mentors own attitude and way of thinking. But I also learnt to question, to doubt, to ask, and to discuss, to follow my own gut feeling when it was necessary, not to always take advice and direction for granted, and to trust myself too. It did help in many occasions along the way, although at times I found myself swimming against the stream in my own school. Altogether these principles generated an important philosophy and shaped my approach to science, something I try to instill to my own students, as I strongly believe it is the only way one can make an impact and leave an imprint behind.

Toward graduation I had to think of the next step: postdoctoral training and planning of my future career as an independent scientist. I was in a dilemma. On the one hand I knew it was important to obtain training somewhere else, under different mentorship, in a different environment, being exposed to a different culture of science. On the other hand I knew for certain that the ubiquitin system was extremely important and that we were seeing only the tip of its iceberg. I therefore wanted to continue my studies in a related field, learning more on regulated proteolysis, but also to continue my own studies on ubiquitin.

I had several ideas in mind of where to go. The choice was quite narrow and also risky, as I did not have any idea of how much independence I could have as a postdoctoral fellow. Searching for a mentor, and with the advice of my colleague Mickey Fry, I looked for scientists whose work was related to regulated proteolysis. I wrote to Günter Blobel in the Rockefeller University, who worked at that time on translocation of proteins to the endothelium reticulum (ER), a process which involves cleavage of the leader peptide by signal peptidase, to Jeffrey Roberts in Cornell, who worked on E. coli RecA protein directed cleavage of phage λ repressor and its requirement for polynucleotide, and to Harvey Lodish at the MIT, who worked, among other subjects, on the processing of viral polyproteins. I am not sure Harvey was that impressed with the ubiquitin system at that time, but he was the only one to respond positively. Typical of his etiquette (as I learnt later), his response was prompt and direct, and he invited me for an interview, after which he accepted me. Günter was kind enough to let me know he did not have space in his laboratory at that time, and Jeffrey never responded.

With two fellowships, one from the Leukemia Society of America and one from the Israel Cancer Research Fund (ICRF), I started a period of three wonderful years (1981– 1984) in Harvey's laboratory in the Department of Biology at MIT. Harvey gave me complete freedom to choose my research subjects. What I had in mind was to take advantage of the exceptional strength of the laboratory and Harvey's unique expertise in cell biology, but in parallel, to continue my own studies on the ubiquitin system.

I realized that Harvey was no longer interested in viral protein processing, and along with Alan Schwartz who was a visiting scientist (from Harvard Medical School) in the laboratory, we started to characterize the transferrin receptor on a human hepatoma cell line with the aim of later studying the mechanism of transferrin and transferrin receptor mediated iron delivery to cells. This collaboration led us, along with another fellow in the laboratory, Alice Dautry-Varsat (from the Pasteur Institute) who joined us later, to the discovery of a fascinating mechanism of how iron is delivered into cells: in the neutral pH of the growth medium, the ironloaded holotransferrin binds to its receptor with a high affinity and is endocytosed into the cell. At the low endosomal pH, the affinity between the iron and transferrin is weakened dramatically. As a result, the iron cation is released, but the apotransferrin, which has high affinity for the receptor at acidic pH, remains bound. Along with the receptor, the apotransferrin recycles to the cell surface. At the neutral pH of the growth medium, the apotransferrin loses its high affinity to the receptor and is released into the extracellular fluid where it can load additional iron ions and then rebind to its receptor with high affinity.

The transferrin/transferrin receptor pH-dependent and iron loading-dependent cycle has become a "classic" in the field of receptor-mediated endocytosis. Based on this, other phenomena related to receptor and ligand recycling to the cell surface or targeting to the lysosome could be explained, which are also due to the pH difference between the external environment and the interior of the endocytic pathway vesicles.

However, throughout this time I lived under the strong feeling that the ubiquitin system had barely started to emerge, with only the basic principles unraveled. I felt compelled to get back and work on it. So gradually I started to "crawl" and return to my "alma mater" research subject.

On one fascinating subject I worked on my own continuing to explore a mysterious finding I discovered during my graduate training and which I did not pursue at the time: when we purified APF-1/ubiquitin in Haifa, we noticed a large discrepancy between its dry weight and the Lowry assay quantitative protein measurement. Avram hypothesized that the protein could be a ribonucleoprotein (RNP), and the remaining mass is that of the nucleic acid component. To test this hypothesis, we added DNase to the crude extract (ATPand ubiquitin-containing) assay in which we monitored degradation of bovine serum albumin (BSA) that was used as one of our model substrates. The enzyme had no effect. We then added RNase A, and to our surprise proteolysis was completely inhibited, even with an extremely small amount a mere few nanograms—of the enzyme added: it looked as though the enzyme exerted its effect by catalysis—RNA degradation.

Avram suggested testing the RNase effect on lysozyme as well-our second model substrate. Here we got no effect, which was kind of a surprise, as proteolysis of the two substrates, BSA and lysozyme, behaved in an identical manner all along the way. ATP as well as all the different factors resolved from the crude extract were all required for the degradation of both proteins. Avram suspected that the RNase effect could be an artifact. Meanwhile, APF-1 was identified by Keith Wilkinson and his colleagues as ubiquitin, and the amino acid sequence/composition of ubiquitin disclosed the "secret" of the dry weight/protein measurement discrepancy-the molecule has a single tyrosine residue, thus eyplaining the low readings at 280 nm and in the Lowry assays. So we decided not to pursue this subject, and the selective inhibitory effect of RNase A on BSA degradation remained an unsolved mystery-for the time.

I had not stopped suspecting however that the findings must represent some true biological phenomenon, and used the opportunity of my independence at Harvey's laboratory to pull out the late 1970s data from my notebook and to start dissecting the RNase effect in a systematic manner. With some advice from Alexander (Alex) Varshavsky (MIT), and a lot of help from Joan Steitz (Yale), Harvey Lodish, and Uttam RajBhandary (MIT), I managed to make some progress. I discovered that the degradation of BSA was completely dependent on specific tRNAs (for Arg and His), and that the destruction of the tRNA led to inhibition of the reaction. The nature of the mechanism of action of the tRNAs and the problem of why the degradation of lysozyme was insensitive to RNase had remained a mystery at that time, which I resolved only when I retuned to Israel and established my own laboratory.

The other ubiquitin subject I was studying involved a collaboration with Alex Varshavsky and his then graduate student, Daniel (Dan) Finley. At that time Alex was studying the role of monoubiquitination of histones (see above for the histone H2A/ubiquitin adduct, also known as protein A24 or uH2A). He noted a series of publications on a temperaturesensitive cell-cycle-arrest mouse mutant cell ts85 that was generated and described by the group of M. Yamada. At the nonpermissive temperature, the cell lost the histone H2A/ ubiquitin adduct. This loss could be due to one of two defects, either loss of ubiquitination, or activated deubiquitination. Alex asked me to collaborate with him and Dan to identify the mutation in this cell. We surmised that the defect in these cells was more likely due to loss rather then to gain of function, and set out to dissect the defect. The idea was that the same defect may also affect protein degradation, although it was clear that the single modification of the histone molecule by ubiquitin does not lead to its targeting to proteolysis.

Identification of the defect in the cells was not too difficult, as we used the isolation technique of the conjugation

enzymes developed in Haifa, and demonstrated that the defect results from a temperature-sensitive ubiquitin-activating enzyme E1, the first enzyme in the ubiquitin system cascade. Importantly, inactivation of the enzyme led to inhibition of ubiquitin conjugation to the general population of cellular proteins, and was not confined to inhibition of conjugation of histone H2A. Consequently, degradation of short-lived proteins was also inhibited, demonstrating that the same enzyme that is involved in ubiquitin activation for histone modification is also involved in activation of ubiquitin for modification of substrates destined for degradation. Identification and characterization of the cell defect further corroborated our earlier general hypothesis that ubiquitination signals proteins for degradation, and that it also occurs in nucleated cells, a finding we had already demonstrated, albthough indirectly, in Haifa, using the anti-ubiquitin antibody. Since the ts85 cell was also a cell-cycle-arrest mutant, we hypothesized, but did not prove experimentally at the time, that the system might be involved in regulating the cell cycle, a hypothesis that later turned out to be correct.

The Return to Israel—Independent Research Career

After three years at MIT (1981–1984), it was time to seek an independent academic position. After many deliberations and despite attractive offers and a big temptation to stay in the US, I decided to return home, to Israel. With the help of Avram, I obtained an independent academic position in the Department of Biochemistry at the Faculty of Medicine of the Technion (where I graduated), and returned home towards the end of 1984, after a productive postdoctoral period. Importantly, I already had a research subject I wanted to pursue, the effect of RNase on ubiquitin-mediated proteolysis.

The years that followed the postdoctoral fellowship (1984–present) have been extremely rewarding. I was happy to return to Israel to my family and friends, to a place I felt I belong. I established my own independent research group and laboratory, obtained extramural competitive funding, and continued my research on the ubiquitin system. I have been lucky to have, through the years, a group of extremely talented graduate students and postdoctoral fellows.

In our first series of studies we elucidated the role of tRNA in the proteolytic process, a subject I discovered as a graduate student and continued to study independently at MIT. Along with one of my first graduate students, Sarah Ferber, we demonstrated that proteins with acidic N-termini, such as Asp or Glu, undergo arginylation at the N-terminus, converting the acidic, negatively charged residue at this site into a positively charged residue. The reaction is catalyzed by arginine tRNA-protein transferase, a known protein with a hitherto unknown function. The enzyme uses charged tRNA^{Arg} as a source of activated Arg. Therefore, digestion of the cell extract RNA with RNase A inhibits this reaction. This finding explained the selectivity of the RNase effect to BSA and not to lysozyme: BSA has an Asp residue at the N-terminus, while lysozyme has lysine in this position. Interest-

ingly, the ligase involved is $E3\alpha$, which we discovered during my graduate studies. The ligase recognizes only proteins with basic termini, but not with acidic N-termini. Thus, what appeared initially as an artifact turned out to be part of the first specific recognition signal in a target substrate.

Parallel to our work on the RNase effect, Avram and his graduate student Yuval Reiss characterized the E3 α ligase and identified on it three distinct substrate binding sites for: 1) basic N-termini (the one involved in recognition of basic and Arg-modified acidic N-termini), 2) bulky-hydrophobic N-terminal, and 3) "body" sites that reside downstream of the N-terminal residue. In parallel and by using a systematic genetic approach in the yeast *S. cerevisiae*, Alex Varshavsky and his colleagues formulated a general rule ("N-end rule") for recognition of all 20 different amino acid residues at the N-terminal site.

Research in the laboratory has also evolved in other directions. We have shown that N- α -acetylated proteins are also targeted by the ubiquitin system. This important finding demonstrated that the N-terminally modified proteins, a group that constitutes the vast majority of cellular proteins, must be targeted by signals that are distinct from the Nterminal residue and reside downstream to it: they do not have free N-termini and therefore cannot be recognized by the N-terminal amino acid residue. Along with the discovery of the "body" site in E3 α , we felt that N-terminal recognition is of minor physiological significance, an exception rather then a rule, and the mode of recognition of the numerous substrates of the system must be broad and diverse: they are recognized by multiple and distinct targeting motifs.

At that point, towards the end of the 1980s, we felt it was time to move from studying model substrates to investigating the fate of specific native cellular substrates. We have shown that an important group of cell regulators-tumor suppressors (e.g. p53) and growth promoters (c-Myc)-are targeted by the ubiquitin cell-free system. We believed that this was true also for the targeting of these substrates in vivo, which later turned out to be correct. We continued and demonstrated that, unlike the thinking in the field until that time, that degradation of proteins in the lysosome proceeds independently from the ubiquitin system, the two proteolytic pathways are actually linked to one another, and ubiquitination is required for stress-induced lysosomal degradation of cellular proteins. This area later evolved in a dramatic manner, and engulfed involvement of the ubiquitin system in receptor-mediated endocytosis and autophagy. Other studies involved elucidation of some of the mechanisms involved in the two-step ubiquitin-mediated proteolytic activation of the transcriptional regulator NF-kB, demonstration of a role for heat-shock proteins in targeting certain protein substrates, and identification of a novel mode of ubiquitination at the N-terminal residue of the protein substrate. This modification is clearly different and distinct from recognition of the substrate by $E3\alpha$ at the N-terminal residue. In the latter case, the ligase binds to the N-terminal residue while ubiquitination occurs on an internal lysine. In N-terminal ubiquitination, modification occurs at the Nterminal residue, while the ligase binds, most probably, to an internal sequence in the protein target molecule. This subject has evolved in a surprising manner and changed another paradigm in the field that ubiquitination is limited to internal lysine(s) of the target substrate. We, and later others, have shown that the phenomenon is not limited to the one protein we identified initially (the muscle-specific transcriptional regulator MyoD), and identified a large group of proteins that undergo N-terminal ubiquitination. This group of proteins contain many that have internal lysine(s), but for some reason these residues cannot be targeted, and interestingly also a large group of proteins (such as p16^{INK4a} that plays an important role in cell-cycle regulation) that are devoid of any lysine residue. To be degraded by the ubiquitin system they must undergo N-terminal ubiquitination.

These years have not been simple, however. The Technion has traditionally been a school of engineering, and life sciences and biomedicine have been foreign to many of its senior faculty members and policy planners: we were treated in many ways like step-children, and thoughts of closing the school have been aired at times. This deeply rooted philosophy, which only now starts to change slowly, has severely hampered development in these fields and had left the body of researchers and infrastructure in these areas small and battling for survival. Through a network of wonderful colleagues all over the world (important among them is my friend Alan Schwartz from Harvard Medical School and then from Washington University in St. Louis) and fruitful collaborations, it was possible to establish an active research group and carry out what I believe was a good and original research program, even under less than optimal, and at times impossible conditions. This was important in balancing my desire to live in Israel, but at the same time to remain at the forefront of the ubiquitin research field that has grown in importance to become an extremely exciting, yet a highly competitive area.

Unpaid Debts

Last but not least, I owe a huge debt which I doubt I shall ever be able to repay to several people who helped me cross critical stormy waterways along my life. My aunt Miriam, who took me to her house after the death of my father and made her home a new home for me, thus enabling me to complete seamlessly my high-school studies without any interruption. My brother Yossi and my sister-in-law Atara, who opened their home for me during the fragile times of my high-school and medical studies, and made sure I would not collapse along the way, emotionally, but also economically. And last, my wonderful wife Menucha and my son Tzachi (Yitzhak, Isaac; named after my late father); they have flooded me with love, care, and deep understanding of my needs, and were always there for me, when I was flying high on the wings of my dreams, not always seeing them or listening to them or being with them, physically and emotionally. Without all these wonderful life partners, I could not have achieved anything.

I also owe special thanks to all my mentors, who each contributed in their own way to my upbringing as a scientist. I

huge debt to Harvey Lodish, who is not only a great cell

biologist, but a wonderful spiritual mentor in a different way

to how we tend to think of mentors. He gave me complete

freedom to choose my own way, but did not let me fall. He

always listened carefully and helped me to analyze data, and

with his deep insight was able to find in the ocean of my

numbers and graphic analyses new routes and pathways that I

could have never seen or thought of. He used to gently

comment on my approach when he felt I derailed, and helped

redirecting me. Yet, he was never imposing: Harvey's active

passive educational approach is truly unique. I owe many

thanks to all my colleagues, in particular Alan Schwartz, Iasha

Sznajder, and Kazuhiro Iwai, who helped me in many ways

along this long voyage. I must also mention my laboratory

have to thank Jacob Bar-Tana and Benjamin Shapira for taking me, hand in hand, through the complex maze of metabolic pathways, thus enabling me to fall in love with Biochemistry. Their enthusiasm and deep thinking convinced me, at a critical stage of my development, to pursue a career in biological sciences. I owe a big debt to my mentor, Avram Hershko, with whom I have come a long way in discovering the ubiquitin system, and from whom I learnt the very basic principles of how to approach a scientific problem. I owe special thanks to Ernie Rose for showing me that ordered thinking is not always necessary in science, and is even interfering at times, and that being erratic and disordered, absent minded at times, collecting sparks from all over the place, can yield wonderful ideas and results. Last, I owe a

Table 1: Tabulated biography of Aaron Ciechanover.

Date and place of birth	October 1, 1947	Haifa, Israel
Education		
Elementary School	1953–1956	"Hashiloach" Elementary School, Haifa
High School	1959–1965	"Hugim" High School, Haifa
University (undergrad- uate studies)	1965–1972	"Hadassah" and the Hebrew University School of Medicine, Jerusalem (MSc, MD)
Clinical internship	1972–1973	"Rambam" Medical Center, Haifa
Military Service	1973–1976	Medical Corps, Israel Defense Forces (military physician)
University (graduate studies)	1976–1981	Faculty of Medicine, Technion, Haifa (DSc). Thesis advisor: Dr. Avram Hershko
Postdoctoral Training	1981–1984	Department of Biology and the Whitehead Institute, Massachusetts Institute of Technology, Cambridge, USA (with Dr. Harvey F. Lodish)
Faculty Position	1984–present	Faculty of Medicine, Technion, Haifa
Degrees		
MSc	1970	Medical Sciences. Faculty of Natural Sciences and the Department of Biochemistry, "Hadassah" and the Hebrew University School of Medicine, Jerusalem
MD	1974	"Hadassah" and the Hebrew University School of Medicine, Jerusalem
DSc	1981	Faculty of Medicine, Technion, Haifa
Academic		
Appointments		
1977–1979	Research Fellow	Department of Biochemistry, Faculty of Medicine, Technion, Haifa
1979–1981	Lecturer	
1984–1987	Senior Lecturer	
1987–1992	Associate Professor	
1992–	Full Professor	
2002–	Distinguished Research Professor	
Administrative		
Appointments		
1993–2000	Director	The Rappaport Family Institute for Research in the Medical Sciences, Technion, Haifa
Military Service		
1974–1977		Military Physician in the Israeli Navy and the Unit for Research and Development, Surgeon General Headquarters; discharged at the rank of Major
Visiting Appointments		
1978, 1979	Visiting Scientist	The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia (with Dr. Irwin A.
1980 1981		Rose)
1985–present	Visiting Professor	The Dana Farber Cancer Institute and Harvard Medical School, Boston; Washington University School of Medicine, St. Louis; University of Kyoto School of Medicine; Northwestern University School of Medicine, Chicago; STINT Fellow. Microbiology and Tumor Biology Center (MTC), The Karolinska Institute, Stockholm, Sweden; City University of Osaka School of Medicine, Osaka, Janan: Rockefeller University. New York
		Japan; Rockefeller University, New York

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research associates, initially Sarah Elias and then Hedva Gonen and Beatrice Bercovich, who have become my eyes and hands since I established my own laboratory. Last but not least, my wonderful graduate students, fellows, and visiting scientists, with whom I discovered new and exciting paths in the rapidly evolving and exciting ubiquitin field.

2. Introduction

The concept of protein turnover is barely 60 years old. Beforehand, body proteins were viewed as essentially stable constituents that were subject to only minor "wear and tear". Dietary proteins were believed to function primarily as energy-providing fuel, which were independent from the structural and functional proteins of the body. The problem was hard to approach experimentally, as research tools were not available. An important research tool that was lacking at that time were stable isotopes. While radioactive isotopes were developed earlier by George de Hevesy (*Nobel Lectures in Chemistry 1942–1962*, World Scientific, **1999**, pp. 5–41), they were mostly unstable and could not be used to follow metabolic pathways.

The concept that body structural proteins are static and the dietary proteins are used only as a fuel was challenged by Rudolf Scheonheimer at Columbia University in New York city. Schoenheimer, like many other Jewish scientists (for example, Albert Einstein), escaped from Germany after the rise of the Nazis, and joined the Department of Biochemistry in Columbia University, founded by Hans T. Clarke.^[1-3] There he met Harold Urey who was working in the Department of Chemistry and who discovered deuterium, the heavy isotope of hydrogen, a discovery that enabled him to prepare heavy water, D₂O. David Rittenberg, who had recently received his PhD in Urey's laboratory, joined Schoenheimer, and together they entertained the idea of "employing a stable isotope as a label in organic compounds, destined for experiments in intermediary metabolism, which should be biochemically indistinguishable from their natural analog".^[1]

Urey later succeeded in enriching nitrogen with ¹⁵N, which provided Schoenheimer and Rittenberg with a "tag" for amino acids and thus for their study on protein dynamics. They discovered that following administration of ¹⁵N-labled tyrosine to rats, only about 50% was recovered in the urine, "while most of the remainder is deposited in tissue proteins. An equivalent of protein nitrogen is excreted".[4] They further discovered that from the half that was incorporated into body proteins "only a fraction was attached to the original carbon chain, namely to tyrosine, while the bulk was distributed over other nitrogenous groups of the proteins",^[4] mostly as an NH₂ group in other amino acids. These experiments demonstrated unequivocally that the body structural proteins are in a dynamic state of synthesis and degradation, and that even individual amino acids are in a state of dynamic interconversion. Similar results were obtained using ¹⁵N-labled leucine.^[5]

This series of findings shattered the paradigm in the field at that time that: 1) ingested proteins are completely metabolized and the products are excreted, and 2) that body structural proteins are stable and static. Schoenheimer was invited to deliver the prestigious Edward K. Dunham lecture at Harvard University where he presented his revolutionary findings. After his untimely tragic death in 1941, his lecture notes were edited by Hans Clarke, David Rittenberg, and Sarah Ratner, and were published in a small book by Harvard University Press. The editors called the book The Dynamic State of Body Constituents,^[6] adopting the title of Schoenheimer's presentation. In the book, the new hypothesis is clearly presented: "The simile of the combustion engine pictured the steady state flow of fuel into a fixed system, and the conversion of this fuel into waste products. The new results imply that not only the fuel, but the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure". However, the idea that proteins are turning over was not accepted easily and was challenged as late as the mid-1950s. For example, Hogness and colleagues studied the kinetics of β-galactosidase in E. coli and summarized their findings:^[7] "To sum up: there seems to be no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover, our experiments have shown that the proteins of growing E. coli are static. Therefore it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a 'dynamic state'". While the experimental study involved the bacterial β galactosidase, the conclusions were broader, and included also the authors' hypothesis on mammalian proteins. The use of the term "dynamic state" was not incidental, as they challenged directly Schoenheimer's studies.

Now, after more then six decades of research in the field and with the discovery of the lysosome and later the complex ubiquitin-proteasome system with its numerous tributaries, it is clear that the area has been revolutionized. We now realize that intracellular proteins are turning over extensively, that this process is specific in most cases, and that the stability of many proteins is regulated individually and can vary under different conditions. From a scavenger, unregulated and nonspecific end process, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process that plays major roles in a broad array of basic pathways. Among these processes are the cell cycle, development, differentiation, regulation of transcription, antigen presentation, signal transduction, receptormediated endocytosis, quality control, and modulation of diverse metabolic pathways. As a result, this development has changed the paradigm that regulation of cellular processes occurs mostly at the transcriptional and translational levels, and has placed regulated protein degradation in an equally important position. With the multitude of substrates targeted and processes involved, it is not surprising that aberrations in the pathway have been implicated in the pathogenesis of many diseases, among them certain malignancies, neurodegeneration, and disorders of the immune and inflammatory system. As a result, the ubiquitin system has become a platform for drug targeting, and mechanism-based drugs are currently developed, one of them is already on the market.

3. The Lysosome and Intracellular Protein Degradation

In the mid-1950s, Christian de Duve discovered the lysosome (see, for example, Refs. [8,9] and Figure 1). The lysosome was first recognized biochemically in rat liver as a vacuolar structure that contains various hydrolytic enzymes which function optimally at an acidic pH. It is surrounded by a membrane that endows the contained enzymes with latency that is required to protect the cellular contents from their action (see below). The definition of the lysosome has been broadened over the years. This is because it has been recognized that the digestive process is dynamic and involves numerous stages of lysosomal maturation together with the digestion of both exogenous proteins (which are targeted to the lysosome through receptor-mediated endocytosis and pinocytosis) and exogenous particles (which are targeted through phagocytosis; the two processes are known as heterophagy), as well as digestion of endogenous proteins and cellular organelles (which are targeted by micro- and macro-autophagy; see Figure 2).

The lysosomal/vacuolar system as we currently recognize it is a discontinuous and heterogeneous digestive system that also includes structures that are devoid of hydrolases, for example, early endosomes which contain endocytosed receptor–ligand complexes and pinocytosed/phagocytosed extracellular contents. At the other extreme it includes the residual bodies—the end products of the completed digestive processes of heterophagy and autophagy. In between these extremes one can observe: primary/nascent lysosomes that have not yet been engaged in any proteolytic process; early



Figure 1. The lysosome: Ultrathin cryosection of a rat PC12 cell that had been loaded for 1 h with bovine serum albumin (BSA)·gold (5-nm particles) and immunolabeled for the lysosomal enzyme cathepsin B (10-nm particles) and the lysosomal membrane protein LAMP1 (15-nm particles). Lysosomes are recognized also by their typical dense content and multiple internal membranes. Scale bar, 100 nm. Printed with permission from Viola Oorschot and Judith Klumperman, Department of Cell Biology, University Medical Centre, Utrecht, The Netherlands.



Figure 2. The four digestive processes mediated by the lysosome: 1) specific receptor-mediated endocytosis; 2) pinocytosis (nonspecific engulfment of cytosolic droplets containing extracellular fluid); 3) phagocytosis (of extracellular particles), and 4) autophagy (micro- and macro-autophagy of intracellular proteins and organelles). Printed from Ref. [83] with permission from Nature Publishing Group.

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autophagic vacuoles that might contain intracellular organelles; intermediate/late endosomes and phagocytic vacuoles (heterophagic vacuoles) that contain extracellular contents/ particles; and multivesicular bodies (MVBs) which are the transition vacuoles between endosomes/phagocytic vacuoles and the digestive lysosomes (Figure 2).

The discovery of the lysosome along with independent experiments that were carried out at the same time and that have further strengthened the notion that cellular proteins are indeed in a constant state of synthesis and degradation (see, for example, Ref. [10]), led scientists to feel, for the first time, that they had at hand an organelle that could potentially mediate degradation of intracellular proteins. The separation of the proteases from their substrates by a membrane provided an explanation for controlled degradation, and the only problem left to be explained was how the substrates are translocated into the lysosomal lumen, where they are degraded by the lysosomal proteases.

An important discovery in this respect was the unraveling of the basic mechanism of action of the lysosome, namely autophagy (reviewed in Ref. [11]). Under basal metabolic conditions, portions of the cytoplasm which contain the entire cohort of cellular proteins, are segregated within a membrane-bound compartment, and are then fused to a primary nascent lysosome and their contents digested. This process was denoted micro-autophagy. Under more extreme conditions (for example, starvation) mitochondria, endoplasmic reticulum membranes, glycogen bodies, and other cytoplasmic entities can also be engulfed by a process called macroautophagy (see, for example, Ref. [12]). The different modes of action of the lysosome in digesting extra- and intracellular proteins are shown in Figure 2.

However, over a period of more than two decades (between the mid-1950s and the late-1970s) it became gradually more and more difficult to explain several aspects of intracellular protein degradation based on the known mechanisms of lysosomal activity. Accumulating lines of independent experimental evidence indicated that the degradation of at least certain classes of cellular proteins must be non-lysosomal. Yet, in the absence of any "alternative", researchers found different explanations, some more substantiated and others less, to defend the "lysosomal" hypothesis.

First was the gradually emerging notion, coming from different laboratories, that different proteins vary in their stability, and their half-life times $(t_{1/2})$ can span three orders of magnitude—from a few minutes to many days. Thus, the $t_{1/2}$ of ornitihine decarboxylase (ODC) is about 10 min, while that of glucose-6-phosphate dehydrogenase (G6PD) is 15 h (for review articles, see, for example, Refs. [13,14]). Also, the rates of degradation of many proteins were shown to change with changing physiological conditions, such as availability of nutrients or hormones. It was conceptually difficult to reconcile the findings of distinct half-lives of different proteins with the mechanism of action of the lysosome, where the micro-autophagic vesicle contains the entire cohort of cellular (cytosolic) proteins that are therefore expected to degrade at the same rate. Likewise, if micro- and macroautophagy had been the mechanisms that mediate intracellular proteolysis, changing pathophysiological conditions, such as starvation or resupplementation of nutrients, would have been expected to affect the stability of all cellular proteins to the same extent. Clearly, this was not the case.

A second source of concern about the lysosome as the organelle in which intracellular proteins are degraded were the findings that specific and general inhibitors of lysosomal proteases have different effects on different populations of proteins, making it clear that distinct classes of proteins are targeted by different proteolytic machineries. Thus, the degradation of endocytosed/pinocytosed extracellular proteins was significantly inhibited, a partial effect was observed on the degradation of long-lived cellular proteins, and almost no effect could be detected on the degradation of short-lived and abnormal/mutated proteins.

Finally, the thermodynamically paradoxical observation that the degradation of cellular proteins requires metabolic energy, and more importantly, the emerging evidence that the proteolytic machinery uses the energy directly, were in contrast with the known mode of action of lysosomal proteases that under the appropriate acidic conditions, and similar to all known proteases, degrade proteins in an exergonic manner.

The assumption that the degradation of intracellular proteins is mediated by the lysosome was nevertheless logical. Proteolysis results from direct interaction between the target substrates and proteases, and therefore it was clear that active proteases cannot be free in the cytosol, which would have resulted in destruction of the cell. Thus, it was recognized that any suggested proteolytic machinery that mediates degradation of intracellular protein degradation must also be equipped with a mechanism that separates—physically or virtually—between the proteases and their substrates, and enables them to associate only when needed. The lysosomal membrane provided a physical fencing mechanism.

Of course, nobody could have predicted that a new mode of posttranslational modification—ubiquitination—could function as a proteolytic signal, and that untagged proteins would remain protected. Thus, while the structure of the lysosome could explain the separation necessary between the proteases and their substrates, and autophagy could explain the mechanism of entry of cytosolic proteins into the lysosomal lumen, major problems have remained unsolved. Important among them were: 1) the varying half-lives, 2) the energy requirement, and 3) the distinct response of different populations of proteins to lysosomal inhibitors.

Nevertheless, scientists tried to "defend" the lysosomal model. According to one model, it was proposed that different proteins have different sensitivities to lysosomal proteases, and their half lives in vivo correlate with their sensitivity to the action of lysosomal proteases in vitro.^[15] To explain an extremely long half-life for a protein that is nevertheless sensitive to lysosomal proteases, or alterations in the stability of a single protein under various physiological states, it was suggested that although all cellular proteins are engulfed into the lysosome, only the short-lived proteins are degraded, whereas the long-lived proteins exit back into the cytosol: "*To account for differences in half-life among cell components or of a single component in various physiological*

states, it was necessary to include in the model the possibility of an exit of native components back to the extralysosomal compartment".^[16]

According to a different model, selectivity is determined by the binding affinity of the different proteins for the lysosomal membrane which controls their entry rates into the lysosome, and subsequently their degradation rates.^[17] For a selected group of proteins, such as the gluconeogenetic enzymes phosphoenolpyruvate carboxykinase (PEPCK), and fructose-1,6-biphosphatase, it was suggested, though not firmly substantiated, that their degradation in the yeast vacuole is regulated by glucose through a mechanism called "catabolite inactivation" that possibly involves their phosphorylation. However, this regulated mechanism for vacuolar degradation was limited only to a small and specific group of proteins (see, for example, Refs. [18],[19]).

More recent studies have shown that at least for stressinduced macro-autophagy, KFERQ, a general sequence of amino acids that in its general structure was identified in many proteins, directs, by binding to a specific "receptor" and in cooperation with cytosolic and lysosomal chaperones, the regulated entry of many cytosolic proteins into the lysosomal lumen. While further corroboration of this hypothesis is still required, it explains the mass entry of a large population of proteins that contain a homologous sequence, but not the targeting for degradation of a specific protein under defined conditions (reviewed in Refs. [20,21]). The energy requirement for protein degradation was described as indirect, and necessary, for example, for protein transport across the lysosomal membrane^[22] and/or for the activity of the H⁺ pump and the maintenance of the low acidic intralysosomal pH that is necessary for optimal activity of the lysosomal proteases.^[23] We now know that both mechanisms require energy. In the absence of any alternative, and with lysosomal degradation as the most logical explanation for targeting all known classes of proteins at the time, Christian de Duve summarized his view on the subject in a review article published in the mid-1960s, saving: "Just as extracellular digestion is successfully carried out by the concerted action of enzymes with limited individual capacities, so, we believe, is intracellular digestion".^[24] The problem of different sensitivities of distinct protein groups to lysosomal inhibitors has remained unsolved, and may have served as an important trigger in the future quest for a non-lysosomal proteolytic system.

Progress in identifying the elusive, non-lysosomal proteolytic system(s) was hampered by the lack of a cell-free preparation that could faithfully replicate the cellular proteolytic events, degrading proteins in a specific and energyrequiring mode. An important breakthrough was made by Rabinovitz and Fisher who found that rabbit reticulocytes degrade abnormal, amino acid analogue containing hemoglobin.^[25] Their experiments modeled known disease states the hemoglobinopathies. In these diseases abnormal mutated hemoglobin chains (such as sickle cell hemoglobin) or excess of unassembled normal hemoglobin chains (which are synthesized normally, but accumulate and found in excess in thalassemias, diseases in which the pairing chain is not synthesized at all or is mutated and rapidly degraded, and consequently the hemoglobin complex is not assembled) are rapidly degraded in the reticulocyte.^[26,27] Reticulocytes are terminally differentiating red blood cells that do not contain lysosomes. Therefore, it was postulated that the degradation of hemoglobin in these cells is mediated by a non-lysosomal machinery.

Etlinger and Goldberg^[28] were the first to isolate and characterize a cell-free proteolytic preparation from reticulocytes. The crude extract selectively degraded abnormal haemoglobin, required ATP hydrolysis, and acted optimally at a neutral pH, which further strengthened the assumption that the proteolytic activity was of a non-lysosomal origin. A similar system was isolated and characterized later by our research group.^[29] Additional studies by our group led subsequently to resolution, characterization, and purification of the major enzymatic components from these extracts, and to the discovery of the ubiquitin tagging system (see below).

4. The Lysosome Hypothesis is Challenged

As mentioned above, the unraveled mechanism(s) of action of the lysosome could explain only partially, and at times not satisfactorily, several key emerging characteristics of intracellular protein degradation. Among them were the heterogeneous stability of individual proteins, the effect of nutrients and hormones on their degradation, and the dependence of intracellular proteolysis on metabolic energy. The differential effect of selective inhibitors on the degradation of different classes of cellular proteins could not be explained at all.

The evolvement of methods to monitor protein kinetics in cells, together with the development of specific and general lysosomal inhibitors, has resulted in the identification of different classes of cellular proteins, long- and short-lived, and the discovery of the differential effects of the inhibitors on these groups (see, for example, Refs. [30,31]). An elegant experiment in this respect was carried out by Brian Poole and his colleagues at the Rockefeller University. Poole was studying the effects on proteolysis of lysosomotropic agents-weak bases such as ammonium chloride and chloroquine-that accumulate in the lysosome and dissipate its low acidic pH. It was assumed that this mechanism also underlies the antimalarial activity of chloroquine and similar drugs, where they inhibit the activity of the parasite's lysosome, "paralyzing" its ability to digest the host's hemoglobin during the intraerythrocytic stage of its life cycle. Poole and his colleagues metabolically labeled endogenous proteins in living macrophages with ³H-leucine and "fed" them with dead macrophages that had been previously labeled with ¹⁴Cleucine. They assumed, apparently correctly, that the dead macrophages debris and proteins will be phagocytosed by the live macrophages and targeted to the lysosome for degradation. They monitored the effect of lysosomotropic agents on the degradation of these two protein populations. In particular, they studied the effect of the weak bases chloroquine and ammonium chloride (which enter the lysosome and neutralize the H⁺ ions), and the acid ionophore X537A which dissipates the H⁺ gradient across the lysosomal membrane.

They found that these drugs specifically inhibited the degradation of extracellular proteins, but not that of intracellular proteins.^[32]

Poole summarized these elegant experiments and explicitly predicted the existence of a non-lysosomal proteolytic system that degrades intracellular proteins: "Some of the macrophages labeled with tritium were permitted to endocytise the dead macrophages labeled with ¹⁴C. The cells were then washed and replaced in fresh medium. In this way we were able to measure in the same cells the digestion of macrophage proteins from two sources. The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins are broken down during protein turnover".^[33]

The requirement for metabolic energy for the degradation of both prokaryotic^[34] and eukaryotic^[10,35] proteins was difficult to understand. Proteolysis is an exergonic process and the thermodynamically paradoxical energy requirement for intracellular proteolysis made researchers believe that energy cannot be consumed directly by proteases or the proteolytic process per se, and is used indirectly. As Simpson summarized his findings:^[10] "The data can also be interpreted by postulating that the release of amino acids from protein is itself directly dependent on energy supply. A somewhat similar hypothesis, based on studies on autolysis in tissue minces, has recently been advanced, but the supporting data are very difficult to interpret. However, the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically, together with the consideration that autolysis in excised organs or tissue minces continues for weeks, long after phosphorylation or oxidation ceased, renders improbable the hypothesis of the direct energy dependence of the reactions leading to protein breakdown". Being cautious, however, and probably unsure about this unequivocal conclusion, Simpson still left a narrow orifice opened for a proteolytic process that requires energy in a direct manner: "However, the results do not exclude the existence of two (or more) mechanisms of protein breakdown, one hydrolytic, the other enrgy-requiring."

Since any proteolytic process must be at one point or another hydrolytic, the statement that makes a distinction between a hydrolytic process and an energy-requiring, yet nonhydrolytic one, is not clear. Judging the statement from a historical point of view and knowing the mechanism of action of the ubiquitin system, where energy is required also in the prehydrolytic step (ubiquitin conjugation), Simpson may have thought of a two-step mechanism, but did not give it a clear description: in retrospect, one can view ubiquitination as a nonhydrolytic, yet energy-requiring process. At the end of this clearly understandable and apparently difficult deliberation, he left us with a vague explanation linking protein degradation to protein synthesis, a process that was known at that time to require metabolic energy: "The fact that a supply of energy seems to be necessary for both the incorporation and the release of amino acids from protein might well mean that the two processes are interrelated. Additional data suggestive of such a view are available from other types of experiments. Early investigations on nitrogen balance by Benedict, Folin, Gamble, Smith, and others point to the fact that the rate of protein catabolism varies with the dietary protein level. Since the protein level of the diet would be expected to exert a direct influence on synthesis rather than breakdown, the altered catabolic rate could well be caused by a change in the rate of synthesis."^[10]

With the discovery of lysosomes in eukaryotic cells it could be argued that energy is required for the transport of substrates into the lysosome or for maintenance of the low intralysosomal pH, for example. The observation by Hershko and Tomkins that the activity of tyrosine aminotransferase (TAT) was stabilized following depletion of ATP^[36] indicated that energy may be required at an early stage of the proteolytic process, most probably before proteolysis occurs. Yet, it did not provide a clue for the mechanism involved: energy could be used, for example, for specific modification of TAT, for example, phosphorylation, that would sensitize it to degradation by the lysosome or by a yet unknown proteolytic mechanism, or for a modification that activates its putative protease. It could also be used for a more general lysosomal mechanism, one that involves transport of TAT into the lysosome or maintenance of the low intralysosomal pH, as it is cleat that ATP depletion also inhibited completely lysosomal degradation. The energy inhibitors inhibited almost completely degradation of the entire population of cell proteins, confirming previous studies (see, for example, Ref. [10]) and suggesting a general role for energy in protein catabolism. An interesting finding was that energy inhibitors had an effect that was distinct from that of protein synthesis inhibitors, which affected only enhanced degradation (induced by steroid hormone depletion), but not basal degradation. This finding ruled out, at least partially, a tight linkage between protein synthesis and all classes of protein degradation.

In bacteria, which lack lysosomes, an argument involving energy requirement for lysosomal degradation could not have been proposed, but other indirect effects of ATP hydrolysis could have affected proteolysis in *E. coli*, such as phosphorylation of substrates and/or proteolytic enzymes, or maintenance of the "energized membrane state". According to this model, proteins could become susceptible to proteolysis by changing their conformation, for example, following association with the cell membrane that maintains a local, energydependent gradient of a certain ion. While such an effect was ruled out,^[37] and since there was no evidence for a phosphorylation mechanism (although the proteolytic machinery in prokaryotes had not been identified at that time), it seemed that at least in bacteria, energy is required directly for the proteolytic process (which later turned out to be correct).

In any event, the requirement for metabolic energy for protein degradation in both prokaryotes and eukaryotes, a process that is exergonic thermodynamically, strongly indicated that in cells proteolysis is highly regulated, and that a similar principle/mechanism has been preserved in the evolution of the two kingdoms. From the possible direct requirement for ATP in the degradation of proteins in bacteria, it was not too unlikely to assume a similar direct mechanism involved in the degradation of cellular proteins in eukaryotes. Supporting this notion was the description of the cell-free proteolytic system in reticulocytes,^[28,29] a cell that lacks lysosomes, which indicated that energy is probably required directly for the proteolytic process, although here too, the underlying mechanisms had remained enigmatic at the time. Yet, the description of the cell-free system paved the road for detailed dissection of the underlying mechanisms involved.

5. The Ubiquitin–Proteasome System

The cell-free proteolytic system from reticulocytes^[28,29] turned out to be an important and rich source for the purification and characterization of the enzymes that are involved in the ubiquitin–proteasome system. Initial fractionation of the crude reticulocyte cell extract on the anion-exchange resin diethylaminoethylcellulose (DEAE) yielded two fractions which were both required to reconstitute the energy-dependent proteolytic activity that was identified in the crude extract: The unadsorbed, flow-through material was denoted fraction I, and the adsorbed proteins which were eluted with a high concentration of salt was denoted fraction II (Table 2).^[38]

Table 2: Resolution of the ATP-dependent proteolytic activity from crude reticulocyte extract into two essentially required complementing activities (adapted from Ref. [38] with permission from Elsevier).

Fraction	Degradatio	Degradation of [³ H]globin [%]	
	-ATP	+ ATP	
lysate	1.5	10.0	
fraction I	0.0	0.0	
fraction II	1.5	2.7	
fraction I + fraction II	1.6	10.6	

This was an important observation and a lesson for the future dissection of the system. For one, it suggested that the system is not composed of a single "classical" protease that has evolved evolutionarily to acquire energy dependence (although such energy-dependent proteases such as the mammalian 26S proteasome and the prokaryotic *Lon* gene product, for example, were described later), but that it is made of at least two components. This finding of a two-component, energy-dependent protease left us with no paradigm to follow, and in attempts to explain the finding, we suggested, for example, that the two fractions could represent an inhibited protease and its activator.

Second, learning from this reconstitution experiment and the essential dependence of the two active components, we continued to reconstitute activity from resolved fractions whenever we encountered a loss of activity in further purification steps. This biochemical "complementation" approach resulted in the discovery of additional enzymes in the system, which are all required to be present in the reaction mixture in order to catalyze the multistep proteolysis of the target substrate. We chose first to purify the active component from fraction I. It was found to be a small, about 8.5 kDa heat-stable protein that was designated ATP-dependent proteolysis factor 1 (APF-1). APF-1 was later identified as ubiquitin (see below; I am using the term APF-1 to the point at which it was identified as ubiquitin, and then change terminology accordingly). In retrospect, the decision to start

the purification efforts with fraction I turned out to be important, as fraction I contained only one single protein, APF-1, that was necessary to stimulate proteolysis of the model substrates we used at the time, BSA and lysozyme, while fraction II turned out to contain many additional active factors. Later studies showed that fraction I contains other components necessary for the degradation of other substrates, but these were not necessary for the reconstitution of the system at that time. This enabled us not only to purify APF-1, but also to quickly decipher its mode of action. If we had started our purification efforts with fraction II, we would have encountered a significantly bumpier road. A critically important finding that paved the road for future developments in the field was that multiple moieties of APF-1 are covalently conjugated to the target substrate when incubated in the presence of fraction II, and the modification requires ATP (Figures 3 and 4).^[39,40] It was also found that the modification is reversible, and APF-1 can be removed from the substrate or its degradation products.^[40]

The discovery that APF-1 is covalently conjugated to protein substrates and stimulates their proteolysis in the presence of ATP and crude fraction II, led in 1980 to the proposal of a model, according to which protein-substrate modification by multiple moieties of APF-1 targets it for degradation by a downstream, at that time as yet unidentified, protease that cannot recognize the unmodified substrate; following degradation, reusable APF-1 is released.^[40] Amino



Figure 3. APF-1/ubiquitin is shifted to high-molecular-mass compound(s) following incubation in an ATP-containing crude cell extract. ¹²⁵I-labeled APF-1/ubiquitin was incubated with reticulocyte crude fraction II in the absence (\odot) or presence (\bullet) of ATP, and the reaction mixtures were resolved by gel-filtration chromatography. The radioactivity measured in each fraction is shown. As can be seen, following addition of ATP, APF-1/ubiquitin becomes associated with some component(s) (another enzyme of the system or its substrate(s)) in fraction II. Printed from Ref. [39] with permission from the National Academy of Sciences.



Figure 4. Multiple molecules of APF-1/ubiquitin are conjugated to the proteolytic substrate, probably signaling it for degradation. To analyze the data described in the experiment depicted in Figure 3 mechanistically and to test the hypothesis that APF-1 is conjugated to the target proteolytic substrate, ¹²⁵I-APF-1/ubiquitin was incubated along with crude fraction II in the absence (lane 1) or presence (lanes 2-5) of ATP and in the absence (lanes 1, 2) or presence (lanes 3-5) of increasing concentrations of unlabeled lysozyme. Reaction mixtures resolved in lanes 6 and 7 were incubated in the absence (lane 6) or presence (lane 7) of ATP, and included unlabeled APF-1/ubiquitin and ¹²⁵Ilabeled lysozyme. C1-C6 denote specific APF-1/ubiquitin-lysozyme adducts in which the number of APF-1/ubiquitin moieties bound to the lysozyme moiety of the adduct increases, probably from 1 to 6. Reaction mixtures were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized following exposure of the gel to an Xray film (autoradiography). Printed from Ref. [40] with permission from the National Academy of Sciences.

acid analysis of APF-1, along with its known molecular mass and other general characteristics, raised the suspicion that APF-1 was ubiquitin,^[41] a known protein of previously unknown function. Indeed, Wilkinson and colleagues confirmed unequivocally that APF-1 was indeed ubiquitin.^[42]

Ubiquitin is a small, heat-stable, and highly evolutionarily conserved protein of 76 residues. It was first purified during the isolation of thymopoietin^[43] and was subsequently found to be ubiquitously expressed in all kingdoms of living cells, including prokaryotes.^[44] Interestingly, it was initially found to have lymphocyte-differentiating properties, a characteristic that was attributed to the stimulation of adenylate cyclase.^[44,45] Accordingly, it was named UBIP for ubiquitous immunopoietic polypeptide.^[44] However, later studies showed that ubiquitin is not involved in the immune response,^[46] and that it was a contaminating endotoxin in the preparation that generated the adenylate cyclase and the T-cell-differentiating activities. Furthermore, the sequence of several eubacteria and archaebacteria genomes as well as biochemical analyses of cell extracts from these organisms (unpublished results) showed that ubiquitin is restricted only to eukaryotes. The finding of ubiquitin in bacteria^[44] was probably due to contamination of the bacterial extract with yeast ubiquitin derived from the yeast extract in which the bacteria were grown. While, in retrospect, the name ubiquitin is a misnomer, as it is restricted to eukaryotes and is not ubiquitous as was previously thought, for historical reasons it has still maintained its name. Accordingly, and in order to avoid confusion, I suggest that the names of other novel enzymes and components of the ubiquitin system, as well as of other systems, should remain as they were first coined by their discoverers.

An important development in the ubiquitin research field was the discovery that a single ubiquitin moiety can be covalently conjugated to histones, particularly to histones H2A and H2B. While the function of these adducts had remained elusive until recently, their structure was unraveled in the mid-1970s. The structure of the ubiquitin conjugate of H2A (uH2A; also designated protein A24) was deciphered by Goldknopf and Busch^[47,48] and by Hunt and Dayhoff,^[49] who found that the two proteins are linked through a forklike, branched isopeptide bond between the carboxy-terminal glycine of ubiquitin (Gly⁷⁶) and the ϵ -NH₂ group of an internal lysine (Lys¹¹⁹) of the histone molecule. The isopeptide bond found in the histone-ubiquitin adduct was suggested to be identical to the bond that was found between ubiquitin and the target proteolytic substrate^[50] and between the ubiquitin moieties in the polyubiquitin chain^[51,52] that is synthesized on the substrate and that functions as a proteolysis recognition signal for the downstream 26S proteasome. In this particular polyubiquitin chain the linkage is between Gly⁷⁶ of one ubiquitin moiety and internal Lys48 of the previously conjugated moiety. Only Lys⁴⁸-based ubiquitin chains are recognized by the 26S proteasome and serve as proteolytic signals.

In recent years it has been shown that the first ubiquitin moiety can also be attached in a linear mode to the Nterminal residue of the proteolytic target substrate.^[53] However, the subsequent ubiquitin moieties generate Lys⁴⁸-based polyubiquitin chains on the first, linearly fused moiety. Nterminal ubiquitination is clearly required for targeting naturally occurring lysine-free proteins for degradation. Yet, several lysine-containing proteins have also been described that traverse this pathway, the muscle-specific transcription factor MyoD, for example. In these proteins the internal lysine residues are probably not accessible to the cognate ligases.

Other types of polyubiquitin chains have also been described that are not involved in targeting the conjugated substrates for proteolysis. Thus, a Lys⁶³-based polyubiquitin chain has been described that is probably necessary for the activation of transcription factors (see Ref. [54]). Interestingly, the role of monoubiquitination of histones has also been identified recently, and this modification is also involved in regulation of transcription, probably by modulation of the structure of the nucleosomes (see, for example, Refs. [55, 56]).

The identification of APF-1 as ubiquitin, and the discovery that a high-energy isopeptide bond, similar to the one that links ubiquitin to histone H2A, links it also to the target proteolytic substrate, resolved at that time the enigma of the energy requirement for intracellular proteolysis and paved the road to the untangling of the complex mechanism of isopeptide-bond formation. This process turned out to be similar to that of peptide-bond formation that is catalyzed by tRNA synthetase following amino acid activation during protein synthesis or during the nonribosomal synthesis of short peptides.^[57] With the unravelled mechanism of ubiquitin activation and using immobilized ubiquitin as a "covalent" affinity bait, the three enzymes that are involved in the cascade reaction of ubiquitin conjugation were purified by us. These enzymes are: 1) E1, the ubiquitin-activating enzyme, 2) E2, the ubiquitin carrier protein, and 3) E3, the ubiquitin protein ligase.^[58,59] The discovery of an E3 which is a specific substrate-binding component indicated a possible solution to the problem of the varying stabilities of different proteins they might be specifically recognized and targeted by different ligases.

Within a short period, the ubiquitin-tagging hypothesis received substantial support. For example, Chin and coworkers injected into HeLa cells labeled ubiquitin and hemoglobin, and denatured the injected hemoglobin by oxidizing it with phenylhydrazine. They found that ubiquitin conjugation to globin is markedly enhanced by denaturation of the hemoglobin, and the concentration of globin-ubiquitin conjugates was proportional to the rate of hemoglobin degradation.^[60] Hershko and colleagues observed a similar correlation for abnormal, amino acid analogue containing short-lived proteins.^[61] A previously isolated cell-cycle-arrest mutant that loses the ubiquitin-histone H2A adduct at the permissive temperature^[62] was found to harbor a thermolabile E1.^[63] Following heat inactivation, the cells failed to degrade normal short-lived proteins.^[64] Although the cells did not provide direct evidence for substrate ubiquitination as a destruction signal, their characterization established the strongest direct linkage between ubiquitin conjugation and degradation.

At this point, the only missing link was the identification of the downstream protease that would specifically recognize ubiquitinated substrates. Tanaka and colleagues identified a second ATP-requiring step in the reticulocyte proteolytic system, which occurred after ubiquitin conjugation,^[65] and Hershko and colleagues demonstrated that the energy is required for conjugate degradation.^[66] An important advance in the field was a discovery by Hough and colleagues, who partially purified and characterized a high-molecular-mass alkaline protease that degraded, in an ATP-dependent mode, ubiquitin adducts of lysozyme, but not untagged lysozyme.^[67] This protease which was later called the 26S proteasome (see below), provided all the necessary criteria for being the specific proteolytic arm of the ubiquitin system.

This finding was confirmed and the protease was further characterized by Waxman and colleagues who found that it is an unusually large, approximately 1.5 MDa enzyme, unlike any other known protease.^[68] A further advance in the field was the discovery^[69] that a smaller neutral multi-subunit 20S protease complex that was discovered together with the larger 26S complex is similar to a "multicatalytic proteinase complex" (MCP) that was found earlier in the bovine pituitary gland by Wilk and Orlowski.^[70] This 20S protease is ATP-independent and has different catalytic activities: cleaving on the carboxy-terminal side of hydrophobic, basic, and acidic residues. Hough and colleagues raised the possibility-although they did not show it experimentally-that this 20S protease can be a part of the larger 26S protease that degrades ubiquitin adducts.^[69] Later studies showed that, indeed, the 20S complex is the core catalytic particle of the larger 26S complex.^[71,72] However, strong evidence that the active "mushroom"-shaped 26S protease was generated through the assembly of two distinct subcomplexes—the catalytic 20S cylinder-like MCP and an additional 19S ball-shaped subcomplex (that was predicted to have a regulatory role)—was provided only in the early 1990s by Hoffman et al.^[73] who mixed the two purified particles and generated the active 26S enzyme.

The proteasome is a large, 26S multicatalytic protease that degrades polyubiquitinated proteins to small peptides (Figures 5 and 6). It is composed of two subcomplexes: a 20S core



Figure 5. The ubiquitin–proteasome proteolytic system: Ubiquitin is activated by the ubiquitin-activating enzyme E1 (1), followed by its transfer to a ubiquitin-carrier protein (ubiquitin-conjugating enzyme, UBC) E2 (2). E2 transfers the activated ubiquitin moieties to the protein substrate that is bound specifically to a unique ubiquitin ligase E3. The transfer is either direct ((3) in the case of RING finger ligases) or via an additional thiol-ester intermediate on the ligase ((4, 4a) in the case of HECT domain ligases). Successive conjugation of ubiquitin moieties to one another generates a polyubiquitin chain that serves as the binding (5) and degradation signal for the downstream 26S proteasome. The substrate is degraded to short peptides (6), and free and reusable ubiquitin is released by deubiquitinating enzymes (DUBs; 7).

particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer α rings and two identical inner β rings. The eukaryotic α and β rings are composed each of seven distinct subunits, giving the 20S complex the general structure $\alpha_{1-7}\beta_{1-7}$ $_{7}\beta_{1-7}\alpha_{1-7}$. The catalytic sites are localized to some of the β subunits. Each extremity of the 20S barrel can be capped by a 19S RP each composed of 17 distinct subunits, 9 in a "base" subcomplex, and 8 in a "lid" subcomplex. One important function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome. Several ubiquitin-binding subunits of the 19S RP have been identified, although their biological roles and mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the



Figure 6. Structure of the proteasome. Printed from Ref. [83] with permission from Nature Publishing Group. a) Electron microscopy image of the 26S proteasome from the yeast *Saccharomyces cerevisiae*; b) schematic representation of the structure and function of the 26S proteasome.

channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP "base" contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin.

6. Concluding Remarks

The evolution of proteolysis as a centrally important regulatory mechanism is a remarkable example of the evolution of a novel biological concept and the accompanying battles to change paradigms. The five-decade journey between the early 1940s and early 1990s began with fierce discussions on whether cellular proteins are static, as had been thought for a long time, or are turning over. The discovery of the dynamic state of proteins was followed by the discovery of the lysosome, that was believed between the mid-1950s and mid-1970s to be the organelle within which intracellular proteins are destroyed. Independent lines of experimental evidence gradually eroded the lysosomal hypothesis and resulted in a new idea that the bulk of intracellular proteins are degraded—under basal metabolic conditions—by a nonlysosomal machinery. This resulted in the discovery of the ubiquitin system in the late 1970s and early 1980s.

With the identification of the reactions and enzymes that are involved in the ubiquitin-proteasome cascade, a new era in the protein-degradation field began in the late 1980s and early 1990s. Studies that showed that the system is involved in the targeting of specific key regulatory proteins, such as lightregulated proteins in plants, and transcriptional factors, cellcycle regulators, and tumor suppressors and promoters in mammalian cells, started to emerge (see, for example Refs. [74–78]). They were followed by numerous studies on the underlying mechanisms involved in the degradation of these specific proteins, each with its own unique mode of recognition and regulation. The unraveling of the human



Figure 7. Some of the different functions of modification by ubiquitin and ubiquitin-like proteins: a) Proteasomal-dependent degradation of cellular proteins (see Figures 5 and 6). b) Mono- or oligoubiquitination targets membrane proteins to degradation in the lysosome/vacuole. c) Monoubiquitination, or d) a single modification by a ubiquitin-like (UBL) protein, for example, SUMO, can target proteins to different subcellular destinations such as nuclear foci or the nuclear pore complex (NPC). Modification by UBLs can serve other nonproteolytic functions, such as protecting proteins from ubiquitination or activation of E3 complexes. e) Generation of a Lys⁶³-based polyubiquitin chain can activate transcriptional regulators, directly or indirectly (through recruitment of other proteins, such as the shown protein Y, or activation of upstream components such as kinases). Ub = ubiquitin. Printed from Ref. [83] with permission from the Nature Publishing Group.

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genome revealed the existence of hundreds of distinct E3s, attesting to the complexity and the high specificity and selectivity of the system.

Two important advances in the field were the discovery of the nonproteolytic functions of ubiquitin, among which are activation of transcription and routing of proteins to the vacuole, and the discovery of modification by ubiquitin-like proteins (UBLs) that are also involved in numerous nonproteolytic functions such as directing proteins to their subcellular destination, protecting proteins from ubiquitination, or controlling entire processes such as autophagy (see, for example, Ref. [79]). Some of the different roles of modifications by ubiquitin and UBLs are shown in Figure 7. All these studies have led to the emerging realization that this novel and general mode of covalent conjugation plays a key role in regulating a broad array of cellular processes-among them cell cycle and division, growth and differentiation, activation and silencing of transcription, apoptosis, the immune and inflammatory response, signal transduction, receptor-mediated endocytosis, various metabolic pathways, and cell-quality control-through proteolytic and nonproteolytic mechanisms. The discovery that ubiquitin modification plays a role in routing proteins to the lysosome/vacuole and that modification by specific and unique ubiquitin-like proteins and the conjugation mechanism controls autophagy, closed an exciting historical cycle, since it demonstrated that the two apparently distinct proteolytic systems communicate with one another.

With the many processes and substrates targeted by the ubiquitin pathway, it was not surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases. While inactivation of a major enzyme such as E1 is obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways, or that affect the involved process only partially, may result in a broad array of phenotypes. Likewise, acquired changes in the activity of the system can also evolve into certain pathologies. The pathological states associated with the ubiquitin system can be classified into two groups: 1) those that result from loss of function-mutation in a ubiquitin system enzyme or in the recognition motif in the target substrate that results in stabilization of certain proteins, and 2) those that result from gain of function-abnormal or accelerated degradation of the protein target.

Aberrations in the ubiquitin system that result in disease states are shown in Figure 8. Studies that employ targeted inactivation of genes coding for specific ubiquitin system enzymes and substrates in animals can provide a more systematic view into the broad spectrum of pathologies that may result from aberrations in ubiquitin-mediated proteolysis. A better understanding of the processes and identification of the components involved in the degradation of key regulatory proteins will lead to the development of mechanism-based drugs that will target specifically only the involved proteins. While the first drug, a specific proteasome inhibitor



Angewandte

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Figure 8. Aberrations in the ubiquitin-proteasome system and pathogenesis of human diseases: Normal degradation of cellular proteins maintains them at a steady-state level, although this level may change under various pathophysiological conditions (right side, top and bottom). When degradation is accelerated because of an increase in the level of an E3 (Skp2 in the case of p27, for example), or overexpression of an ancillary protein that generates a complex with the protein substrate and targets it for degradation (for example, the human papillomavirus E6 oncoprotein that associates with p53 and targets it for degradation by the E6-AP ligase, or the cytomegalovirus-encoded ER proteins US2 and US11 that target MHC class I molecules for endoplasmic reticulum-associated degredation, ERAD), the steady-state level of the protein decreases (top left). A mutation in a ubiquitin ligase (such as occurs in adenomatous polyposis coli, or EG-AP in Angelmans' syndrome), or in the substrate's recognition motif (such as occurs in β -catenin or in ENaC), will result in decreased degradation and accumulation of the target substrate (bottom left).

is already on the market,^[80] it appears that one important hallmark of the new era we are entering now will be the discovery of novel drugs based on the targeting of specific processes such as inhibiting aberrant Mdm2- or E6-AP-mediated accelerated targeting of the tumor suppressor p53 which will lead to regeneration of its lost function.

Many reviews have been published on different aspects of the ubiquitin system. The purpose of this article was to bring to the reader several milestones on the historical pathway along which the ubiquitin system has evolved. For additional reading on the ubiquitin system the reader is referred to the many reviews written on the system, such as Refs. [81,82]. Some parts of this Review, including several figures, are based on another recently published review article (Ref. [83]).

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