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Discovery of Cellular Regulation by Protein Degradation

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I was born and educated in Russia and was 30 at the time of emigration to the United States, in 1977. Over the last three decades, studies by my laboratory encompassed several fields. Although the title of this article is about our main contribution to date, I describe other significant advances as well, in addition to new biochemical and genetic methods introduced by the lab. A condensed summary, below, is followed by an account of specific discoveries and inventions in their historical contexts.

In 1978–1979, we discovered the first "exposed," nuclease-hypersensitive region in chromosomes (1, 2). Studies of these dynamic structures have become a large field, as the exposed regions are critical signposts of chromosome organization, being a part of transcriptional promoters, replication origins, and other functionally important segments of chromatin.

Another early work, in 1980–1981, was the discovery of the first pathway of chromosome segregation, through the decatenation of multiply intertwined (multicatenated) daughter chromatids in replicated chromosomes (Fig. 1) (3–5). In the 1990s, several groups identified a "complementary" pathway of chromosome segregation in which proteins called cohesins form circles around replicated chromosomes. Both multicatenanes (Fig. 1) and ring-shaped cohesins hold sister chromatids together through a topological confinement, in two entirely different ways. The multicatenane-mediated (3–5) and cohesin-mediated pathways are essential for chromosome segregation in both mitosis and meiosis, and are closely coordinated, in ways that remain to be understood. Acquired or inherited perturbations of these pathways lead to a decreased fidelity of chromosome segregation and other genomic instabilities. These instabilities are among the causes of cancer.

The third major advance, a set of interconnected studies between 1984 and 1990, revealed the manifold biological significance of ubiquitin (Ub) conjugation and Ub-mediated proteolysis (Fig. 2) (6–22). The field of Ub and regulated protein degradation was created in the 1980s, largely through the complementary discoveries by the laboratory of Avram Hershko at the Technion (Israel) and by my laboratory, then at Massachusetts Institute of Technology (MIT). I describe below the elegant insights, in 1978–1984, by Hershko and his colleagues that yielded the initial understanding of the Ub-mediated protein degradation in cell extracts, including the identification of E1, E2, and E3 enzymes (23–27).

These mechanistic (enzymological) advances were followed in 1984–1990 by genetic and biochemical discoveries in my laboratory that revealed the biology of the Ub system, *i.e.* its necessity for the bulk of protein degradation in living cells (6, 7) and its specific functions, in the cell cycle (6, 13), DNA repair (12), protein synthesis (17), transcriptional regulation (21, 22), and stress responses (8, 10, 11). During that time, we also discovered the first degradation signals in short-lived proteins (9); the first complete pathway of the Ub system (termed the N-end rule pathway) (Fig. 3) (9, 14, 16); the first specific poly-Ub chains, their isopeptide bonds through Lys^{48} and Gly^{76} of adjacent Ub moieties, and the essential function of poly-Ub

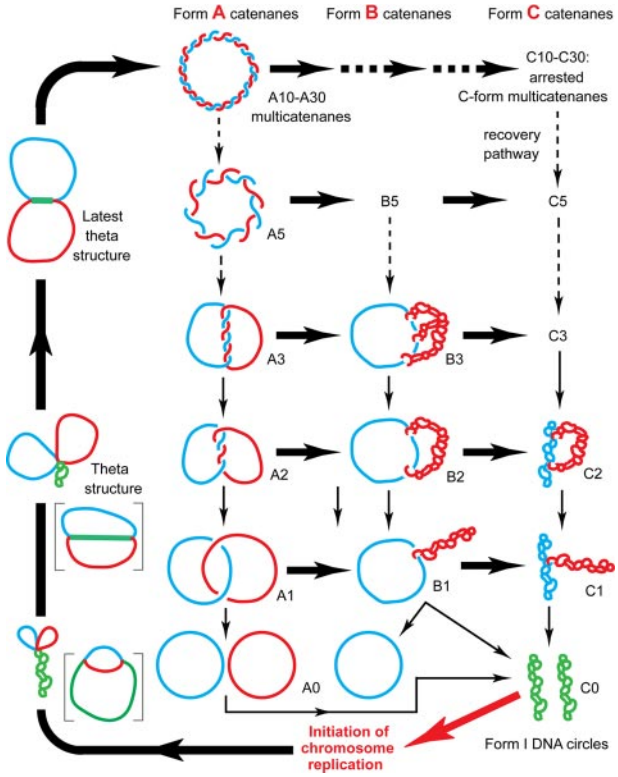

FIGURE 1. **The multicatenane pathway of chromosome segregation (3–5).** *Thick horizontal arrows* denote pathways of DNA synthesis that convert gapped or nicked DNA circles into covalently closed circles. *Thin vertical arrows* denote pathways of decatenation of the intertwined daughter chromosomes. Daughter DNA duplexes are in *blue* and *red*, whereas parental double-stranded DNA is in *green*. The monomeric (completely decatenated) and supercoiled daughter circles are also in *green*, as they become parental DNA in the next round of replication (*red arrow*). The *A*, *B*, and *C* notations of multicatenated DNA dimers correspond to both circles relaxed (nicked or gapped), one circle covalently closed (and therefore supercoiled), and both circles covalently closed, respectively. *Numbers , e.g. A10–A30*, are catenation linking numbers, *i.e.* the levels of intertwining of double helices in the topologically linked circles. The theta structures (replication intermediates) in *brackets* are diagrams of thetas in which the parental (*green*) DNA is nicked and therefore relaxed as histone-free DNA. The indicated multicatenanes such as *B5*, *C3*, *C5*, etc., are not depicted explicitly, given their highly "entangled" configurations as free DNA. Among the technical innovations in these 1980–1981 studies (3–5) was the discovery that hypertonic treatment of SV40-infected green monkey CV1 cells arrested replicated chromosomes at the stage of decatenation. During arrest, highly intertwined nicked (or gapped) A-type multicatenanes were eventually converted (via B-type intermediates) to the equally highly intertwined C-type multicatenanes (*C10–C30*), in which both circles were covalently closed and supercoiled (*thick dashed arrows* at the top). The supercoiled state stemmed from the presence of nucleosomes in the minichromosomes, prior to removal of histones for analyses of DNA topology. Upon release from the decatenation block, the highly intertwined C-type multicatenanes were decatenated to supercoiled monomers (*vertical dashed arrows*; "recovery pathway"). The actual paths taken during chromosome segregation (in the absence of decatenation arrest) include the entire matrix of transitions shown in the diagram. The relative rates of flow along specific paths depend on the rates of gap-filling DNA synthesis (converting a nicked or gapped circle into a covalently closed circle) *versus* the rates of decatenation of multiply intertwined chromosomes by topoisomerase II (3, 4).

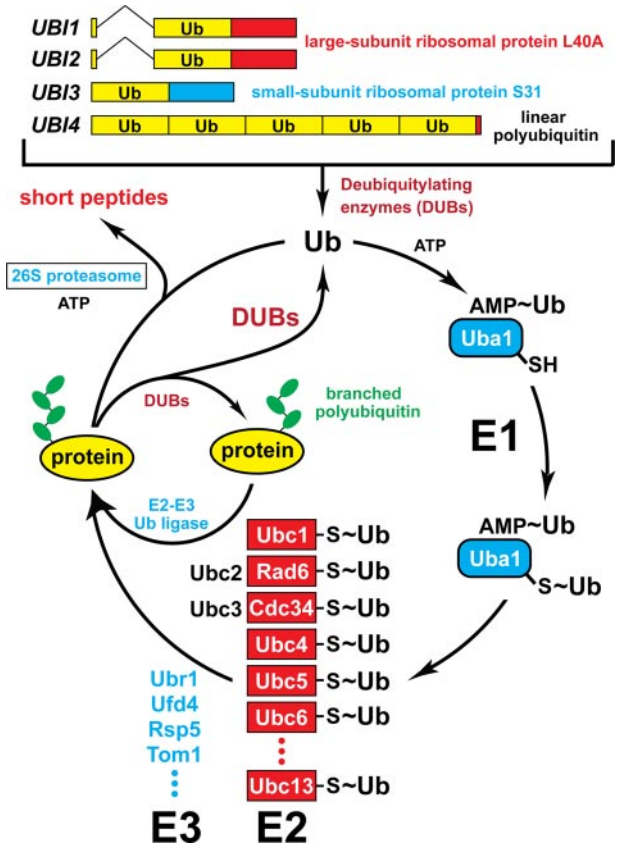

FIGURE 2. **The Ub system of the yeast *S. cerevisiae*.** The fundamental design of this system is conserved among eukaryotes. The yeast Ub genes *UBI1–UBI4*, two of which contain introns, encode fusions of Ub either to itself or to one of two ribosomal proteins. These fusions are cleaved by DUB enzymes, yielding mature Ub. Thioester bonds between Ub and the active-site Cys residues of Ub-specific enzymes are indicated (~). The conjugation of Ub to other proteins involves a preliminary ATP-dependent step in which the last residue of Ub (Gly^{76}) is joined, via a thioester bond, to a Cys residue of the E1 (Ub-activating) enzyme, encoded by *UBA1*. The activated Ub is transferred to a Cys residue in one of several Ub-conjugating (E2) enzymes, encoded by the *UBC* family genes, and from there to a Lys residue of an ultimate acceptor protein. E2 enzymes function as subunits of E2-E3 Ub ligase holoenzymes. The functions of E3 include the initial recognition of a substrate's degradation signal (degron) and the E2/E3-mediated formation of substrate-linked poly-Ub chains (*green ovals*). The names of some of the ~200 E3 enzymes of *S. cerevisiae* are indicated as well. A targeted ubiquitylated protein is processively degraded to short peptides by the ATP-dependent 26 S proteasome.

chains (15); the first physiological substrate of the Ub system (MATα2 repressor) (21, 22); the first nonproteolytic function of Ub (its role as a cotranslational chaperone) (17); and the subunit selectivity of Ub-mediated proteolysis, a fundamental capability of the Ub system that makes possible protein remodeling (19, 21). We also cloned the first Ub genes, discovering their divergent functions (8, 10, 11, 17), as well as the first genes for deubiquitylating (DUB) enzymes and Ub-activating enzyme (28–30). In 1990, we identified, cloned, and analyzed the first specific E3 Ub ligase, UBR1 (18). This advance opened up a particularly large field, as individual mammalian genomes are now known to encode at least a *thousand* (!) Ub ligases. The targeting of distinct degradation signals in cellular proteins by this immensity of E3 enzymes enables the

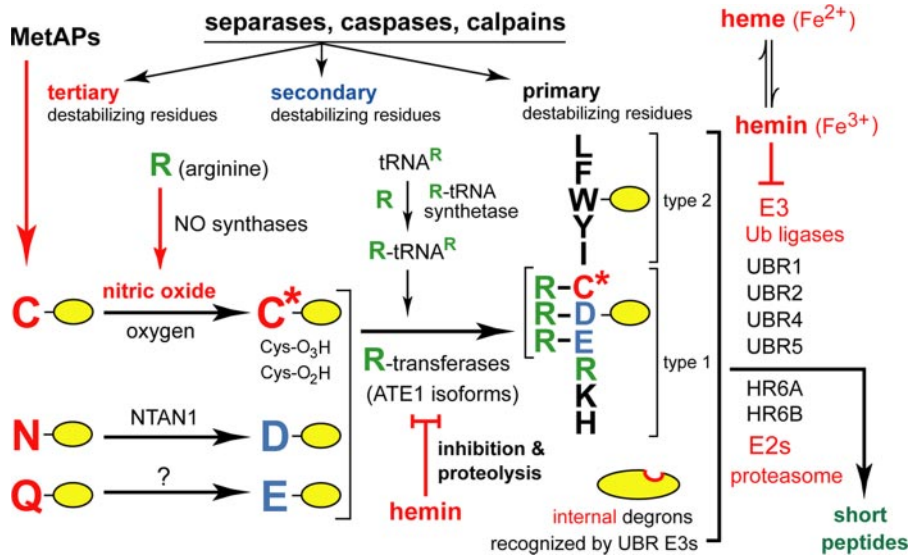

FIGURE 3. **The mammalian N-end rule pathway.** N-terminal residues are indicated by single-letter abbreviations for amino acids. *Yellow ovals* denote the rest of a protein substrate. A sign, above *hemin* in the middle of diagram, is a modified "down-regulation" sign that denotes, specifically, a down-regulation mediated, at least in part, by target's degradation. *C** denotes oxidized Cys, either Cys sulfinate or Cys sulfonate, produced in reactions mediated by NO, O_2, and their derivatives. Oxidized N-terminal Cys is arginylated by *ATE1*-encoded isoforms of R-transferase. Type 1 and 2 primary destabilizing N-terminal residues are recognized by the pathway's E3 Ub ligases, called N-recognins. Through their other substrate-binding sites, these E3 enzymes also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by the *larger oval*. As shown in the diagram, hemin (Fe^{3+}-heme) interacts not only with ATE1 (R-transferase) but with UBR family Ub ligases as well, and down-regulates at least some E3 enzymes of this family (89).

unprecedented functional reach of Ub-mediated pathways.

By revealing the fundamental and broad roles of the Ub system in cellular physiology, our biological studies of 1984–1990 (6–22) produced the overall discovery of the *physiological regulation by intracellular protein degradation*. Ramifications of this advance became the beginnings of several biomedical fields. This discovery has also transformed our understanding of the logic of biological circuits, as it became clear that the control through regulated protein degradation rivals, and often surpasses in significance, the classical regulation through transcription and translation.

What follows is a story of my lab's adventures, including the inventions of new biochemical and genetic methods. This account stems, in part, from previous descriptions of the early history of the Ub field (31, 32). Another antecedent is an interview I gave to Dr. Istvan Hargittai, a distinguished Hungarian chemist. It describes my life and science, including the early years in Moscow, the 1977 escape from the former Soviet Union, the essentially accidental hiring of me by MIT, and the work that ensued (33). The narrative below borrows from these sources and mentions our more recent contributions as well.

Beginnings

I grew up in a scientist's family, so my interest and later love for science were a case of "nature" and "nurture" together. My father, Yakov Varshavsky, who is now 91 and lives with my mother Mary in Utah (near my sister's family there), was a physical chemist in Moscow, where he developed methods for the industrial-scale production of heavy water. That work, a blend of fundamental and applied physical chemistry, was a part of Russia's atomic bomb project. In the 1950s, he became interested in DNA, joining other physicists and chemists who were leaving their fields at that time for the nascent world of molecular biology. I was born in 1946, a year after the end of World War II, in a country devastated by it and by the inefficiencies (let alone cruelties) of Stalin's dictatorship. Stalin died when I was 6. He was soon denounced by Khrushchev, a sidekick who clawed his way to the top and began a less bloody rule. In 1964, I was admitted as an undergraduate to the Chemistry Department of Moscow University. In 1970, I began working at Moscow's Institute of Molecular Biology, in the laboratory headed by Georgi Georgiev. We studied chromosomes and RNA, amid shortages of nearly everything, including good reagents, equipment, and contacts with Western researchers. What sustained me was youth, love of science, and ambition, a heady mix. It kept me working and hoping that things might improve, despite evidence to the contrary. The time was the mid-1970s, a couple of years away from a chance to escape it all. A convoluted path to that escape, which involved help from friends and a lot of luck, is described in Ref. 33.

I left Russia in the fall of 1977, and ended up in Boston/Cambridge, MA. A month later, I was a faculty member in the Biology Department of MIT, before I knew what exactly grants were (and before the colleagues who hired me became aware of that fact) (33). In Moscow, I studied chromosome structure and regulation of gene expression.

During my first year at MIT, I worked alone and continued research that began in Russia.

I used the SV40 viral minichromosomes isolated from virus-infected green monkey (CV1) cells as models of cellular chromosomes and tried to address the problem of nucleosome arrangement. (Nucleosomes, the repeating "subunits" of chromosomes that comprise two superhelical turns of DNA wrapped around the octamer of histones H2A, H2B, H3, and H4, were discovered in 1974 by Roger Kornberg (34).) Were the nucleosomes distributed in a pattern that was specific vis-á-vis DNA sequence? Or was their arrangement quasi-random, in addition to being dynamic? To reduce potential nucleosome "sliding" (nothing was known about it at the time), I cross-linked the isolated minichromosomes with formaldehyde, which formed both protein-protein and DNA-protein cross-links. The resulting structures were treated with restriction endonucleases that cut SV40 DNA either once or at multiple specific sites.

At first I learned little, but later saw that one site in the minichromosome was much more susceptible to cleavage than any other site. This "hypersensitive" site resided in a regulatory region that contained both the origin of replication and transcriptional promoters of SV40. Soon thereafter, an analogous experiment with the endonuclease HaeIII hit the jackpot: the entire ~400-bp regulatory region could be "excised" from the formaldehyde-fixed minichromosome as a fragment of *histone-free* DNA, in contrast to the rest of the structure, which was still an intramolecular aggregate (despite multiple HaeIII cuts in DNA), held together by formaldehyde-produced DNA-histone and histone-histone cross-links.

This and related advances yielded two insights. The regulatory region of the SV40 minichromosome was strikingly more exposed (more susceptible to endonuclease cuts) than the rest of the minichromosome; moreover, the nucleosomes (including histones) were either absent from the exposed region or in a configuration that precluded DNA-histone cross-links. Our first results were published in 1978 (1), and a more detailed account in 1979 (2). Two other groups, Carl Wu and Sarah Elgin at Harvard, and Walter Scott at the University of Florida, independently discovered nuclease-hypersensitive regions in chromosomes, using a different approach that involved (relatively) nonspecific nucleases such as DNase I. These discoveries became a major part of the modern understanding of chromosome organization, as later studies by many groups, including my lab, have shown that the exposed (nuclease-hypersensitive) regions, which allow access to DNA in the otherwise tightly coiled chromosomal fibers, are a universal feature of chromosomes at replication origins, transcriptional promoters, and other functionally important sites.

Discovery of the Multicatenane Pathway of Chromosome Segregation

One day in the fall of 1978, I was reading a paper on minichromosomes and noticed a faint "ladder" of bands in a pattern of electrophoretically fractionated SV40 DNA. The paper's authors did not comment on the ladder. At that time, Olof Sundin, a graduate student who recently joined the lab, began to study replication of the SV40 minichromosomes and saw what appeared to be a similar set of bands. Neither of us suspected that we were beginning a 3-year study that would lead, in 1980–1981, to a fundamental discovery: the first pathway of chromosome segregation (Fig. 1) (3–5).

Briefly, when a circular chromosome such as SV40 begins its replication, two replication forks run from the origin of replication in opposite directions, meeting halfway around the circle and leaving behind two daughter minichromosomes. Analogous processes take place during replication of cellular chromosomes, which are larger and linear, and contain multiple origins of replication, except that in this case, a replication fork meets a fork running "toward" it from an adjacent origin of replication. (In a mechanistically distinct but topologically equivalent model, it is the chromosomal fiber that moves, with replication forks being spatially fixed in the nucleus.) These pictures of chromosome replication had a difficulty that was not even recognized as a problem at that time: how do the two converging replication forks (large structures containing polymerases, helicases, and other proteins) replicate the last several hundreds of nucleotide pairs that the forks themselves occupy?

We discovered that before the replicated SV40 daughter chromosomes separate from each other to yield individual circles, they go through a remarkable topological dance. It begins with *multicatenated* minichromosomes, in which two relatively small (~5 kb pairs) circular chromatids (daughter circles) are intertwined around each more than 30 times! It was the set of SV40 multicatenanes that formed those faint electrophoretic ladders that initially attracted our attention. The multicatenation stems from the *in vivo* conversion, by advancing replication forks, of the Watson-Crick intertwinings between two single strands of the parental DNA double helix into the topologically equivalent (but geometrically distinct) intertwinings of two double helices of the daughter circles. This insight revealed, for the first time, *a pathway of chromo-*

some segregation (Fig. 1) (3–5). Multicatenanes were a new form of double-stranded DNA at the time, as only singly intertwined DNA circles were detected before 1980. It is possible that the multicatenanes we observed were formed not only at the termination of chromosome replication but during replication as well, a subtle and important issue that awaits further research.

We found that chromosome segregation *in vivo* was not a linear sequence of steps but a matrix of transitions that involved not only multicatenations and gradual decatenations but also the superimposed, quasi-independent sealing of gapped or nicked DNA duplexes in sister chromatids, so that the final products were separate circles of the daughter chromosomes containing covalently closed DNA (Fig. 1). As shown by Roger Kornberg in 1974 (34), the bulk of DNA *in vivo* is wrapped around histone octamers in the nucleosomes. Because of this and other structural confinements of DNA, chromosomes in which the DNA is nicked or gapped in living cells are conformationally similar to their covalently closed counterparts. In contrast, histone-free DNA circles *in vitro* are relaxed if they contain a nick or a single-stranded gap but supercoiled if they are covalently closed, because the latter form of DNA cannot dissipate its torsional strain through rotation of the double helix (Fig. 1). The disposition with linear chromosomes is fundamentally the same, except that the number of replication forks is larger than two. Also, the topologically isolated domains ("loops") of a linear chromosome are based on DNA-protein interactions, in contrast to DNA circularity in SV40. In addition to the discovery of the multicatenane segregation pathway, our 1980–1981 *Cell* papers (3, 4) introduced new two-dimensional electrophoretic methods for analyzing replication intermediates, including multicatenanes. These methods made the discovery possible and are still in use today.

At the time of this work, type II topoisomerases, which mediate the segregation of chromosomes, were a novelty, having been identified, in the form of DNA gyrase, by the laboratory of Martin Gellert in the late 1970s and characterized by his and other laboratories. One aspect of our contribution was that topoisomerase II enzymes were now expected to be essential for decatenation of the *multiply intertwined* chromosomal fibers that formed during chromosome replication and functioned as segregation intermediates (3–5). Genetic evidence that topoisomerase II was indeed required for separating chromosomes by the multicatenane pathway (Fig. 1) was produced around 1985 through a collaboration between the laboratories of David Botstein and James Wang (35), and independently by Mitsuhiro Yanagida (36) and Rolf Sternglanz (37). In 1982, Goto and Wang (38) showed that purified topoisomerase II could catalyze both decatenation and unknotting of duplex DNA rings. In 1984, Steck and Drlica (39) isolated a temperature-sensitive mutant of DNA gyrase in *Escherichia coli* and demonstrated a double-nucleoid chromosome segregation arrest of this mutant at nonpermissive temperature. These and other findings showed that the multicatenane segregation pathway (Fig. 1) was present and essential in all organisms, from bacteria to yeast and mammals.

In the 1981 paper on chromosome segregation, Sundin and I proposed that the multicatenane pathway acts on multiply intertwined sister chromatids in ways that are likely to be controlled *spatially*, *i.e. separately* at specific regions such as, for example, the centromere *versus* the arms of a chromosome, and *temporally* as well, *i.e.* at specific stages of mitosis and meiosis (4). Recent work (Refs. 40–48 and references therein), including the evidence for a *decatenation checkpoint*, strongly supports these early conjectures and has revealed several (still incompletely understood) interactions between the multicatenane pathway (Fig. 1) and other processes, including checkpoints that monitor the completion of specific steps in the cell cycle. As one would expect, the multicatenane pathway and its aberrations are relevant to human diseases and their therapies. For example, topoisomerase II inhibitors are prominent among anticancer drugs (49). BRCA1, a major tumor suppressor, plays a role in chromosome decatenation (50).

The multicatenane pathway of chromosome segregation (Fig. 1) formally suffices to account for the sister chromatids staying together until their multiple catenations have been resolved, stepwise, by topoisomerase II during mitosis or meiosis. Nevertheless, a different, complementary system was identified during the 1990s that achieves a similar topological purpose through a distinct mechanism. The second segregation pathway, mediated by proteins termed cohesins, was discovered and analyzed by several groups, particularly those of Douglas Koshland, Kim Nasmyth, Mitsuhiro Yanagida, and Tatsuyo Hirano, with contributions by other labs as well. These findings included the identification of multisubunit cohesins, which were later shown to form circles around replicating chromosomes (Refs. 51–55 and references therein). In 1999, Frank Uhlmann, Kim Nasmyth, and their colleagues in the Nasmyth laboratory discovered a critical aspect of the cohesin pathway: a protease, termed separase, cleaves SCC1, a specific subunit of cohesin, and thereby allows the separation of sister chromatids (56), provided that their

multiple intertwinings had been resolved by the multicatenane pathway (Fig. 1). This brief description of cohesin mechanisms does not do justice to their intricate design and regulation (for example, the activation of separase is controlled by a specific pathway of the Ub system (51–55)). There is profound complementarity between the multicatenane-mediated (Fig. 1) and cohesin-mediated pathways of chromosome segregation. Either one of the two pathways provides mechanisms for the adhesion and release of sister chromatids, yet both pathways are essential. It remains to be determined, in functional and mechanistic detail, how the two segregation pathways interact. This is an area of intense research, and much will be learned in the coming years.

Our Ub studies, which began in 1978, eventually supplanted all other work in the laboratory. With reluctance and regret, I stopped working on chromosome segregation in 1983 and did not expect to return to that endeavor, which grew from our elucidation of the multicatenane segregation pathway. But fate held a surprise. In 1999, Hai Rao (then a postdoctoral student) and I saw, in an article by the Nasmyth lab (56), that the C-terminal fragment of the separase-cleaved SCC1 subunit of cohesin bore an N-terminal arginine, which our previous work had shown to be a degradation signal in substrates of the Ub-dependent N-end rule pathway (Fig. 3) (57). Rao and I decided to determine whether the separase-produced fragment of cohesin was actually short-lived *in vivo* and, if so, whether its degradation was functionally important. By 2001, in collaboration with Uhlmann and Nasmyth, we demonstrated that the N-end rule pathway targeted cohesin's fragment for degradation. This degradation was shown to be required for the proper functioning of cohesin machinery and the high fidelity of chromosome segregation, thus identifying one major function of the N-end rule pathway (58).

In summary, my laboratory's studies of chromosome segregation underlie the understanding of this fundamental process at three levels: (i) through the discovery, in 1980–1981, of the multicatenane pathway of chromosome segregation (Fig. 1) (3–5); (ii) through the genetically based 1984–1988 discovery, in both yeast and mammals, that the Ub system is an essential part of the cell cycle (see below) (6, 13); and (iii) through the discovery, in 2001, that the degradation of the separase-produced fragment of cohesin by the N-end rule pathway is required for high fidelity of chromosome segregation (58).

Recent work by Dr. Jianmin Zhou and colleagues in my lab, in a collaboration with Dr. Debananda Pati (University of Texas, Houston), has shown that the function of the N-end rule pathway in high fidelity chromosome segregation, first observed with the yeast *Saccharomyces cerevisiae* (58), is relevant to multicellular eukaryotes as well (J. Zhou, D. Pati, and A. Varshavsky, unpublished data). We employed mouse strains previously constructed in the lab by Dr. Yong Tae Kwon and colleagues (59) that lacked the *ATE1*-encoded Arg-tRNA-protein transferase (R-transferase). The latter is a component of the N-end rule pathway that arginylates the N-terminal Asp, Glu, or (oxidized) Cys residues of N-end rule substrates, thereby making them "recognizable" by Ub ligases of this pathway (Fig. 3). The absence of R-transferase is lethal in mice: $ATE1^{-/-}$ embryos die in midgestation, with cardiovascular and other defects (59). In both $ATE1^{-/-}$ embryos and $ATE1^{-/-}$ fibroblasts, the normally short-lived (separase-produced) fragment of the RAD21/SCC1 subunit of cohesin was shown to become a long-lived protein (J. Zhou, D. Pati, and A. Varshavsky, unpublished data). This result is in agreement with the inference, from findings by the laboratory of Jan-Michael Peters (60), that in humans, mice, and other mammals, the cleavage of RAD21/SCC1 by separase would be expected to produce fragments that bear N-terminal Glu, a substrate of R-transferase (Fig. 3).

Fittingly, $ATE1^{-/-}$ fibroblasts exhibit much higher chromosome instability than their $ATE1^{+/+}$ counterparts (J. Zhou, D. Pati, and A. Varshavsky, unpublished data), a phenotype similar to that of an *S. cerevisiae* mutant that lacks the N-end rule pathway (58). The identity of the residue that becomes the N-terminal residue upon the cleavage of cohesin by separase is not conserved in evolution. For example, this (eventually N-terminal) residue is Arg in *S. cerevisiae* but Glu in mammals. Remarkably, however, in all eukaryotes examined, this residue is a *destabilizing* one in the N-end rule (Fig. 3), indicating the importance of maintaining, during evolution, a short *in vivo* half-life of the cohesin fragment. Because this fragment was found to interact with the rest of the cohesin complex (58), one explanation of the demonstrated importance of "proactively" destroying the cohesin fragment (presumably directly on chromosomes) is that its spontaneous dissociation from the rest of cohesin may be too slow for the optimal kinetics of cohesin functioning.

A decade before our findings with cohesin (58), Erica Johnson (then a graduate student), David Gonda (then a postdoctoral student), and I discovered that the N-end rule pathway can destroy just one subunit of an oligomeric protein while sparing the other subunits (19). The subunit-selective degradation of cohesin's fragment is the first "physiological" example of *protein remodeling* by the N-end rule pathway. (Its subunit selectivity was discov-

ered in 1990 using engineered N-end rule substrates (19).) This function of the N-end rule pathway involves oligomeric proteins (including cohesins) that are conditionally cleaved by nonprocessive proteases such as separases, caspases, and calpains. Any cleaved subunits with C-terminal fragments that bear destabilizing N-terminal residues (Fig. 3) would then be targeted by the N-end rule pathway for processive degradation in a subunit-selective manner, *i.e.* modifying an oligomeric protein without destroying it. A number of previously described conditional cleavages of oligomeric proteins remain to be examined for a possible involvement of protein remodeling by the N-end rule pathway.

Discovery of Regulation by Protein Degradation and Its Major Roles in Cellular Physiology

I began working at MIT in the fall of 1977, shortly after leaving the former Soviet Union. The libraries, which I loved in Russia, were just as quiet and pleasant in Cambridge as in Moscow, and a library at MIT soon became my second home. Reading there, I came across a curious paper by Ira Goldknopf and Harris Busch (61). They found a DNA-associated protein that had one C terminus but two N termini, an unprecedented structure. The short arm of that Y-shaped protein was joined, through its C terminus, to an internal Lys residue of histone H2A, a component of nucleosomes. The short arm was soon shown by others to be Ub, a 76-residue protein of unknown function that had been identified (as a free protein) in 1975.

I became interested in that first Ub conjugate, Ub-H2A. In 1975, when I was still in Russia, we developed a technique for fractionation of nucleosomes that was based on gel electrophoresis of DNA-protein complexes at a low ionic strength (62, 63). At MIT, Louis Levinger (then a postdoctoral student) and I advanced this method by adding electrophoresis in the second dimension (64, 65). A first-dimension gel separated mononucleosomes into three subspecies. Besides an ~150-bp fragment of DNA and histones H2B, H3, and H4, these subspecies contained two molecules of histone H2A, one molecule each of H2A and Ub-H2A, and two molecules of Ub-H2A, respectively (66). Fractionated nucleosomes were dissociated *in situ* and electrophoresed in the second dimension through an SDS-containing gel, followed by Southern hybridization with DNA probes (64). In 1982, we applied this new technique to *Drosophila* nucleosomes and discovered that a transcribed gene such as *HSP70* was greatly enriched in nucleosomes that contained one or two molecules of Ub-H2A, whereas the centromeric heterochromatin (specifically its regions containing the 1.688 satellite DNA) was devoid of ubiquitylated nucleosomes (65).

These 1982 findings, two years prior to our genetic papers that initiated the biological understanding of the Ub system (6–22), were the first evidence bearing on the physiological functions of both Ub and modified histones. Years later, in the current field of histone modifications, ubiquitylated histones H2A and H2B are a part of fascinating patterns that include not only ubiquitylation but also acetylation, methylation, sumoylation, ADP-ribosylation, and citrullination of specific histones. All or most of these changes are reversible *in vivo*. Histone modifications are "read" by chromatin-associated proteins of many kinds, including enzymes that modify histones as a function of their previous modification states and specific locations in chromosomes. Our mapping of *Drosophila* nucleosomes 26 years ago and the conclusion (based on *HSP70 versus* heterochromatin patterns) that ubiquitylation of histones plays a role in transcriptional regulation (65) are in agreement with insights by several recent studies (Ref. 67 and references therein).

In the late 1970s, Avram Hershko, his graduate student Aaron Ciechanover, and their colleagues in the Hershko laboratory at the Technion were studying ATP-dependent protein degradation in extracts from rabbit reticulocytes. In 1978, they discovered that a small protein, termed APF-1 (ATP-dependent proteolytic factor 1), was covalently conjugated to proteins before their degradation in the extract (23). In 1980, they suggested that APF-1 linked to a protein served as a signal for a downstream protease (24) and began dissecting the enzymology of APF-1 conjugation. In 1981–1984, Hershko and co-workers identified a set of three enzymes involved, termed E1 (Ub-activating enzyme), E2 (Ub carrier protein or Ub-conjugating enzyme), and E3 (an accessory component that appeared to confer specificity on E2) (25, 26). A large ATP-dependent protease that mediates the processive degradation of Ub-protein conjugates (27) was characterized by several laboratories much later, in the 1990s, and is now called the 26 S proteasome (for recent reviews of the proteasome, see Refs. 68 and 69).

Although our studies of Ub began in 1978, I did not know about the APF-1 results of Hershko and co-workers, as the identity of APF-1 and Ub was unknown, at that time, to them as well. This changed in 1980, when APF-1 and Ub were shown to be the same protein (70) by Keith Wilkinson, Michael Urban, and Arthur Haas, who worked in the laboratory of Irwin Rose, a collaborator of Hershko during his stays at the Philadelphia Fox Chase Cancer Center. When I read the 1980 paper of Hershko *et al.* (24) that described the APF-1 conjugation to proteins in reticulocyte extracts and the paper of Wilkinson *et al.* (70) that

described the identity of APF-1 and Ub, two previously independent realms, protein degradation and ubiquitylated nucleosomes, came together for me, suggesting a regulatory system of great complexity and broad, still to be discovered biological functions. I decided to find genetic approaches to the entire problem, because a system of such complexity was unlikely to be understood through biochemistry alone. In 1980, reverse-genetic techniques were about to become feasible with *S. cerevisiae*, but were still a decade away in mammalian genetics. I kept reading, as widely as I could. Near the end of 1980, I came across a brief paper by Masa-atsu Yamada and colleagues that described a conditionally lethal, temperature-sensitive mouse cell line called ts85. The researchers showed that a specific nuclear protein disappeared from ts85 cells at elevated temperatures and suggested that this protein may be Ub-H2A (71). Glancing at their data, I had to calm down to continue reading, being virtually certain that the protein was Ub-H2A: in the preceding two years, we had learned much about electrophoretic properties of this Ub conjugate. On the hunch that mouse ts85 cells might carry a mutation in a component of the Ub system, I wrote to Yamada and received from him, in 1981, both ts85 and the parental ("wild-type") cell line.

Daniel Finley (then a graduate student) joined my lab at that time to study regulation of gene expression. He did not need much convincing to switch to ts85 cells. A few months into the project, Finley and I made the critical observation that Ub conjugation in an extract from ts85 cells was temperature-sensitive, in contrast to an extract from parental cells. While this was going on, I met Aaron Ciechanover, who came from the Hershko laboratory in Israel for a postdoctoral stint at MIT, and was studying growth factor receptors in the laboratory of Harvey Lodish. Presuming that Ciechanover was still interested in Ub (very few people were), I told him about our results with ts85 cells and invited him to join, part-time, Finley and me to complete the ts85 study. Ciechanover joined us; the work continued; and in 1984, we published two papers that described two logically independent discoveries (6, 7):

1) Mouse ts85 cells have a temperature-sensitive (*ts*) Ub-activating (E1) enzyme.

2) In contrast to parental cells, these cells *stop degrading the bulk of their short-lived proteins at nonpermissive temperature*.

This was the first evidence that Ub conjugation was *required* for protein degradation *in vivo*. (The earlier studies by Hershko and co-workers were carried out with extracts from reticulocytes.) Our results (6, 7) also explained the disappearance of Ub-H2A from ts85 cells at nonpermissive temperature: thermal inactivation of the mutant (*ts*) Ub-activating enzyme in these cells stops the *de novo* formation of Ub-H2A, whereas pre-existing Ub-H2A is gradually deubiquitylated by DUB enzymes. (In a paper published in 1983 (72), Yamada and co-workers confirmed their initial conjecture about the identity of Ub-H2A as a protein that disappeared from ts85 cells, but pinpointed neither the cause of this effect (a *ts* E1 enzyme), nor, most importantly, the crucial property of these cells: the inability, at nonpermissive temperature, to degrade proteins that are normally short-lived.)

Our discoveries with ts85 cells (6, 7) also indicated that Ub conjugation was essential for cell viability, the first hint of the enormous, many-sided biological importance of the Ub system. In addition, ts85 cells were preferentially arrested in the G_2 phase of the cell cycle, and the synthesis of heat stress proteins was strongly induced in these cells at the nonpermissive temperature (in contrast to parental cells), suggesting that Ub conjugation was involved in cell cycle progression and stress responses (6, 7). Although these conclusions about *specific* functions of the Ub system proved correct, they were still preliminary in 1984, given the limitations of mammalian somatic cell genetics. Therefore, we decided to turn to *S. cerevisiae*, where such issues could be addressed more rigorously. As described below, our 1987–1988 discoveries with the yeast RAD6, CDC34, and UBI4 proteins (10, 12, 13) produced the first specific and definitive evidence that the Ub system is of central importance for both cell cycle progression and stress responses, thus validating the indirect evidence with ts85 cells. In 1983, the laboratory of Tim Hunt, a pioneer in studies of the cell cycle, identified unusual proteins in sea urchin and clam embryos. These proteins, which he called cyclins, were degraded at the end of mitosis (73). We suggested in 1984 that cyclins were destroyed by the Ub system (6, 7), a hypothesis shown to be correct in 1991 by Michael Glotzer, Andrew Murray, and Marc Kirschner (74) and independently by Hershko *et al.* (75).

It may be helpful to place the above advances in historical context. Despite some evidence to the contrary, until the 1984 *Cell* papers about mouse ts85 cells (6, 7) and our subsequent genetic discoveries with *S. cerevisiae*, the prevailing view was that intracellular protein degradation was a simple and even mundane process, serving largely to dispose of "aged" or otherwise damaged proteins. Cellular regulation was believed to be a separate affair, mediated primarily by repressors and activators of gene expression, which were assumed, often tacitly, to be long-lived. Among the reasons for this lopsided perspective was the difficulty in connecting the long-recognized proteolytic

system in the lysosomes to specific pathways of intracellular regulation. Thus, most people studying gene expression in the 1960s and 1970s assumed that the regulatory circuits they cared about did not involve short-lived proteins. As we now know, just the opposite proved true, especially in eukaryotes, where most regulators of transcription are short-lived proteins whose levels in a cell are determined at least as much by the rates of their Ub-dependent destruction as by the rates of their synthesis. Given the presumed stability of transcriptional regulators, it is ironic that the first physiological (as distinguished from artificial) substrate of the Ub system was MATα2, a transcriptional regulator that Mark Hochstrasser (then a postdoctoral student) and I demonstrated in 1990 to be a short-lived protein *in vivo* (21).

In addition to having been a breakthrough that indicated the requirement of the Ub system for intracellular proteolysis, the ts85 papers were also the first to address the *in vivo* workings of this system. In 2004, this pair of papers (6, 7) was selected for republication by the editors of *Cell* as being among the most important papers that had been published in its 30-year history. In a review accompanying republication, Cecile Pickart, one of the early pioneers in the Ub field, summed up the papers' contribution: *"The two papers . . . led to a new worldview: not only was the ubiquitin/proteasome pathway a major proteolytic mechanism in the average mammalian cell, but it was also likely to regulate cell cycle progression. These conclusions are so well accepted today that it is difficult to appreciate the magnitude of their impact at the time the two papers appeared"* (76).

In 1983, even before the ts85 work was completed, Finley, Engin Özkaynak (then a postdoctoral student), and I, together with other colleagues in the lab, began a systematic analysis of the Ub system in the genetically tractable *S. cerevisiae* (Fig. 2), a project that soon expanded to occupy the entire laboratory. Between 1983 and 1990, this work revealed the first specific biological functions of Ub conjugation and discovered the first degradation signals in short-lived proteins. Briefly mentioned below are key advances of those early years that established the physiological fundamentals of the Ub field.

Polyubiquitin Gene and Resistance to Stress—In 1984, Özkaynak, Finley, and I cloned the first Ub gene. Unexpectedly, it encoded not a single Ub moiety but a head-to-tail poly-Ub precursor protein (8). By 1987, we showed that this gene, *UBI4*, was induced by a variety of stresses and that a deletion of *UBI4* resulted in *hypersensitivity* to these stresses (*ubi4*Δ cells were viable under normal conditions, as they still contained other Ub genes) (10, 11). These genetically based results validated and deepened our earlier indirect evidence with mouse ts85 cells (6), thus identifying stress responses as one important and broad function of the Ub system.

RAD6, the Ubiquitin System, and DNA Repair—In 1987, Stefan Jentsch (then a postdoctoral student), John McGrath (then a graduate student), and I discovered that RAD6, a protein known to yeast geneticists as an essential component of DNA repair pathways, is a Ub-conjugating (E2) enzyme, *the first Ub-conjugating enzyme with a specific physiological function* (12). In addition to learning that the Ub system is required for DNA repair, we noticed that the sequence of RAD6 was weakly similar to that of CDC34, an essential cell cycle regulator (of unknown biochemical activity) that had been defined genetically in *S. cerevisiae* by Lee Hartwell and had been shown to be required for the G_1-S phase transition in the cell cycle.

CDC34, the Ubiquitin System, and the Cell Cycle—To verify the exciting possibility that CDC34 may be a distinct Ub-conjugating enzyme, Jentsch, McGrath, and I collaborated with Mark Goebl in the laboratory of Breck Byers (University of Washington, Seattle). In 1988, this collaboration demonstrated that CDC34 is indeed a specific Ub-conjugating (E2) enzyme (13), thereby producing the first rigorous proof that *the Ub system is an integral part of the cell cycle*. (This function was suggested but not proven by our 1984 study with mouse ts85 cells (6, 7).)

The critical discovery with CDC34 (13) gave rise to a major field, as it became clear (in general terms right away and in detail over the next decade) that proteolysis by the Ub system underlies *biological periodicity*, from the cell cycle to circadian rhythm oscillators. Other connections between the Ub system and the cell cycle, including the 1991 demonstration by the Kirschner and Hershko laboratories that cyclins are degraded by the Ub system (74, 75), were established after the 1987–1988 discoveries with RAD6 and CDC34 (12, 13).

Degradation Signals in Short-lived Proteins—Through the 1986 invention of the Ub fusion technique, Andreas Bachmair (then a postdoctoral student), Finley, and I discovered specific *degradation signals* that target proteins for Ub conjugation and proteolysis, including the set of signals that give rise to the N-end rule of protein degradation (9). By identifying the first "primary" degradation signals in short-lived proteins, this advance solved the fundamental problem of *selectivity* in the Ub system. Primary degradation signals are specific features of proteins that make them targets for ubiquitylation and subsequent degradation. (In most settings, Ub itself is a "secondary" signal for proteolysis in that Ub is conjugated to a protein that

contains a primary degradation signal.) The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (9, 14). The underlying N-end rule pathway (Fig. 3) was the first of two specific pathways of the Ub system to be discovered, years before a multitude of other Ub-mediated pathways came into view.

The Ubiquitin Fusion Degradation Pathway—The second pathway of the Ub system, which we termed the Ub fusion degradation (UFD) pathway, was discovered also in 1986, simultaneously with the N-end rule (9). The UFD pathway was subsequently characterized by Erica Johnson, Bonnie Bartel (then graduate students), and myself (77, 78). Substrates of this pathway include a subset of Ub fusions in which the N-terminal Ub moiety cannot be cleaved off *in vivo* by DUB enzymes either at all or rapidly enough (9, 77, 78). An impediment to cleavage by DUB enzymes can be a Pro residue at the Ub-protein junction (9) or an alteration of Ub structure. Retention of the N-terminal Ub moiety was shown to result in the recognition of this moiety as a primary degradation signal by Ub ligases of the UFD pathway (77, 78). Detailed studies of this pathway, particularly by Stefan Jentsch and colleagues (*e.g.* Ref. 79), are now an active and important field.

The N-end Rule Pathway in Prokaryotes—The functional and mechanistic understanding of the N-end rule pathway (Fig. 3) eventually became a major focus of my laboratory. In 1991, John Tobias (then a graduate student), Thomas Shrader (then a postdoctoral student), and I extended the Ub fusion technique (9) to prokaryotes. We discovered that although *E. coli* lacked the Ub system, it still had the N-end rule pathway (80). In contrast to many physiological functions of eukaryotic N-end rule pathways that were identified over the last decade (see below), the physiology of prokaryotic N-end rule pathways remains a fascinating enigma.

Terminology for Degradation Signals—The term "degron," shorthand for "degradation signal," was suggested in 1991 and gradually became a standard notation (81). N-terminal degrons of the N-end rule pathway (Fig. 3) are called N-degrons (81). One advantage of degron (a mnemonic and compact term) is that it can denote any specific degradation signal through the use of distinct prefixes, superscripts, or subscripts.

Determinants of Degradation Signals—In 1989, Bachmair and I carried out the first dissection of a degradation signal and found that an N-degron consisted of three determinants: a *destabilizing* N-terminal residue of a protein substrate (Fig. 3), at least one of the substrate's internal Lys residues (the site of formation of a poly-Ub chain), and a nearby conformationally disordered region (14). A decade later, Tetsuro Suzuki (then a postdoctoral student) and I extended this analysis by identifying N-degrons through a novel genetic screen in a two-residue (Lys-Asn) sequence space. We found that N-degrons can consist of compositionally simple sequences that contain just Asn and Lys (82).

The multideterminant organization of N-degrons recurs in other (subsequently identified) degradation signals. Key differences among degrons include their first determinant, which is recognized by a specific E3 Ub ligase. In an N-degron, this determinant is a destabilizing N-terminal residue (Fig. 3), whereas in another degron it can be, for example, an internal sequence motif. By revealing *the basis of specificity* of intracellular protein degradation, the 1986 discovery of the UFD degron and N-degrons (9) has founded the field of degradation signals. This is a major arena nowadays, because a number of distinct, often conditional degrons in the great multitude of cellular proteins are targeted by specific and exquisitely controlled E3 enzymes of the Ub system.

N-terminal Arginylation as Part of the N-end Rule Pathway—In the early 1970s, Kaiji *et al.* (83) and Soffer (84) identified a new reaction, the N-terminal *arginylation* of proteins by the enzyme R-transferase. In our 1986 paper about degradation signals in short-lived proteins, we proposed that R-transferase and N-terminal arginylation are, most likely, a part of the N-end rule pathway (Fig. 3) (9). This suggestion pinpointed the previously enigmatic biological function of R-transferases, identifying them as components of a proteolytic system (9). Both our later work (16) and a 1987 study by Ferber and Ciechanover (85) confirmed the above conjecture (9). In 1990, Mordechai Choder (then a postdoctoral student) collaborated with the laboratory of André Goffeau (University of Louvain, Louvain, Belgium) to identify and clone the yeast *ATE1* gene, encoding R-transferase (20). More recent work with mammalian R-transferases (59, 86–90) is described below.

A Nonproteolytic Function of Ubiquitin—In 1987, Özkaynak, Finley, and I cloned *S. cerevisiae UBI1–UBI3*, the Ub genes other than the (stress-specific) poly-Ub gene *UBI4* (11). In 1989, Finley, Bartel, and I identified the nature of non-Ub parts of the UBI1–UBI3 proteins and discovered a new function of Ub in this setting. *UBI1–UBI3* were found to encode fusions of Ub to one protein of the large ribosomal subunit and one protein of the small ribosomal subunit, an arrangement conserved from yeast to mammals (17). Remarkably, genetic analyses with precursors of ribosomal proteins encoded by *UBI1–UBI3* indicated that the presence of Ub in front of a specific

ribosomal protein, despite being transient *in vivo* (Ub is removed by DUB enzymes), is *required* for efficient biogenesis of ribosomes (17). Apart from discovering a specific role of the Ub system in the translation apparatus and protein synthesis, these results showed that in certain contexts Ub acts not as a degradation signal but as a cotranslational chaperone. This first nonproteolytic function of Ub (17) appeared to be an exceptional case until years later, when Linda Hicke and Howard Riezman demonstrated that ubiquitylation of an integral membrane protein signals its endocytosis (91). Ub is now recognized to have numerous nonproteolytic functions.

Specific Polyubiquitin Chains—In 1989, using new approaches that included two-dimensional protein mapping assays, Vincent Chau and other colleagues in my laboratory demonstrated that Ub-protein conjugation *in vivo* produces a substrate-linked poly-Ub chain of a specific topology, with the isopeptide bonds between adjacent Ub moieties through Lys^{48} of Ub (15). In contrast to linear precursors of Ub that are encoded by poly-Ub genes, a posttranslationally produced poly-Ub chain is based on repeating, branch-generating isopeptide bonds between the adjacent Ub moieties in a chain. This study also demonstrated that a poly-Ub chain linked to a substrate is required for the substrate's degradation (15). We suggested that a major function of poly-Ub chains is to bind targeted substrates to the proteasome (15), a hypothesis confirmed by others, in part through the identification of poly-Ub-binding proteins as components of the 26 S proteasome. Later work has shown that poly-Ub chains can also be of different "local" topologies, with adjacent Ub moieties in a chain linked through Lys residues other than Lys^{48}. Most of the "alternative" poly-Ub chains have nonproteolytic functions. The 1989 discovery of specific poly-Ub chains (15) illuminated the mechanistic role of Ub in proteolysis and was the beginning of another major field in Ub studies.

Physiological Substrate of the Ubiquitin System—In 1990, Mark Hochstrasser and I identified the first short-lived *physiological substrate* of the Ub system, the transcriptional repressor MATα2, and delineated its degradation signals (21, 22). The 1990–1991 MATα2 papers were also the first direct evidence for the involvement of the Ub system in transcriptional regulation. Many specific functions of Ub-dependent processes in transcription and its control have been discovered since 1990. The MATα2 work (21, 22) was also the beginning of insightful independent studies by Hochstrasser that employed MATα2 both for its own sake and as a fulcrum to dissect specific aspects of the Ub system. A mitotic cyclin was the second physiological substrate of this system to be characterized, in 1991, by the Kirschner and Hershko laboratories (74, 75). Today, we know hundreds of such substrates. Moreover, it has become clear that most cellular proteins, including those that travel through or reside in membrane-enclosed compartments, can become physiological substrates of the Ub system at certain stages of their *in vivo* existence.

Subunit Selectivity of Proteolysis—The above section about multicatenanes already introduced the 1990 discovery by Johnson, Gonda, and myself of *subunit selectivity* in the Ub-mediated protein degradation (19). This fundamental capability of the Ub system underlies *protein remodeling, i.e.* the replacement of specific subunits in oligomeric complexes of innumerable kinds. Also in 1990, Hochstrasser and I detected subunit selectivity in the degradation of the MATα2 repressor, the first physiological substrate of the Ub system (21). Subunit-selective proteolysis is a critical property of this system, a feature both powerful and flexible in that it enables protein degradation to be wielded as an instrument of protein remodeling for either positive or negative regulation. Through its ability to make protein machines compositionally dynamic, the subunit selectivity of degradation (19, 21) literally enables the vast physiology of Ub pathways. Some better known examples of subunit selectivity are the degradation of cyclin subunits in the CDK kinases (a process that drives the cell cycle oscillator), the degradation of the inhibitory IκB subunit in the IκB·NF-κB complex (a transition that activates NF-κB, a major transcriptional regulator), and the degradation of a separase-produced cohesin fragment by the N-end rule pathway, a step that underlies the high fidelity of chromosome segregation (58).

Cloning and Analysis of Specific E3 Ubiquitin Ligase—In 1990, Bartel, Ingrid Wünning (then a postdoctoral student), and I employed genetic and biochemical approaches to identify, clone, and analyze *the first specific E3 Ub ligase,* termed UBR1 (18). This multifunctional 225-kDa protein is the sole E3 of the *S. cerevisiae* N-end rule pathway. (The term "Ub ligase" denotes either an E2–E3 holoenzyme or its E3 component.) This advance (18) opened up a particularly large field, as individual mammalian genomes are now known to encode at least a *thousand* distinct Ub ligases. Through their regulated targeting of specific degrons in conditionally or constitutively short-lived proteins, these E3 enzymes underlie the enormous functional reach of the Ub system.

In summary, the complementary discoveries in the 1980s by Hershko's and my laboratories revealed three sets of previously unknown facts.

1) ATP-dependent protein degradation involves a new protein modification, Ub conjugation, which is mediated by specific enzymes, termed E1, E2, and E3 (23–26, 31).

2) The selectivity of Ub conjugation is determined by specific multipartite degradation signals (degrons) in short-lived proteins, including degrons that give rise to the N-end rule (9, 14, 16, 18).

3) Ub-dependent processes play a strikingly broad, previously unsuspected part in cellular physiology, primarily by controlling the *in vivo* levels of specific proteins. Ub conjugation was shown by us to be required for protein degradation *in vivo* (6, 7), for cell viability, and also, specifically, for the cell cycle (6, 13), DNA repair (12), protein synthesis (17), transcriptional regulation (21), and stress responses (8, 10). Ub-dependent proteolysis was also discovered to involve an essential, substrate-linked poly-Ub chain of unique topology (15). In addition, the Ub system was found to possess the critical property of subunit selectivity, *i.e.* the ability to destroy a specific subunit of oligomeric protein while leaving the rest of the protein intact and thereby making possible protein remodeling (19, 21).

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Our function-based studies in the 1980s yielded the overall discovery of the *physiological regulation by intracellular protein degradation*. The complementary "chemical" and "biological" insights by Hershko's and my laboratories caused a massive expansion of the Ub field in the 1990s. It became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. Because perturbations of the cell cycle, DNA repair and stress response pathways are hallmarks of malignant transformation, our 1987–1988 discoveries with CDC34, RAD6, and UBI4 (10, 12, 13) opened up Ub studies in cancer research as well.

The work by many excellent laboratories that entered the Ub field over the last 15–20 years has uncovered, among other things, the existence of more than ten *Ub-like* conjugation pathways. These pathways involve Ub-like proteins (called SUMO, NEDD8, etc.) and specific enzymes that are similar to the enzymes of Ub conjugation. Ub-like pathways have a number of functions, mostly (though not exclusively) nonproteolytic ones. These pathways interact with the Ub system, the largest one and the first to be characterized. The resulting "meta-set" of the Ub and Ub-like systems has an astonishing functional range and mechanistic complexity (for recent reviews, see Refs. 68, 69, and 92–101).

Our discovery of the biological fundamentals of the Ub system (6–22) has yielded the modern paradigm of cellular physiology, in which regulated proteolysis is of central importance. These advances, together with later studies by many groups, have shown that *the control through regulated protein degradation rivals, and often surpasses in significance, the classical regulation through transcription and translation*. This changed understanding of the logic of biological circuits is of importance for medicine, given the immense functional range of the Ub system and the multitude of ways in which Ub-mediated processes can malfunction in disease or in the course of aging, from cancer and neurodegenerative syndromes to perturbations of immunity and many other illnesses, including birth defects. A number of pharmaceutical companies are developing compounds that target specific components of the Ub system. The fruits of their labors have already become or will soon become clinically useful drugs. Efforts in this arena may yield not only "conventional" inhibitors or activators of enzymes but also more sophisticated drugs that would direct the Ub system to target, destroy, and thereby down-regulate any specific protein. I feel privileged having been able to contribute to the birth of this field two decades ago and to partake in its later development. The dynamism and surprises of this endeavor remain undiminished even today.

Multidrug Transporters, Gene Amplification, and AT-DNA-binding Proteins

MDR1, a Multidrug Transporter—In 1984, Igor Roninson (then a postdoctoral student) and I collaborated with the laboratory of David Housman at MIT to identify and clone the first mammalian gene that encoded a major multidrug transporter, termed MDR1 (102). This transmembrane protein was the first member of a large family of transporters, some of which are induced by stress. One function of MDR-type transporters is to mediate the export of toxic (largely hydrophobic) compounds. Increased activity and/or abundance of multidrug transporters in cancer cells is a major cause of drug resistance in cancer therapy. By making possible both the dissection of MDR1 and the cloning of other MDR-type transporters, the 1984 breakthrough (102) gave rise to a large field in cancer research.

Induced Gene Amplification—Gene amplification in mammalian cells was discovered in 1978 by Frederic Alt and Robert Schimke. In 1981, I found that growth factors such as hormones or tumor promoters can greatly increase the *frequency* of gene amplification under conditions of cytotoxic stress (103). (The lab was small then, and I could still work at the bench.) A follow-up study in 1983 with James Barsoum (then a graduate student) confirmed and extended these insights (104). Analogous gene ampli-

fication events contribute to rapid evolution of cancer cells in a tumor and to the emergence of drug-resistant cells during anticancer therapy. Thea Tlsty and Robert Schimke independently demonstrated, also in 1981, the same phenomenon of induced (accelerated) gene amplification.

High Mobility Group (HMG) I/Y, Datin, and D1 as AT-DNA-binding Proteins—In the 1982 study of *Drosophila* nucleosomes that revealed their preferential ubiquitylation in a transcribed region such as the *HSP70* gene (65), we noticed that nucleosomes containing an ~50-kDa protein called D1 (105) were entirely devoid of Ub-H2A. Another 1982 study by Levinger and myself showed that the D1 protein preferentially binds to (A + T)-rich DNA *in vitro*, including the 1.672 and 1.688 satellite DNA repeats, and is a component of isolated nucleosomes containing these centromeric satellite DNAs (106). Later studies (107, 108) indicated that D1, which contains 10 copies of the "AT-hook" domain, a motif that interacts with the minor groove of AT-rich DNA, is a part of heterochromatin-associated complexes that mediate transcriptional repression.

Work on D1 increased my interest in AT-DNA-binding proteins, in part because of their relevance to sequence-specific nucleosome arrangements in chromatin. Our projects in this arena led to discoveries of two new proteins. In 1984, Francois Strauss (then a postdoctoral student) and I identified and purified a green monkey protein, eventually termed HMG-I/Y (its initial name was α-protein), that bound to three specific AT-rich sites in the nucleosome-sized DNA repeat of the monkey α-satellite DNA (109). Later studies by others showed that HMG-I/Y, which contains AT-hook domains, plays a role in the assembly and function of DNA-associated multiprotein complexes that mediate transcriptional activation (Refs. 107 and 108 and references therein).

In 1989, Edward Winter (then a postdoctoral student) and I searched for AT-DNA-binding proteins in yeast and identified a 248-residue protein, named datin, that specifically bound to DNA containing oligo(dA)-oligo(dT) tracts (110). Such tracts are common in the intergenic regions of *S. cerevisiae* and other organisms. The discovery of datin was our last contribution to chromosome research for many years to come; by that time, Ub studies supplanted all other work in the laboratory.

New Biochemical and Genetic Methods

A scientific study is constrained by the quality and sophistication of its tools. Hence my lifelong interest in new methods. I mentioned the development, by Sundin and myself, of two-dimensional electrophoretic techniques for analyzing DNA replication intermediates. These methods made possible the discovery of the multicatenane pathway of chromosome segregation (Fig. 1) (3–5) and are still in use today.

Low Ionic Strength Gel Electrophoresis and Gel Shift Assay—In 1975, when I was still in Russia, my colleagues and I introduced a method for gel electrophoresis in buffers of low (~10 mM) ionic strength (62, 63). Decreased levels of counterions increased both stability and mutual repulsion of, for example, negatively charged nucleoprotein complexes and thereby greatly reduced their aggregation during electrophoresis. In a 1976 application of this technique, the forerunner of the gel shift assay, we employed it to fractionate isolated nucleosomes at a previously unattainable resolution (63). In 1981, the laboratories of Donald Crothers (111) and Arnold Revzin (112) independently employed a similar nondenaturing electrophoresis, termed later the gel shift assay (113), to detect and measure the interactions of specific DNA-binding proteins, such as the Lac repressor, with their cognate DNA operators. In 1984, Strauss and I demonstrated that this assay, until then used exclusively with purified proteins, can detect specific DNA-binding proteins *in the presence of nonspecifically binding ones*, provided that a sample contains a sufficient excess of "nonspecific" DNA (109). This elaboration of the gel shift assay made possible the detection of sequence-specific DNA-binding proteins in crude cell extracts. It soon became, and continues to be, one of the most widely practiced procedures in chromosome research.

Chromatin Immunoprecipitation Assay—In 1988, Mark Solomon (then a graduate student), Pamela Larsen (then a postdoctoral student), and I introduced the chromatin immunoprecipitation (ChIP) assay (114). In this technique, intact cells are covalently (and extensively) "fixed" with a cross-linking reagent such as formaldehyde, followed by disruption of the fixed cells, fragmentation of their chromosomes, and immunoprecipitation of cross-linked chromatin fragments with an antibody to a protein of interest. Subsequent thermal reversal of cross-links makes possible the identification (through hybridization or DNA sequencing) of specific genomic sites that are bound, *in vivo*, to a protein of interest under different physiological conditions. Since 1988, the ChIP assay (named "ChIP" by later users of this method) (114) has become a major, strikingly versatile tool in chromosome research. One of many examples is the central role of ChIP assays in studies on the functions of histone modifications. The use of formaldehyde, both in our 1978–1979 discovery of the first exposed region in chromosomes (1, 2) and

in the 1988 invention of the ChIP assay (114), stemmed from my earlier chromosome studies in Russia (115).

Hypersensitivity to Heavy Water, a New Conditional Phenotype—One of our last "non-Ub" contributions in the 1980s was the invention, by Bartel and myself, of a new way to make conditional mutants (116). Although *ts* mutants were a mainstay of genetic analysis for decades, my first encounters with them, in the early 1980s, suggested that this approach could stand an improvement. One problem is the leakiness of *ts* mutants, *i.e.* non-zero activity of a *ts* protein at nonpermissive temperature. In addition, producing a *ts* version of a protein of interest is often an iffy affair that requires extensive mutagenesis. Might there be other, also generally applicable, types of conditional mutants? Might there be a perturbant as pervasive as temperature? One evening in 1986, I realized that the answer might be "yes": heavy water! (My father's studies of D_2O in the 1950s and my recollection of his work were the likely impetus of that thought.) Biologically, D_2O would be indistinguishable from H_2O in all respects except toxicity. The idea was simplicity itself: determine a maximum concentration of D_2O that is still compatible with a near-normal growth of an organism of interest, such as yeast. Thereafter, mutagenize the cells and carry out a screen for mutants that are *hypersensitive* to D_2O, *i.e.* for mutants (we called them *ds*, for D_2O-sensitive) that cannot grow at a concentration of D_2O that is still nontoxic to wild-type cells. If such mutants can be obtained, straightforward assays can be used to determine whether most of the organism's essential genes can be mutated to a conditionally lethal *ds* phenotype. I did preliminary experiments that suggested the *ds* technique may be feasible. Bonnie Bartel got interested in this approach and soon demonstrated that yeast *ds* mutants could be easily produced and that apparently any or almost any gene could be mutated to a *ds* allele. Moreover, we found that most *ds* mutants were not *ts* and vice versa. In other words, the *ds* and *ts* techniques tapped entirely different sets of mutant alleles. Although the *ds* method (116) is technically straightforward and possibly superior to the *ts* method in some settings, the *ts* technique is still the predominant approach, in part because of its comparative ease and familiarity. As described below, yet another technique for producing conditional mutants, invented by us in 1994 (117), proved to be more in tune with current trends in genetics.

Ubiquitin Fusion Technique—In 1986, Bachmair, Finley, and I invented the Ub fusion technique, which led to the discovery of the N-end rule pathway (9). DNA-encoded linear Ub fusions to other proteins mimic natural Ub fusions that are encoded by Ub genes (10, 11). Ub fusions were found to be cotranslationally cleaved at the Ub-protein junction by DUB enzymes irrespective of the identity of a residue at the junction's C-terminal side, with Pro being a single exception (9, 14). The resulting Ub fusion approach, in which site-directed mutagenesis is used to alter a residue at the Ub-protein junction, is still the method of choice for placing, *in vivo*, a desired residue at the N terminus of a protein of interest (9, 118). The requirement for a "technique" to alter the N termini of proteins stems from constraints of the genetic code, as all nascent proteins start with the Met residue. Met aminopeptidases (MetAPs) would cleave off the N-terminal Met only if a residue at position 2, to be made N-terminal after cleavage, has a small enough side chain (118). Hence the necessity of a "bypass" method to alter N-terminal residues at will *in vivo* (9). Several descendants of the Ub fusion technique that were developed in the lab over the last two decades are briefly described below.

Ubiquitin Reference Technique—This 1996 method, by Frédéric Lévy (then a postdoctoral student), other colleagues in the lab, and myself, utilizes Ub fusion constructs encoding both a protein of interest and a "reference" protein, with two proteins cotranslationally coexpressed at (initially) equimolar levels (118, 119). Through a "built-in" reference protein, this method greatly increases the accuracy of pulse-chase assays (82, 120).

Heat-activated Degron (ts-Degron)—This 1994 method, by Jürgen Dohmen and Pei Pei Wu (then postdoctoral students) and myself, allows one to produce *ts* alleles of specific proteins without the (usual) resort to random mutagenesis and without altering a protein's amino acid sequence (117). The *ts*-degron method involves a "predesigned," portable N-degron that can be *activated* by increased temperature. A protein of interest is linked to such a degron and becomes short-lived *in vivo* at the nonpermissive temperature (117, 121). In contrast to our under-utilized D_2O (*ds*) technique for making conditional mutants (116), the *ts-degron* method (117, 121) is widely employed by other laboratories, in a range of projects, as an alternative to conventional *ts* mutants.

Ubiquitin Translocation Technique—This 1994 method, by Nils Johnsson (then a postdoctoral student) and myself, allows one to probe, *in vivo*, kinetic aspects of protein translocation across membranes (122). A fusion in which the Ub moiety is placed between a signal sequence and a reporter domain is cleaved by DUB enzymes in the cytosol unless the fusion can "escape" into a compartment such as the endoplasmic reticulum (ER). The critical step involves rapid folding of the newly formed Ub moiety, which precludes its translocation and makes possible the cleavage by

DUB enzymes after its last residue. However, if a sufficiently long spacer is present between the signal sequence and Ub, then by the time the Ub polypeptide emerges from the ribosome and is ready to fold, the ribosome *is already docked* at the transmembrane channel, allowing the translocation of *both* the Ub moiety and the downstream reporter domains of a fusion into the ER. These mutually exclusive outcomes make it possible to probe, by varying the length of the spacer and other parameters, both kinetic and stochastic aspects of targeting in protein translocation and to compare the strengths of different signal sequences *in vivo*. The Ub translocation technique (122) is also applicable to non-ER translocation.

Split-ubiquitin Assay for In Vivo Protein Interactions—This 1994 method, by Johnsson and myself (123), makes possible both the detection of a protein-protein interaction and its monitoring as a function of time at the natural sites of this interaction in living cells. The latter feature of the split-Ub technique is one of its advantages over methods such as the classical two-hybrid assay, where interacting protein domains must "make" it to the nucleus and transcriptional promoter settings. We found that when an ~40-residue C-terminal half of the 76-residue Ub (C_{ub}) was expressed as a fusion to a reporter protein, the fusion was cleaved only if the N-terminal half of Ub (N_{ub}) was also expressed in the same cell. Thus, the two halves of a single-domain protein coalesced *in vivo* to produce a quasi-native Ub moiety, thereby allowing the cleavage by DUB enzymes at the Ub-reporter junction. This reconstitution of quasi-native Ub from its fragments was not observed with a specific missense mutant of N_{ub}. However, if C_{ub} and the altered N_{ub} were each linked to polypeptides that interacted *in vivo*, the cleavage of the reporter fusion containing C_{ub} was restored. This approach yielded an assay for *in vivo* protein interactions (123). One difference between the split-Ub method and the earlier α-complementation with LacZ (β-galactosidase) (124) is that the latter assay involves a reconstitution of oligomeric protein through contacts between its subunits (or their derivatives) that were separate molecules to begin with, whereas the split-Ub assay involves a split *single-domain* protein. Variants of the split-Ub assay were developed after 1994 and employed to dissect specific protein interactions *in vivo* or to screen for new interactions (Refs. 125–127 and references therein). The invention of the split-Ub technique (123) gave rise to the field of *protein fragment complementation assays* and a number of useful designs that include split dihydrofolate reductase (128) and split green fluorescent protein (129).

Ubiquitin Sandwich Technique—"Cotranslational degradation" refers to destruction of a polypeptide *in vivo* while it is still part of a ribosome-associated peptidyl-tRNA. How to detect and measure such degradation? In the 2000 Ub sandwich technique, by Glenn Turner (then a graduate student) and myself (130), the polypeptide to be examined for cotranslational degradation, termed **B**, is sandwiched between two reporter domains, **A** and **C**. The **A**, **B**, and **C** domains are connected by Ub moieties, yielding a linear **A**Ub-**B**Ub-**C**Ub fusion. DUB enzymes cotranslationally cleave this fusion, generating **A**Ub, **B**Ub, and **C**Ub. After the initial cleavage at the **A**Ub-**B**Ub junction, the activity of DUB enzymes results in a kinetic competition between two *mutually exclusive* fates during the synthesis of **A**Ub-**B**Ub-**C**Ub: cotranslational cleavage at the **B**Ub-**C**Ub junction, which produces the long-lived **C**Ub module, or, alternatively, cotranslational processive degradation of the entire **B**Ub-**C**Ub nascent chain by the proteasome that destroys the Ub moiety between **B** and **C** before it can be recognized by DUB enzymes. The resulting decrease in **C**Ub relative to **A**Ub reflects the presence and extent of cotranslational degradation of the **B** domain (130). Using this method, we found that >50% of nascent protein molecules bearing an N-degron can be degraded cotranslationally, never reaching their mature size before their destruction by processive proteolysis (130). Thus, the folding of nascent proteins, including abnormal ones, may be in kinetic competition with pathways that target these proteins for degradation cotranslationally.

Recent Studies

Our current focus is the N-end rule pathway. We study it in yeast (*S. cerevisiae*), mammals (*Mus musculus*), and prokaryotes such as *E. coli* and the human pathogen *Vibrio vulnificus*. Over the last decade, this work led to discoveries of the pathway's functions in eukaryotes, whereas the physiology of bacterial N-end rule pathways is still unknown, despite advances in their mechanistic understanding (80, 131–134). Our studies of the mouse N-end rule pathway were led by Dr. Yong Tae Kwon, who now works in his own laboratory at the University of Pittsburgh. Thus far, Kwon's and my laboratories are the only ones that focus on the N-end rule pathway.

In 2000, Youming Xie (then a postdoctoral student) and I discovered that UBR1 (the E3 of the N-end rule pathway) and UFD4 (the E3 of the UFD pathway) interact with specific proteins of the 19 S regulatory particle of the 26 S proteasome (135). In addition to the possibility that these interactions underlie a route for delivery of ubiquitylated substrates to the proteasome, our results suggested that at least some Ub ligases may target their substrates while in a

complex with the 26 S proteasome. Other groups reported that several other Ub ligases also interacted with the 26 S proteasome. In 2002, Xie and I showed that a mutant *S. cerevisiae* UFD4 Ub ligase that lacked its proteasome-binding region could still mediate ubiquitylation of UFD substrates but that their proteolysis was impaired, strongly suggesting that the proteasome-UFD4 interaction is functionally relevant (136).

Also in 2002, Jun Sheng (then a postdoctoral student) and I dissected the degradation signal of c-MOS, a kinase and regulator of oocyte maturation (137). Data obtained by others suggested that the N-terminal Pro residue of c-MOS may be a destabilizing residue that targets c-MOS for destruction in frog oocytes. Our degradation assays involved microinjection of plasmids that expressed wild-type or mutant c-MOS in *Xenopus* oocytes. We found that the N-terminal Pro residue of c-MOS is dispensable for c-MOS degradation if Ser^2 (encoded Ser^3) of c-MOS is replaced by a small non-phosphorylatable residue such as Gly. These and other results (137) showed that the dependence of c-MOS degradation on the N-terminal Pro residue is caused by a Pro-mediated down-regulation of the net phosphorylation of Ser^2, a modification that halts c-MOS degradation. Thus, the N-terminal Pro of c-MOS is not a ligand for an E3 Ub ligase. In other words, the N-terminal Pro is, operationally, a stabilizing residue in the N-end rule, in agreement with our earlier findings (137).

The N-end rule has a hierarchic structure (Fig. 3). In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation to yield the secondary destabilizing N-terminal residues Asp and Glu (138, 139). The destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by *ATE1*-encoded R-transferases of the N-end rule pathway (Fig. 3).

In 1999, Kwon, Anna Kashina (then a postdoctoral student), and I cloned mouse *ATE1* and found that it encodes at least two splicing-derived isoforms of R-transferase (86). In 2006, Rong-Gui (Cory) Hu, Christopher Brower (postdoctoral students in the lab), and their colleagues identified and characterized six splicing-derived isoforms of mouse R-transferase (88), a pattern that remains to be understood functionally. In mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated N-terminal residues contains not only Asp and Glu but also N-terminal Cys (16), which is arginylated after its oxidation to Cys sulfinate or Cys sulfonate, as shown in 2002 by Kwon, Kashina, Hu, Ilia Davydov (then a postdoctoral student), Fangyong Du (then a graduate student), and myself (Fig. 3) (59).

In 2005, Hu, Sheng, and I discovered that the *in vivo* oxidation of N-terminal Cys *requires* NO as well as oxygen (O_2) or its derivatives (Fig. 3) (87). The N-end rule pathway is thus a sensor of NO, through the ability of this pathway to destroy proteins with N-terminal Cys, at rates controlled by NO, O_2, and their derivatives. This work also identified G-protein regulators RGS4, RGS5, and RGS16 as conditionally short-lived proteins that are targeted by the arginylation branch of the N-end rule pathway through their (conditionally oxidized) N-terminal Cys (87). The Kwon laboratory independently showed that RGS4 and RGS5 are physiological N-end rule substrates (90). (In 2000, Davydov and I found that RGS4 was degraded by the N-end rule pathway in reticulocyte extracts (140), before we learned that this degradation requires NO and oxygen (87, 90).) Through the conditional degradation of RGS4, RGS5, and RGS16, the N-end rule pathway regulates G-proteins and thus the signaling by transmembrane receptors.

An N-degron is produced from a pre-N-degron through a proteolytic cleavage. MetAP proteases remove Met from the N terminus of a newly formed protein only if the residue at position 2, to be made N-terminal after cleavage, has a small enough side chain. Consequently, of the 13 destabilizing residues in the mammalian N-end rule (Fig. 3), only Cys can be made N-terminal by MetAPs (87). Individual mammalian genomes encode ~250 proteins with Cys at position 2, *i.e.* the proteins that would be expected to contain N-terminal Cys *in vivo*. Thus, RGS4, RGS5, and RGS16, the currently known N-end rule substrates of this class, are but a small subset of potential N-end rule substrates whose conditional degradation would be regulated by NO and oxygen. Note that *any destabilizing residue*, including Cys, can be made N-terminal through internal cleavages of proteins by other proteases such as separases, caspases, and calpains (Fig. 3). In other words, the above number (~250) is an upper limit only for those N-terminal Cys-bearing proteins that are produced by MetAPs. There are many more potential N-end rule substrates that may be produced by other proteolytic cleavages in cellular proteins.

In *S. cerevisiae*, the N-terminal amidase NTA1 (identified, cloned, and analyzed in 1995 by Rohan Baker, then a postdoctoral student in the lab) (138) mediates deamidation of N-terminal Asn and Gln, converting them into Asp and Glu, the substrates of R-transferase. In multicellular eukaryotes, the deamidation branch of the N-end rule pathway bifurcates: a distinct N-terminal amidase,

NTAN1, is specific for N-terminal Asn, whereas another amidase (which remains to be identified) deamidates N-terminal Gln (Fig. 3). In 1996, Sergei Grigoryev (then a postdoctoral student) and colleagues in the lab collaborated with the laboratory of Ralph Bradshaw (University of California, Irvine) to clone and characterize the mouse NTAN1 amidase (141). The biological role of yeast NTA1 remains to be discovered, as *S. cerevisiae nta1*Δ, which lacks N-terminal deamidation, has no discernible abnormal phenotypes other than its inability to degrade reporter N-end rule substrates with N-terminal Asn or Gln (138). In contrast, $NTAN1^{-/-}$ mice, which lack the Asn-specific N-terminal deamidation, are behaviorally abnormal (139).

E3 Ub ligases of the N-end rule pathway, called N-recognins (57), recognize (bind to) primary destabilizing N-terminal residues (Fig. 3). At least four N-recognins, including UBR1, underlie the N-end rule pathway in mammals (142–145). In *S. cerevisiae*, the N-end rule pathway is mediated by a single 225-kDa N-recognin, UBR1 (18), which functions in a complex with RAD6, a Ub-conjugating (E2) enzyme (57, 146). UBR1 contains at least three substrate-binding sites, which were identified and analyzed over the years by colleagues in the lab, most recently in 2008 by Zanxian Xia, Ailsa Webster, and Michel Ghislain (then postdoctoral students), Konstantin Piatkov (a postdoctoral student in the lab), Du, and myself (147). The type 1 site of UBR1 is specific for basic N-terminal residues of protein substrates (Arg, Lys, His), whereas the type 2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile) (Fig. 3). The third binding site of UBR1 targets proteins through their internal (non-N-terminal) degrons.

A physiological function of this multisite design of UBR1 was discovered in 1998–2002 by Turner, Du, Christopher Byrd (then a graduate student), and myself (120, 148, 149). In 1998, Byrd, Turner, and I identified the yeast transcriptional repressor CUP9 as a short-lived substrate of the N-end rule pathway and a down-regulator of *PTR2*, which encodes the major transporter of short peptides (148). This result explained the earlier finding by the laboratory of Jeffrey Becker (University of Tennessee, Knoxville) that the import of peptides in *S. cerevisiae* stops in the absence of UBR1, *i.e.* in the absence of the N-end rule pathway (150). In 2000–2002, Turner, Du, and I discovered that the degradation of CUP9 is conditional: a distinct substrate-binding site of UBR1 that targets CUP9 through its internal degron is *autoinhibited*. This site of UBR1 can be allosterically activated through a conformational change that is caused by the binding of short peptides (bearing destabilizing N-terminal residues) to the other two (types 1 and 2) binding sites of UBR1 (120, 149). The resulting reversal of UBR1 autoinhibition by imported peptides accelerates the UBR1-mediated ubiquitylation of CUP9, leads to its faster degradation and consequently to a derepression of the transporter-encoding *PTR2* gene (120, 149). This positive feedback circuit, mediated by short peptides and the N-end rule pathway, allows cells to detect the presence of extracellular peptides and to react by increasing their uptake.

Between 1996 and 2003, Kwon and colleagues in my lab constructed and analyzed mouse strains that lacked specific components of the N-end rule pathway (Fig. 3), such as NTAN1 (Asn-specific N-terminal amidase), ATE1 (R-transferase), UBR1 (one of the mouse N-recognins), and UBR2 (another N-recognin) (59, 139, 142, 151). These studies and our later collaborations with the Kwon laboratory have revealed informative phenotypes of mouse mutants that further expanded the pathway's functional range.

In 2008, Hu, Haiqing Wang (then a postdoctoral student), Xia, and I discovered that the N-end rule pathway is a sensor of heme (89). Low micromolar levels of hemin (Fe^{3+}-heme) inhibit arginylation by the ATE1 R-transferase in both yeast and mammals (Fig. 3). In addition, hemin induces the proteasome-mediated degradation of R-transferase *in vivo*. Thus, remarkably, hemin acts as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. This proteolytic system, a sensor of short peptides, NO, and oxygen, is now a sensor of heme as well (89). One function of the N-end rule pathway may be to coordinate the activities of effectors, both reacting to and controlling the redox dynamic of heme, oxygen, NO, and thiols, in part through the conditional degradation of specific transcriptional regulators (*e.g.* CUP9 and c-Fos) and regulators of G-proteins (*e.g.* RGS4, RGS5, and RGS16).

The functions of the N-end rule pathway include the sensing of NO, oxygen, short peptides, and heme; maintenance of the high fidelity of chromosome segregation in eukaryotes from fungi to mammals through the degradation of a separase-produced fragment of the cohesin subunit; regulation of peptide import through the degradation (modulated by peptides) of *S. cerevisiae* CUP9, a transcriptional repressor of the peptide transporter; regulation of signaling by transmembrane receptors, through the NO/O_2-dependent degradation of specific RGS proteins that down-regulate G-proteins; regulation of apoptosis, through the degradation of a caspase-processed inhibitor of apoptosis; a multitude of functions mediated by the mammalian transcription factor c-Fos, a conditional sub-

strate of the N-end rule pathway; regulation of the human immunodeficiency virus replication cycle; regulation of meiosis, spermatogenesis, neurogenesis, and cardiovascular development in mammals; and regulation of leaf senescence in plants (Refs. 58, 59, 87, 89, 90, 142, 144, and 151–154, and references therein).

In 2005, a collaborative study with Kwon's and my laboratories that was led by Dr. Martin Zenker's laboratory at the University of Erlangen-Nuremberg (Germany) showed that mutations in human UBR1 (Fig. 3) are the cause of Johansson-Blizzard syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis (154). The abnormalities of the previously constructed *UBR1*$^{-/-}$ mice (151) include pancreatic insufficiency (154), a less severe counterpart of this defect in human JBS (*UBR1*$^{-/-}$) patients.

Despite their multiplicity and broad range, the known functions of the N-end rule pathway are still the tip of the iceberg. Several fascinating phenotypes of mouse N-end rule mutants remain to be understood. One example: *NTAN1*$^{-/-}$ mice, which lack the Asn-specific N-terminal deamidation (Fig. 3), are fertile, do not exhibit gross abnormalities, and are seemingly indistinguishable from their *NTAN1*$^{+/+}$ littermates (139). However, behavioral tests with *NTAN1*$^{-/-}$ mice revealed significant perturbations in their spontaneous activity, spatial memory, and social behavior (139). Discovering the relevant physiological N-end rule substrates would be essential for identifying brain circuits that are perturbed in *NTAN1*$^{-/-}$ mice. This is also true for other, more overt and severe defects in other mouse N-end rule mutants and in human patients with JBS (154).

In 2004, my interest in useful terminology (*e.g.* "degron" (81)) led to the suggestion of two terms, *sequelog* and *spalog* (155). They were proposed to address a disconnect between the high rigor of statistical methods for comparing sequences or structures and the often unjustified employment of the assumption-laden terms "homolog," "ortholog," and "paralog." The new terms, *sequelog* and *spalog*, denote a sequence that is similar, to a specified extent, to another sequence and a three-dimensional structure that is similar, to a specified extent, to another three-dimensional structure, respectively (155). Besides their usefulness as separate terms for sequence *versus* spatial similarities, the rigor-conferring advantage of sequelog and spalog is their *evolutionary neutrality*, in contrast to often unproven evolutionary links implied by homolog, ortholog, and paralog. The latter terms are compatible with the sequelog/spalog terminology and can be employed to convey understanding about functions and common descent if this (additional) information is available. Helpful derivatives of, for example, sequelog are *sequelogy* (sequence similarity) and *sequelogous* (similar to another sequence). *Spalog* and *sequelog* fill a lacuna in the existing terminology. These terms would clarify and streamline discourses about similarity (155).

New Approaches to Therapy

A subject I am particularly interested in is the invention of multitarget conditional drugs, devices that "think" before they "decide" to act. The coming revolution in medicine will involve not only qualitatively better ways to do surgery but also drug-based therapies that will take into account, at last, the massive interconnectedness and redundancy of molecular circuits in living cells. Single-compound (and even multi-compound) drugs of today are incapable of such finesse. Therefore, even otherwise useful drugs exhibit undesirable side effects. Yet another problem is our continuing helplessness in containing (let alone curing) major human cancers once they spread beyond a surgeon's knife. The problem is exacerbated by the genomic instability of many, possibly most, cancers. This property increases the heterogeneity of malignant cells in the course of tumor progression or anticancer treatment and is one reason for the failure of most cancer therapies. A few relatively rare cancers can often be cured through chemotherapy but require cytotoxic treatments of a kind that cause severe side effects and are themselves carcinogenic. Recent advances, including the use of anti-angiogenic compounds and inhibitors of specific kinases, hold the promise of curative therapies. Nevertheless, major human cancers are still incurable once they have metastasized.

In 1995, I suggested a design of protein-based "multitarget" reagents based on multiple degrons and their conditional masking by specific cellular proteins (156). A 1998 article described another design, of small compounds whose activity can be modulated by more than one protein ligand at the same time (157). We eventually decided not to implement these approaches in the lab because I thought, perhaps mistakenly, that the resulting drugs would not be efficacious enough in a clinical setting.

In 2007, I proposed a new approach to cancer therapy that involves homozygous DNA deletions (158). Such deletions are present in many (possibly most) cancers and differ from any other attribute of a cancer cell by the fact that a homozygous deletion (HD) *cannot revert*. Hence the idea of a treatment that exclusively homes in on cells that *lack* specific DNA sequences that are present in normal cells. If this proves possible, a resulting therapy may be

entirely specific for cancer cells and thus not only curative but substantially free of side effects as well. The difficulty here is that an HD is an "absence," and therefore it cannot be a conventional molecular target. Nevertheless, an HD-specific regimen is feasible, on paper so far (158). This strategy, termed *deletion-specific targeting* (DST), employs HDs (not their effects on RNA/protein circuits, but deletions themselves) as the targets of cancer therapy. The DST strategy brings together, in a novel way, both existing and new methodologies, including the Ub fusion technique, the split-Ub assay, zinc-finger DNA-recognizing proteins, and split restriction nucleases. The DST strategy also employs a new feedback mechanism that receives input from a circuit operating as a Boolean OR gate and involves the activation of split nucleases, which destroy the DST vector in normal (non-target) cells. The logic of DST makes possible an incremental and essentially unlimited increase in the selectivity of treatment (158).

If the substantial complexity of DST-type strategies is unavoidable (this remains to be determined), approaches of this kind might be a harbinger of therapies to come. The virtues of simplicity notwithstanding, a complex problem, such as an assured cure of cancer, or a selective elimination of damaged (*e.g.* aged) mitochondria in cells of a patient, or other such feats may require commensurately sophisticated solutions. Can small compounds, with their inherently low informational content, ever enable a definitive cure of cancer that is free of collateral damage? The notion that underlies (and motivated) the DST strategy (158) is that a curative, side effect-free treatment may require polymer-scale, multitarget, Boolean-type circuits, *i.e.* that simpler (smaller) drugs may ultimately not suffice, particularly in regard to side effects. The task at hand is to address the validity of this assumption. Work on the DST strategy is under way in the lab.

Epilogue

My laboratory has been at Caltech for the past 16 years. The decision to move here from MIT was prompted by an unexpected invitation. I showed it to my wife Vera, and we visited Caltech in February 1991. The scientific quality of Caltech, the charms of Pasadena's subtropical climate, and the warm reception by colleagues were compelling to both of us. Caltech is a great place, similar to MIT in all respects but smaller. Vera and I live in La Canada, a hamlet on a mountainside near Pasadena.

At 61, I have already traveled through the bulk of life span allotted to us by evolution. Curiously, the Ub system plays a major role in determining that life span. Moreover, Ub-dependent processes underlie just about everything a living cell does. An account of our discoveries through which this fact became known and understood is the main chapter of this recollection. A scientist's life, its adventures and misadventures, its loves and conflicts, is largely incidental, tangential to one's contribution, in a profession that tends to swallow a person whole. Our propensity to be curious about other people obscures the fact that it is essentially immaterial that Isaac Newton was not a kind man or that Johannes Kepler had to defend his mother in court against the charge of being a witch (she was acquitted). To think of Newton or Kepler is to think of their work. The same applies to all of us in this remarkable guild, irrespective of the scale of one's accomplishment.

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