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Concise Original Report

Targeted protein degradation: from small molecules to complex organelles—a Keystone Symposia report

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Targeted protein degradation is critical for proper cellular function and development. Protein degradation pathways, such as the ubiquitin proteasomes system, autophagy, and endosome–lysosome pathway, must be tightly regulated to ensure proper elimination of misfolded and aggregated proteins and regulate changing protein levels during cellular differentiation, while ensuring that normal proteins remain unscathed. Protein degradation pathways have also garnered interest as a means to selectively eliminate target proteins that may be difficult to inhibit via other mechanisms. On June 7 and 8, 2021, several experts in protein degradation pathways met virtually for the Keystone eSymposium "Targeting protein degradation: from small molecules to complex organelles." The event brought together researchers working in different protein degradation pathways in an effort to begin to develop a holistic, integrated vision of protein degradation that incorporates all the major pathways to understand how changes in them can lead to disease pathology and, alternatively, how they can be leveraged for novel therapeutics.

Keywords: aggregation; autophagy; lysophagy; proteasome; protein degradation; ubiquitin

Introduction

Protein quality control is critical to maintain proper cellular function and development. Accumulation and subsequent aggregation of misfolded proteins is a hallmark of several neurodegenerative diseases. Dysfunction in protein quality control systems has been implicated in other pathologies, including with age-related diseases and cancer.[1,2] Maintaining proper protein homeostasis is also key during development. As cells divide and differentiate, the proteome is remodeled to reflect the changing cellular state. One of the most dramatic examples of this is in red blood cells (RBCs), which eliminate unnecessary proteins during the erythroblast phase such that hemoglobin represents 98% of the proteome in mature RBCs.[3] Cells have devised several mechanisms to identify misfolded proteins and either attempt to refold them or target them for degradation, including the ubiquitin proteasomes system (UPS) and autophagy pathway. Understanding the mechanisms of these protein clearance pathways is key to not only understand how their dysfunction is involved in disease pathology but also to harnessing these systems for therapeutic applications.

On June 7 and 8, 2021, experts in protein degradation pathways met virtually for the Keystone eSymposium "Targeting protein degradation: from small molecules to complex organelles." One of the key goals of the event was to bring together researchers working to understand autophagy and proteasomal degradation. While much progress has been made in defining the mechanisms for these pathways separately, there is a clear need in the field to understand how these pathways interact at both the cellular and molecular levels. Ultimately, a holistic, integrated vision of protein degradation that incorporates all the major pathways is key to understanding how these pathways lead to disease pathology.

Speakers discussed bacterial and mammalian protein degradation systems and how pathogens co-opt protein degradation pathways in host cells. Several speakers also focused on specialized protein degradation pathways, such as lysophagy and mitophagy. In addition, they showed how components of the autophagy pathway and UPS recognize target proteins, how proteins destined to be degraded are steered toward distinct degradation pathways, how proteomes are remodeled during development and differentiation, and how protein degradation pathways can be leveraged to design novel therapies for previously undruggable targets.

Regulation of cullin-RING ligases

In eukaryotic systems, ubiquitination of target proteins is the key step that tags them for recognition and degradation by the proteasome complex. Cullin-RING ligases (CRLs) is the largest family of E3 ubiquitin ligases. CRLs are multisubunit, modular assemblies typically consisting of four main entities: a cullin core that acts as a central scaffold, a RING-finger protein that binds to a ubiquitin carrying enzyme (either an E2 ubiquitin conjugating enzyme or an ARIH-family E3 ligase), a substrate receptor that binds to the target protein, and an adaptor protein (or adaptor protein complex). There are approximately 250 CRLs, as defined by their substrate receptors. CRLs are tightly regulated by post-translational modification and changes in subunit composition.[4,5] In particular, the modification of cullin by the ubiquitin-like protein NEDD8 is required for CRL activity.[6]

Cullin specificity for NEDD8 regulation

Brenda A. Schulman from the Max Planck Institute for Biochemistry presented work on understanding how NEDD8 regulates cullins. Schulman's group determined the structure of CRLs to understand how cullin neddylation activates CRLs in different contexts. Schulman focused on the SKP1–CUL1–F-box CRL, which interacts with ARIH1, an RBR-type E3 ligase, to form an E3-E3 superassembly in which neddylated CRL activates ubiquitin transfer from an E2 ligase to an ARIH-RBR E3 ligase, and finally to the substrate.[7] Proteomics analyses in collaboration with David Rhee in Wade Harper's laboratory at Harvard Medical School and Arno Alpi's group at University of Dundee, showed that ARIH1 binds neddylated SCFs and many other CRLs. Knocking down ARIH1 mRNA stabilized SCF E3 substrates, indicating that it is important for SCF function. Similar interactions were observed between ARIH2 and cullin 5–containing CRLs.[7,8] To understand how neddylation activates SCF activity, Schulman's group generated chemically stable mimics of the transition state during ubiquitin transfer from the E2 ligase to ARIH1 and from ARIH1 to the substrate. ARIH1 has a four-helix bundle domain (the Ariadne domain) that binds to CUL1 and RBX1 in a similar manner in both transition states, thus serving to anchor ARIH1 to the complex. Structural studies of ARIH1 alone show that it is autoinhibited via several mechanisms, e.g., the catalytic cysteine residue is blocked, the E2 ubiquitin binding site is misaligned, and the E3 ubiquitin binding site is too far from the catalytic cysteine.[9] Cryo-electron microscopy (cryo-EM) of the two transition states provides key insights into how NEDD8 activates ARIH1. Binding to neddylated-SCF releases the catalytic domain of ARIH1 and aligns the E2-ubiquitin intermediate with E2 ubiquitin binding site, thus activating ubiquitin transfer from the E2 ligase to ARIH1.[10] Schulman also described unpublished cryo-EM structures of neddylated CRL5 interacting with ARIH2 and showed that neddylation mediates CUL5 activation via a different mechanism. This cullin-specific allosteric activation may offer opportunities for therapeutic targets.

The role of CAND in substrate receptor exchange

Deneddylation of CRLs is important for substrate receptor exchange and subsequent cycles of ubiquitination. Work from several laboratories has shown that binding of substrate to a cullin-associated receptor blocks deneddylation. After substrate degradation, the CRL is deneddylated by CSN. Deneddylation allows the CRL to interact with CAND, which promotes the disassociation of the existing substrate receptor and facilitates the binding of a substrate-bound receptor.[4,5]

Wade Harper from Harvard Medical School presented unpublished work by David Rhee using embryonic stem cell (ESC)-based models of human embryogenesis to understand the role of CAND in promoting substrate receptor exchange during development and lineage differentiation. ESCs lacking CAND activity fail to efficiently differentiate to multiple germ cell lineages. This is associated with alterations in large-scale turnover of CRL targets, defects in assembly of specific substrate receptors with the core SCF complex, and alterations in the transcriptional programs during endoderm differentiation as assessed by single-cell RNA sequencing. This suggests that CRL substrate receptors are instrumental in resculpting the proteome during changes in cell state.

Structural insights on proteasome assembly

John Hanna from Harvard Medical School presented structural analysis of proteasome

intermediates to understand proteasome assembly. The central core particle of the proteasome does not assemble spontaneously. Rather, assembly occurs in a step-wise fashion and requires the activity of five protein chaperones: the heterodimers Pba1/2 and Pba3/4, and Ump1. The first step is the assembly of an α-subunit ring facilitated by Pba1/2 and Pba3/4 followed by the consecutive addition of β subunits, facilitated by Ump1. Completion of the β-subunit ring creates a half proteasome, which combines with another half to form a complete proteasome barrel. Cleavage of the N-terminus from active site subunit propeptides, degradation of Ump1, and release of Pba1/2 result in a functioning proteasome. Because proteasome intermediates are low in abundance and transitory in nature, high-resolution structural information on proteasome intermediates has been difficult to acquire. Using proteasome mutants that stall the assembly process, Hanna's group has been able to determine the structure of two intermediates: the 13S complex, which contains the α ring, β2-4, Ump1, and Pba1/2; and a pre-15S intermediate that contains the α ring, β2-6, Ump1, and Pba1/2. The structures reveal several insights into proteasome assembly and clear up several previous misconceptions.[11] Most notably, the structures show that Ump1, which was previously believed to be unstructured and extend out of the nascent core particle, adopts an extended helical conformation and forms extensive contacts throughout the core particle, including with several β-propeptides and Pba1. In addition, the structures reveal novel interactions between the N-terminus of Pba1 and the interior of the core particle. These interactions help to explain how Pba1/2 preferentially bind to the immature core particle and are released at the end of assembly.[12] Overall, these structures reveal how the activity of multiple chaperones is tightly coordinated to orchestrate proteasome assembly.[12]

Proteasomal systems: contributors to pathogenicity

Pathogenic bacteria are faced with a range of stresses from their host. Stresses like elevated temperature, oxidative stress, low nutrients, extreme pH, and antibiotics can cause bacterial proteins to misfold and aggregate. Bacteria, therefore, have evolved robust protein quality control systems to efficiently remove misfolded proteins and enable them to cope with outside stresses.[13,14]

A ubiquitin proteasome system-like pathway in mycobacteria

Eilika Weber-Ban from ETH Zurich discussed proteasomal degradation in mycobacteria, including *Mycobacterium tuberculosis*. Mycobacteria encode a proteasome and ubiquitin-like modification pathway that parallels eukaryotic systems. Covalent attachment of the intrinsically disordered protein prokaryotic ubiquitin-like protein (Pup) targets proteins to mycobacterial proteasome ATPase (Mpa), an ATPase that sits atop the proteasome and feeds substrates into the proteasomal core for degradation.[15–17] Structures of Mpa bound to Pup show that Pup forms a long helix that interacts with the N-terminal coiled–coiled domain in Mpa. This positions the first 15 residues of Pup, which remain unstructured, near the entrance of the proteasomal core. The structure thus suggests that Pup acts not only as a recruitment tag but also as a threading initiator for proteasomal substrates.[17–19] Weber-Ban presented unpublished cryo-EM studies of substrate-engaged mycobacterial Mpa–proteasome complex stalled at early stages of initiation to understand how Mpa engages with the Pup substrate and feeds it into the proteasome core.

Phospho-arginine as a degradation tag in Gram-positive bacteria

Tim Clausen from the Research Institute of Molecular Pathology, IMP discussed the protein degradation system of Gram-positive bacteria. Clausen focused on McsB, a protein arginine kinase that his group previously showed regulates the transcription factor (TF) CtsR, which is involved in the heat-shock response. Arginine phosphorylation of CtsR destabilizes its interaction with DNA, thus preventing its repressive effect on gene expression.[20] During his talk, Clausen discussed a second role for McsB: labeling aberrant proteins for degradation by the bacterial proteasome, ClpCP. They showed that phospho-arginine is a degradation tag that is recognized by the substrate receptor of ClpCP, leading to the activation of the protease machine.[21]

Clausen showed that McsB activity is regulated at several levels. First, structural studies revealed an allosteric binding site for phospho-arginine that stimulates McsB kinase activity.[22] Second, McsB can form octameric structures that are

maintained by internal phosphorylated arginine residues and thus regulated by McsB autophosphorylation. In the octameric structure, the active sites are sequestered within an inner chamber, enabling McsB to selectively phosphorylate unfolded proteins. Clausen proposed a model, wherein dimeric and—to a lesser extent—open multimeric McsB have promiscuous kinase activity and regulate TFs like CtsR. Under conditions of stress, McsB expression is upregulated, promoting the formation of closed octamers that selectively target unfolded proteins.[23]

Clausen also presented work on reprogramming ClpCP to degrade novel substrates. Bacterial proteolysis targeting chimeras (bacPROTACs) are small molecules that contain a ClpCP binding site consisting of a phospho-arginine mimetic and a binding site for a protein of interest. bacPROTACs can, therefore, bring novel substrates to ClpCP and induce their degradation. Clausen's group has conducted proof-of-principle studies of bacPROTACs *in vitro* and *in vivo*.[24] They hope that bacPROTACs may represent a novel, broadly applicable antibiotic strategy.

The proteasome as a pathogenic entity in Plasmodium falciparum *infection*

Michal Sharon from the Weizmann Institute of Science presented work done in collaboration with Neta Regev-Rudzki on the role of the 20S proteasome in *Plasmodium falciparum* infection, the parasite that causes malaria. Sharon focused on the blood stage of the parasite's complicated life cycle in which it infects RBCs. *P. falciparum* induces infected RBCs to secrete extracellular vesicles, which have been shown to promote parasitic growth. Atomic force microscopy data showed that these extracellular vesicles disrupt the cytoskeleton and reduce membrane stiffness in healthy uninfected RBCs, which facilitates *P. falciparum* infection. To understand what components of the extracellular vesicles are responsible for these effects, Sharon's group analyzed the cargo of *P. falciparum*-induced extracellular vesicles. The vesicles contained both human and *P. falciparum* proteasome subunits as well as several kinases. Sharon showed that *P. falciparum*–induced extracellular vesicles transmit active host 20S proteasome complexes and parasitic kinases to uninfected RBCs (Fig. 1). The 20S proteasome complex is able to directly degrade proteins with unstructured elements without the need for ubiquitination. Sharon identified four phosphorylated cytoskeleton host proteins that are direct degradation substrates of the delivered 20S proteasome. The data suggest that phosphorylation facilitates conformational transitions that lead to degradation by the 20S proteasome. The loss of cytoskeleton proteins thus alters the mechanical properties of the membrane and facilitates *P. falciparum* infection. Treatment with the proteasome inhibitor bortezomib abrogated the effect of *P. falciparum*–induced extracellular vesicles on parasite growth and RBC membrane and cytoskeletal properties.[25]

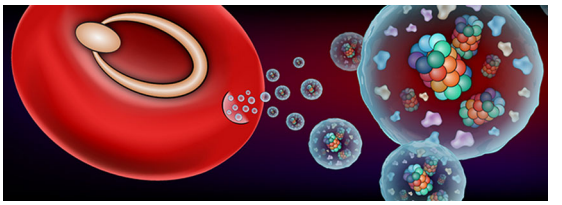

Figure 1. **Schematic representation of a red blood cell infected by a ring-shaped malaria parasite that releases extracellular vesicles containing active 20S proteasomes.**

Autophagy: substrate recognition, autophagosome formation, and autophagosome trafficking

The autophagy process facilitates the targeting of cytoplasmic material or cargo to the lysosome for degradation. Proteasomal degradation can only degrade unfolded proteins. If the proteasome is overwhelmed or proteins are unable to unfold, this can lead to the accumulation of protein aggregates and condensates. These species can subsequently be removed via autophagy, effectively serving as a backup system for the proteasome. During autophagy, a double-membrane vesicle, known as the autophagosome, forms around a cargo and is trafficked to and fuses with the lysosome. Autophagy has principally been studied with respect to starvation, wherein cells form autophagosomes to generate the building blocks needed for survival. However, selective autophagy pathways that play key roles in cellular homeostasis and tissue remodeling have been identified. In these pathways, cargo receptors target specific proteins for degradation.[26]

The role of cargo receptors in autophagosome formation

Sascha Martens from the University of Vienna discussed how the three cargo receptors, p62, NBR1, and TAX1BP1, orchestrate the formation of the autophagosome. p62 binds to ubiquitinated proteins via its UBA domain and forms a phase-separated condensate.[27]

Martens's group has shown that p62 is both necessary and sufficient for the formation of ubiquitin-containing condensates in cells. Martens proposed that aggregation of misfolded proteins crosslink with p62 oligomers and form condensates. A second cargo receptor, NBR1, aids in condensate formation by interacting with p62 via its PB1 domain, which caps the size of p62 filaments, and with ubiquitin via its UBA domain, which has a higher affinity for ubiquitin than p62. In cells, depleting NBR1 reduced the number of p62-containing condensates; both the NBR1 PB1 and UBA domains were necessary for proper condensate formation. NBR1 also plays an important role in recruiting TAX1BP1 to condensates. TAX1BP1 in turn recruits FIP200, which mediates the formation of the isolation membrane. Martens work suggests that rather than bringing cargo to a preformed isolation membrane, autophagosomes form *de novo* around a cargo via the cooperation of p62, NBR1, and TAX1BP1.[28–30]

In vitro *reconstitution of autophagosomes to understand autophagosome formation*

Chunmei Chang from James Hurley's laboratory at the University of California, Berkeley described how cargo receptors cooperate with ATG proteins to make an autophagosome. Chang is investigating autophagosome formation *in vitro* using reconstituted unilamellar vesicles that mimic the autophagosome membrane with addition of cargo signal, receptor, protein kinases, lipid kinases, and LC3 conjugation machinery. Chang's talk focused on the mechanism of LC3 lipidation in autophagosomes, which is important for membrane expansion. Using the reconstituted system, Chang showed that all three cargo receptors (NDP52, TAX1BP1, and OPTN) were able to induce the robust LC3 lipidation but that they interact with distinct subsets of autophagy proteins. For example, both NDP52 and TAX1BP1 are dependent on the ULK1 complex to trigger LC3 lipidation, while OPTN is not. These data support a model whereby cargo induces the formation of LC3-lipidated membranes, in contrast to earlier hypotheses that cargo receptors engage with preformed LC3-lipidated membranes.[31]

Autophagosome trafficking to lysosomes

Autophagosomes can develop anywhere within a cell, though lysosomes–where phagosomes are ultimately headed–are typically located near the nucleus. Therefore, autophagy is dependent on the ability of autophagosomes to migrate toward the nucleus.

Malene Hansen from the Sanford Burnham Prebys Medical Discovery Institute (but moved her laboratory to the Buck Institute for Aging Research in August 2021), discussed the regulatory mechanisms for how autophagosomes traffick within the cell (Fig. 2). Hansen showed that the autophagosome-associated protein LC3B/Atg8 plays a key role in transport. Hansen's laboratory previously published that phosphorylation of LC3B by STK3 and STK4 is critical for autophagy, and that absence of phosphorylation causes an accumulation of lysosomes in a perinuclear fashion.[32] In their new study, Hansen's group further delineated the mechanism by which phosphorylation promotes autophagy. They identified FYCO1 as a binding partner of LC3B,[33] consistent with a recent study.[34] FYCO1 is a protein involved in carrying autophagosomes along microtubules toward the cell periphery.[35–37] Hansen showed that FYCO1 preferentially binds to unphosphorylated LC3B,[33] which is consistent with the structure of the binding site.[38] LC3B phosphorylation is required for perinuclear localization of autophagosomes and for retrograde trafficking of autophagosomes, which ultimately enables autophagosomes to fuse with lysosomes. Hansen put forth a model in which FYCO1 binding to unphosphorylated LC3B connects the autophagosome to the transport machinery and facilitates trafficking toward the cell periphery.[39] Moreover, phosphorylation by STK3/4 reduces the interaction between FYCO1 and LC3B and enables autophagosomes to travel toward the lysosome near the nucleus. They proposed phosphorylation of LC3B as a possible autophagy switch that regulates the subcellular localization of autophagosomes.[33] Hansen's group is ultimately interested in the role of autophagy in aging, including how changes in vesicle transport may play a role in the decline observed in autophagy during aging.[40,41]

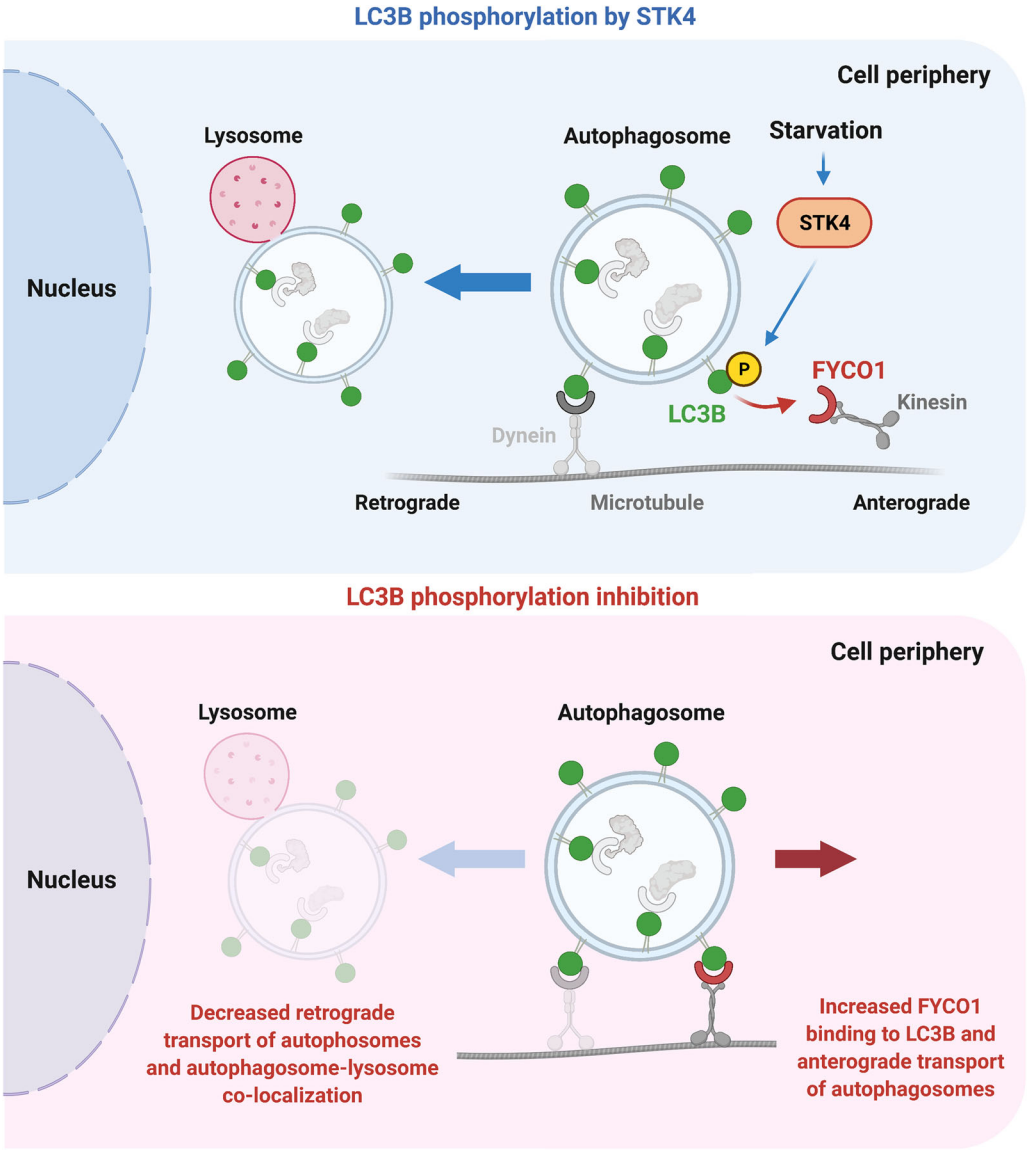

Figure 2. LC3B phosphorylation by STK4 decreases FYCO1 binding to LC3B and promotes retrograde transport of autophagosomes towards lysosomes (top panel). In turn, when LC3B phosphorylation is blocked, FYCO1 and LC3B associate more strongly, and autophagosome transport toward the nucleus is reduced (bottom panel). From Nieto–Torres et al., 2021, DOI: 10.1080/15548627.2021.1961073, with permission.

Lipid-binding proteins in promoting mitophagy

Autophagic membranes are largely devoid of transmembrane proteins.[42] Therefore, it stands to reason that lipid-binding proteins may play a key role in regulating autophagy.

Anne Simonsen from the University of Oslo described her group's screen for lipid-binding proteins in mitophagy, a form of autophagy that selectively degrades damaged or surplus mitochondria. Simonsen's group focusses on PINK1/Parkin-independent mitophagy, which is generally induced by hypoxia and HIFα activity.[43] They used the iron chelator deferiprone (DFP) to induce HIFα-mediated mitophagy, and conducted an siRNA screen to identify lipid-binding proteins that affected mitophagy. Several candidates were found to upregulate or downregulate mitophagy.

Simonsen focused on two kinases that positively regulated mitophagy, GAK and PRKCD. GAK is a cyclin G–associated kinase linked to Parkinson's disease, and PRKCD has been identified as a tumor suppressor. Simonsen showed that the kinase activities of GAK and PRKCD are required for DFP-induced mitophagy but not for Parkin-dependent mitophagy or starvation-induced autophagy. They found that GAK regulates mitochondrial and lysosomal morphology, though the mechanism remains unclear. PRKCD, which localizes to mitochondria and is turned over by mitophagy, facilitates the recruitment of the autophagic markers ATG13 and ULK1 to mitochondria. *In vivo* data in zebrafish confirm the importance of PRKCD in mitophagy.[44] While the PRKCD targets that mediate this effect are unknown, Simonsen proposed that PRKCD may phosphorylate mitophagy receptors and recruit FIP200. Alternatively, PRKCD may act in a manner analogous to PINK in PINK/Parkin-dependent mitophagy, which phosphorylates ubiquitinated outer mitochondrial proteins, leading to the recruitment of the autophagy machinery.

Autophagy-mediated removal of protein aggregates

Liang Ge from Tsinghua University presented unpublished work on the degradation of large protein aggregates. Toxic protein aggregation is a hallmark of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases. Selective autophagy is important to clear these protein aggregates and has been proposed as a potential therapeutic target for neurodegenerative disorders.[45] It is known that autophagy receptors, especially ubiquitin-binding receptors, are important for linking protein aggregates to the autophagosome membrane and for determining autophagy selectivity. Ge's group has identified a new role for chaperonins as autophagy receptors to mediate protein aggregate degradation independent of ubiquitin.

Mechanisms regulating lysosome biogenesis

Jonathan M. Goodwin from Casma Therapeutics presented work in collaboration with Oliver Florey of the Babraham Institute on a new regulatory mechanism for TFEB activation. TFEB is a TF that regulates lysosomal biogenesis and autophagy. In lysosomes, TFEB can be activated by the TRPML1, a nonselective ion channel that participates in Ca^{2+}-dependent processes like membrane fusion and controls prolonged homeostasis of the lysosomal network via TFEB activation. Casma is pursuing TRPML1 as a therapeutic target across several disease indications.

Activation of TRPML1 by small molecule agonists activates TFEB while also inducing the formation of LC3-containing punctae that colocalize with lysosomes via a mechanism dubbed conjugation of ATG8 to single membranes (CASM). Goodwin showed that the CASM machinery (e.g., ATG proteins), but not the autophagy machinery, is required for TRPML1-mediated activation of TFEB and that TFEB-dependent lysosomal biogenesis.

Goodwin showed that the regulation of TFEB by ATG proteins involves GABARAP, which binds to the FLCN–FNIP complex. FLCN–FNIP serves as a negative regulator of TFEB activation by acting as a GTPase accelerating protein (GAP) for the small cytosolic GTPases RagC and RagD. TRMPL1 activation induces relocalization of FLCN from the cytoplasm to the lysosome, which in turn sequesters GABARAP near the lysosome and prevents it from interacting with RagC. In the GDP-bound form, RagC binds to and inhibits TFEB. However, when it is unable to interact with its GAP, RagC remains GTP bound and is unable to inhibit TFEB, which can then localize to the nucleus and promote the expression of genes involved in lysosomal biogenesis. Goodwin's group mapped the binding interaction between GABARAP and FLCN/FNIP and showed that mutants that disrupt the interaction led to relocalization of FLCN/FNIP, enabling it to regulate cytosolic RagC, leading to TFEB inhibition. These data support a new mechanism for TFEB activation. Goodwin showed that this GABARAP/FNIP-dependent mechanism for TFEB activation occurs in several settings in addition to TRPML1 activation, including mitophagy, xenophagy, and in pancreatic cancer cells, where it may serve as a potential target.[46]

Degradation of membrane proteins in the endo-lysosomal system

Richa Sardana from Scott Emr's laboratory at Cornell University discussed the quality control system for membrane proteins targeted for the endosome/lysosome pathway. Ubiquitination of internalized membrane proteins leads them to the endosome membrane. They are eventually

transferred to the lumen of the endosome, and, upon fusion of the endosome with the lysosome, released into the lysosome lumen. If the cargo proteins are not properly sorted into the endosomal lumen, they can persist on the endosome membrane. When the endosome subsequently fuses with the lysosome, accumulation of improperly sorted cargo proteins can negatively affect lysosome function. In yeast, Rsp5 is the E3 ubiquitin ligase that ubiquitinates cargo destined for the endosome/lysosome pathway. It interacts with several adaptor proteins at the cell, endosome, and lysosome membranes to ensure that cargo proteins are properly ubiquitinated. Loss of the endosomal adaptor Ear1or lysosomal adaptor Ssh4 caused cargo proteins to accumulate on the lysosomal membrane. Sardana showed that the cytosolic tail of cargo proteins is critical for proper sorting. There is no specific sequence motif that regulates sorting. Instead, Rsp5 recognizes multiple unmasked lysine residues found within a specific distance from the membrane, dubbed the ubiquitination zone. This system thus incorporates multiple quality control steps along the endocytic pathway to enable Rsp5 to routinely monitor a diverse repertoire of plasma membrane proteins and prevent the accumulation of aberrant membrane proteins on the lysosomal membrane.[47]

Proteome remodeling and proteostasis

Proteome remodeling in RBCs

RBCs make up almost 70% of all cells and are continually being renewed from erythroid progenitor cells, with approximately 2 million RBCs produced every second. RBCs are specialized cells whose main job is to transport oxygen throughout the body. To achieve this, it undergoes a massive remodeling of the proteome such that approximately 98% of soluble protein consists of globulin.[3]

Daniel J. Finley from Harvard Medical School presented work toward understanding the reshaping of the proteome in RBCs to achieve such a high concentration of globulin. While several theories have been proposed, Finley is investigating the role of the ubiquitin pathway in this global remodeling. This is supported by the fact that numerous ubiquitinating enzymes are induced during late erythroid differentiation.[47] Finley's group has determined the role of some of these enzymes; for example, the ubiquitin-conjugating enzyme UBE20 mediates the elimination of ribosome proteins during terminal erythroid differentiation.[48] During his talk, Finley focused on another protein induced during erythroid differentiation, TBCEL, which Nicholas Cowan's group identified and linked to tubulin in 2005 by virtue of its sequence similarity to tubulin-specific molecular chaperones.[48] TBCEL's similarities to tubulin chaperones include the presence of a ubiquitin-like protein (UBL) domain,[48] suggesting that it could be a component of the ubiquitin pathway. Finley showed unpublished proteomic analysis from Miguel Prado and Bryan Seguinot demonstrating a role for TBCEL in reshaping the RBC cytoskeleton by eliminating several cytoskeleton proteins.

Efficient degradation of P granules by autophagy

Hong Zhang from the Chinese Academy of Sciences described the importance of phase separation in regulating autophagy in *C. elegans*. During *C. elegans* embryogenesis, specialized protein aggregates known as P granules exclusively localize to the germ cell lineages. P granules are derived from the oocyte and originally evenly disperse throughout the newly fertilized embryo. During cell division, P granules partition into both daughter cells but are quickly removed from somatic cells. Two components of P granules, PGL-1 and PGL-3, are degraded by autophagy. In autophagy mutant embryos, a large number of PGL-1/PGL-3 granules, termed PGL granules, accumulate in somatic cells. Using genetic screens, Zhang's laboratory has identified a set of metazoan-specific autophagy genes, named *epg*, involved in autophagic degradation of PGL granules as well as several genes that specifically mediate the degradation of PGL granules.[49] Zhang showed that the receptor protein SEPA-1 is required for both formation and degradation of PGL granules via direct interactions with the PGL-granule component PGL-3 and LGG-1/Atg8. Expression of SEPA-1 is temporally regulated—expression is low during early stages of embryogenesis, high expression at the ∼200 cell stage, and virtually nonexistent in late embryogenesis.[50] EPG proteins, in contrast, are required only for degradation, not formation, of PGL granules. Loss-of-function of EPG proteins causes the accumulation of PGL granules in somatic cells with no defect in degradation of other types of protein aggregates. Zhang proposed

that EPG-2 links PGL granules with the autophagic machinery,[49] while EPG-11 regulates association of PGL granules with EPG-2 by arginine methylation of PGL-1 and PGL-3.[51]

While the players involved in PGL granule formation and degradation are identified, less is known about the dynamics of PGL granule formation. Zhang's group has been working to understand how PGL proteins are efficiently degraded from the beginning of embryogenesis given that the expression of SEPA-1 and EPG3 displays distinct temporal expression patterns. P granules have been shown to form gel-like condensates via liquid–liquid phase separation.[52,53] Zhang showed that during *C. elegans* embryogenesis, SEPA-1, EPG-2, and post-translational arginine modification modulate phase separation and transition of PGL granules between liquid-like and gel-like phases. The biophysical properties of the phase-separated aggregates can affect their degradation. Zhang argued that the less dynamic gel-like state may act as a more stable platform for autophagosomal membranes.[53] An essential role for a gel-like state in triggering degradation by autophagosomes has been observed in other systems as well.[54]

The role of Hsp90 in stress granule disassembly

Serena Carra from the University of Modena and Reggio Emilia discussed the role of heat shock proteins in stress granule disassembly. Stress granules are dynamic RNA–protein complexes that form under conditions of stress and rapidly disassemble during stress recovery. Some neurological disorders, such as amyotrophic lateral sclerosis and frontotemporal dementia, are characterized by stress granules that refuse to disassemble and mature into aggregates, contributing to cell toxicity.[55–57] Stress granule disassembly and quality control is regulated by chaperones and autophagy receptors, including Hsp70, VCP, and p62.[58] These prevent the accumulation of misfolded proteins inside stress granules and target aberrant stress granules for degradation. Carra described how Hsp90 promotes stress granule disassembly and regulates stress granule dynamics. Inhibiting or depleting Hsp90 delayed stress granule disassembly (Fig. 3).[59] Hsp90 is an essential chaperone for kinases. Carra's group identified DYRK3 as a new client for Hsp90. DYRK3 is a kinase that promotes stress granule disassembly.[59,60] They showed that Hsp90 interacted directly with DYRK3 and inhibiting Hsp90 destabilized DYRK3 and resulted in its rapid degradation by the proteasome and stress granule persistence. While DYRK3 is recruited inside stress granules and other biomolecular condensates, such as splicing speckles, Hsp90 is not. Carra showed that during stress recovery, DYRK3 continuously shuttles from stress granules to the surrounding cytosol, where it is stabilized and activated by Hsp90. In addition, a DYRK3 construct that is unable to partition inside condensates formed aggregates upon Hsp90 inhibition. Carra, therefore, proposed that targeting DYRK3 to condensates upon stress protects it from aggregation. Upon stress recovery, DYRK3 leaves stress granules and is stabilized by Hsp90.[59]

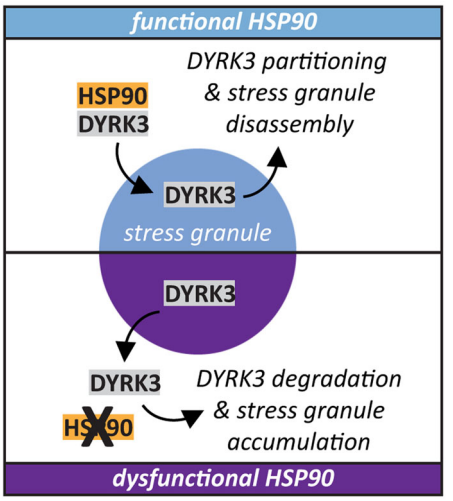

Figure 3. **Hsp90 promotes stress granule disassembly and regulates stress granule dynamics.**

Protein homeostasis in aging

A decline in protein homeostasis and the resulting protein aggregation is a feature of both normal aging and age-related neurodegenerative diseases.[61–63]

Della C. David from Eberhard Karls Universität Tübingen presented work on understanding the mechanisms that regulate proteostasis with the hope that preventing aggregation can promote healthy aging. David focused on protein aggregation control mechanisms in the intracellular and

extracellular space in *C. elegans*. Protein aggregation contributes to functional decline in *C. elegans*, including deficiencies in motility and feeding capacity. David showed that knocking down the protein quality control systems—chaperones, the proteasome, and macroautophagy machinery—led to tissue-specific changes in protein aggregation. While protein aggregation increased in the body wall muscle, it decreased in the pharyngeal muscles. David showed that defects in the protein quality control system trigger a safety mechanism in the pharynx that prevents accumulation of newly synthesized aggregation-prone proteins by targeting them for degradation via the lysosome.[64,65]

RNA-seq analysis identified several genes that may be participating in this safety mechanism, including genes involved in the response to intracellular pathogens.[66] David put forth a model wherein the proteome naturally becomes unstable and aggregates as part of normal aging. If the protein quality control system is also impaired, aggregation accelerates in tissues like the body muscles. However, in the pharyngeal muscles, a safety mechanism is triggered in which unstable aggregation-prone proteins are directly targeted to the lysosome.[64,65]

David also described work on understanding the mechanisms for extracellular proteostasis. Extracellular proteins are subject to damage via oxidation and mechanical stress; however, little is known about the extracellular protein quality control components, partly because of a lack of good models available for extensive genetic screens in which to evaluate it. David's laboratory has developed a model to study extracellular protein aggregation in *C. elegans*. They focused on the secreted protein LBP-2, which is diffusely located in young worms but forms extracellular punctae during aging. A systematic RNAi screen targeting predicted secreted proteins identified 57 putative regulators of LBP-2 aggregation. David described one of these, C36C5.5, a novel extracellular holdase chaperone that directly binds to and stabilizes LBP-2. David stressed that extracellular proteostasis may play a role in aging and systemic defense. Overexpressing extracellular regulators was associated with life span extension and increased survival in response to an intervention that mimics a pathogenic attack.[67]

Epigenetic factors that contribute to life span

While genetics play a role in determining life span,[68–70] it is clear that there are other, stochastic factors at play as well. For example, in *C. elegans*, isogenic organisms grown under identical conditions demonstrate a range of life spans.

Ursula Jakob from the University of Michigan discussed work on understanding what contributes to this stochasticity in life span, focusing on the role of reactive oxygen species (ROS). ROS can act as signaling molecules and influence redox-active proteins involved in metabolism, growth, cell differentiation, and gene expression. Using *in vivo* redox-sensitive proteins[71,72] to monitor the redox state of *C. elegans* in real time, Jakob's group showed that there was high interindividual variance in redox state in *C. elegans* larvae early in development. This variance correlated with future redox states as well as life span. Larvae that had a more oxidized state were more likely to have a reduced state as adults, were more stress resistant, and had longer life spans than larvae that had a more reduced state. In addition, altering the redox state during early development affected life span. While the source of this variability in redox states in larvae is unknown, Jakob noted that younger maternal age is associated with a higher oxidized state among offspring.[73]

Jakob showed that larvae with a higher oxidized state had lower levels of H3K4 methylation. This epigenetic mark is established during the early larval stage in *C. elegans* and is reflective of transcriptional memory with few global changes in gene expression.[73] Previous work has shown that deficiencies in H3K4 methylation are associated with longer life span.[74,75] Jakob showed that H3K4 methylation abrogates the redox-dependent stress resistance and life span observed, suggesting that downregulation of H3K4me3 is likely necessary and sufficient for these effects. They put forth a model in which naturally occurring increases in ROS during early development are associated with downregulation of H3K4 methylation among a subpopulation, which ultimately results in increased life span. Similar redox regulation of histone modifications has been observed in mammalian systems.[73] Jakob's group is currently investigating whether strategies that extend life span can also slow aging-related diseases using *C. elegans* as a model for neurodegenerative diseases.

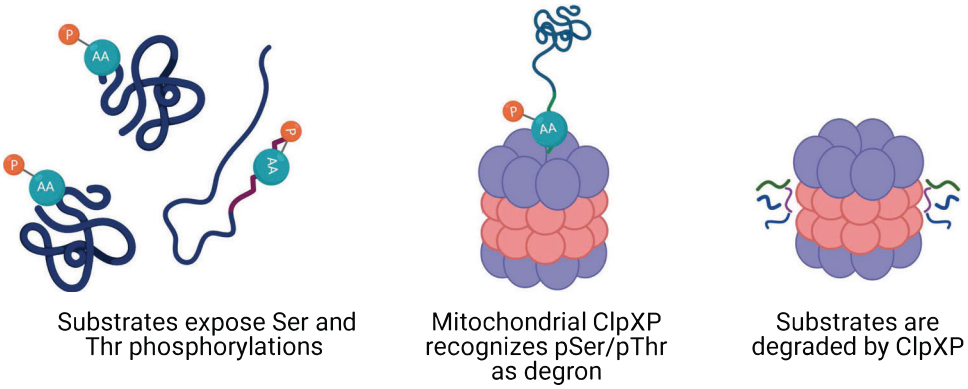

Figure 4. **Model of mitochondrial ClpXP degradation.**

Organelle-specific protein degradation pathways

Regulation of inner nuclear membrane protein degradation

Yangnan Gu from the University of California, Berkeley presented work to understand the regulation of inner nuclear membrane (INM) protein degradation. INM proteins can transduce signals from the cytoplasm to the chromatin, thus impacting chromatin organization and gene expression. Overaccumulation of these proteins has been implicated in rare genetic diseases.[76] Therefore, INM proteins must be carefully maintained, with excess proteins removed in a timely manner. Using proximity labeling in plants, Gu's group has shown that the INM proteome contains multiple members of the ubiquitin degradation pathway as well as CDC48,[77] which facilitates the degradation of endoplasmic reticulum proteins by extracting ubiquitinated proteins from the membrane.[78] They also identified several plant ubiquitin regulatory X (PUX) domain-containing proteins. Gu put forth a model in which INM proteins are polyubiquitinated by an E3 ligase that interacts with the INM. Polyubiquitination recruits the CDC48 complex, which mediates substrate degradation via the 26S proteasome. PUX 3, 4, and 5 also associate with the INM and serve as a negative regulator of substrate degradation, though the mechanism is unclear.[77]

Substrate specificity for the mitophagy-specific proteasome-like complex ClpXP

Protein quality control in mitochondria is essential to remove damaged proteins and maintain the integrity of the respiratory chain function. Mitochondria contain four proteasome-like complexes: i-AAA and m-AAA, which are involved in inner membrane and matrix protein quality control, respectively; LONP1, which degrades oxidatively damaged proteins, and ClpXP, a serine protease located in the matrix. ClpXP is overexpressed in multiple malignancies. Both inhibition and hyperactivation of ClpXP have been shown to impair oxidative phosphorylation and have anticancer effects.[79] **Yue Feng** from Aaron Schimmer's laboratory at the University of Toronto presented unpublished work on identifying marks for degradation of the mitochondrial ClpXP (Fig. 4) to understand how it maintains substrate specificity.

Cargo receptors in lysophagy

Vinay V. Eapen from Wade Harper's laboratory at Harvard Medical School discussed that defective lysosomes are repaired. Lysosomes are subjected to several sources of damage, including pathogens, aggregates, aging, and iron overload. If left unchecked, lysosomal damage can trigger an inflammatory response and result in damage to other organelles. While limited damage to the lysosome membrane can be resealed by the ESCRT proteins, more extensive damage leads to the selective autophagy of lysosomes, that is, lysophagy. In this process, galectins recognize glycan moieties on the luminal side of transmembrane lysosomal proteins, leading to the ubiquitination of lysosomal proteins, recruitment of autophagy receptors, and formation of an autophagosome around the damaged lysosome.[80,81] Eapen is working to better understand the factors required during lysophagy. Taking an unbiased mass spectrometry-based approach,

Eapen identified the factors recruited to damaged lysosome, including galectins and several autophagy receptors. Using a novel technique that quantitatively assesses lysophagic flux, they showed that the kinase TBK1 is required for lysophagy. They also elucidated the roles of autophagy receptors TAX1BP1 and OPTN. While both these receptors link ubiquitin to the autophagic machinery, Eapen showed that the ubiquitin binding capacity of TAX1BP1 is only partially required for TAX1BP1 recruitment, suggesting that it could be recruited via a ubiquitin-independent process. In contrast, ubiquitin binding is required for OPTN recruitment. Eapen's work helps to fill in some of the gaps during lysophagy and provides clear evidence of a role for TAX1BP1 and TBK1 in lysophagic flux.[82]

Cargo receptors in ER-phagy

ER-phagy is a highly conserved process in which portions of the ER are degraded via the autophagy-lysosome system.[83] Several ER-resident proteins have been identified as ER-phagy receptors that target portions of the ER to autophagosomes via their LIR domain, including FAM134B.[84]

Alessio Reggio from the Telethon Institute of Genetics and Medicine discussed the role of FAM134 proteins in ER-phagy. FAM134 is a family of proteins consisting of three paralogs, FAM134A, B, and C. While the role of FAM134B in autophagy is well recognized, it was unknown whether FAM134A and C are involved as well. Reggio characterized the FAM134 paralogs and showed that all three have a highly conserved LIR domain and that they are involved in ER-phagy. In cells, the three paralogs colocalized with the autophagy protein LC3B and are recruited to autophagosomal membranes under starvation. Disrupting the LIR sequence in any of the FAM134 proteins abrogated starvation-induced autophagy. Reggio concluded that the FAM134 proteins regulate ER shape via ER-phagy by delivering portions of the ER to the lysosome for degradation.[85]

Maintaining protein quality control during translation

Eric J. Bennett from the University of California, San Diego discussed the role of ubiquitylation during ribosome-associated quality control. The ribosome sits at the center of the protein homeostasis network. There are many quality control issues that arise during mRNA translation requiring efficient removal of either damaged nascent polypeptide, truncated mRNA, or defective ribosomes. Bennett's group is working to understand how the cell recognizes and deals with stuck or defective ribosomes. Work in his laboratory, as well as other laboratories, has documented that site-specific ubiquitylation of the eS10 and uS10 40S ribosomal proteins are key early events that help triage collided elongating ribosomes. While several players in this process have been identified, the precise mechanistic role ubiquitin plays during quality control is still unclear.

Research in Bennett's lab has identified additional lysine residues that are ubiquitylated in response to diverse protein homeostasis stressors. The 40S ribosomal proteins uS5 and uS3 are ubiquitylated in response to both translation elongation inhibition and activation of the integrated stress response. Compared to eS10 and uS10 ubiquitylation, uS5 and uS3 ubiquitylation occurs by a separate E3 ligase, RNF10. In addition, blocking ubiquitylation of uS3 and uS5 does not impact the canonical ribosome-associated quality control pathway.[86] Structural insights on collided ribosomes show that uS3 and uS5 are located at the ribosome collision interface.[87,88] This, and other observations, led to the hypothesis that uS3 and uS5 ubiquitylation may be involved in 40S quality control events that occur prior to translation initiation.

Translation occurs via a series of sequential steps. In brief, the 43S preinitiation ribosome complex binds to the 5′ end of mRNA and scans along it until it encounters a start codon. At the start codon, various factors dissociate from the ribosomal complex, and the 40S subunit is joined by the 60S subunit, forming the 80S ribosome, which initiates translation elongation. Research in Bennett's lab showed that uS3 and uS5 ubiquitylation is enhanced upon pharmacological inhibition of 43S scanning, as well as introduction of an elongation inhibitor that specifically affects 80S ribosomes at the start codon. In addition, high doses of elongation inhibitors, which would be expected to stall all ribosomes and block elongation collisions, resulted in uS3 and uS5, but not eS10 or uS10 ubiquitylation. Conditions that stimulated constitutive uS3 and uS5 ubiquitylation result in 40S, but not 60S, ribosomal protein degradation in a manner that does not depend upon the canonical autophagy

pathway. Together, the results suggest that a separate arm of the ribosome quality control pathway, initiation ribosome-associated quality control (iRQC), targets either terminally stalled isolated preinitiation complexes or collided preinitiation complexes for degradation.[86,89,90]

Interplay between refolding and ubiquitin-mediated degradation for cytosolic and nuclear proteins

Judith Frydman from Stanford University discussed how the chaperone and ubiquitin machineries communicate with each other to determine whether a misfolded protein is degraded or refolded. Frydman's group has identified chaperone/E3 ligase circuits that cooperate to target misfolded proteins for degradation by the UPS. They showed that there are distinct circuits for cytoplasmic and nuclear protein quality control and identified key differences in how clearance of misfolded proteins is achieved in these two compartments. For example, clearance of cytoplasmic misfolded proteins requires mixed-chain K11 and K48 ubiquitin linkages, while clearance of nuclear misfolded proteins requires K48 linked chains and depends on the ubiquilin Dsk2.[91] During her talk, Frydman focused on unpublished work that builds on these previous findings to understand how the protein quality control machinery is spatially organized within the cell and how the nuclear and cytoplasmic systems coordinate with each other.

Targeting protein degradation pathways for cancer therapies

Novel mechanisms for proteasome inhibition

The proteasome is a target for anticancer therapies. Most agents, like the proteasome inhibitor bortezomib, target the proteasome core.[92]

Kylie J. Walters from the National Cancer Institute described work to identify small molecules that target proteasome ubiquitin receptor, Rpn13.[93,94] *In vitro* studies suggest that Rpn13 may be a viable target for anticancer therapies. A class of small molecules, including RA190, that bind hRpn13[95–97] and an Rpn13-binding peptoid, KDT-11,[98] decreased cancer cell survival, induced apoptosis, and restricted mouse xenograft models of ovarian cancer and multiple myeloma. However, there is conflicting evidence on whether the physiological target of RA190 and KDT-11 is indeed Rpn13. RA190, which attaches to cysteine residues, was shown to be very promiscuous, interacting with dozens of cellular proteins.[99] Changing the level of Rpn13 was found to have no effect on RA190[99] or KDT-11[100] activity in cancer cell lines in two studies, whereas in others, Rpn13 (*ADRM1*) deletion by gene editing revealed a requirement for Rpn13 in RA190-induced apoptosis.[101,102] Walters's group is working to identify more specific Rpn13-targeting molecules and the mechanism of action for Rpn13-dependent induction of cell death. This group and another have previously solved the structure of Rpn13 bound to its proteasomal docking site on Rpn2.[103,104] During her talk, Walters presented unpublished data characterizing the interaction between novel small molecules and Rpn13 and the mechanism by which Rpn13 targeting leads to apoptosis.[105] The Rpn13-binding molecules were identified by using a virtual screen based on the Rpn13:Rpn2 structure coupled with biophysical screening. These compounds were expanded to include E3 ligase warheads, generating Rpn13 PROTAC molecules.

Targeting the DUB USP7 reveals tumor vulnerabilities and informs combination therapies

Ingrid E. Wertz from Bristol Myers Squibb presented work from her time at Genentech on how targeting the DUB USP7 has provided valuable insights on tumor vulnerabilities and effective treatment combinations. USP7 regulates the ubiquitination status of MDM2, a ubiquitin ligase that regulates p53 activity. Inhibition of USP7 promotes the degradation of MDM2, thus stabilizing p53 and increasing the activation of cell cycle arrest and cell death pathways.[106–108] Genentech, in partnership with Almac Therapeutics, identified an oral USP7 inhibitor with robust USP7 inhibition that resulted in increased MDM2 ubiquitination in cells.[109] In mice, however, USP7 inhibition resulted in gastrointestinal tract and hematopoietic toxicity. Wertz showed that these effects were on-target toxicities associated with USP7 inhibition and that they were only partially dependent on p53, suggesting that USP7 inhibition affects p53-independent pathways. To enhance efficacy and limit toxicity, the group investigated synergistic combination treatments. Wertz and colleagues profiled compound libraries in a variety of solid tumor cell lines, and

found that PI3K inhibition synergized with USP7 inhibition. Combining the two agents promoted significant tumor growth inhibition, but did not ameliorate toxicity in mice. In order to better understand the mechanisms of USP7-induced toxicity, the group used RNA-seq, pharmacogenomics, and proteomics approaches to compare the impact of USP7 inhibition and MDM2 inhibition in several cell lines, given that both types of inhibitors stabilize p53 via different mechanisms and result in hematopoietic and gastrointestinal toxicity. They found that USP7 uniquely perturbs pathways involved in polycomb repressive complex (PRC) derepression, cell cycle arrest, and DNA damage response, whereas MDM2 antagonists induce cell cycle arrest and DNA damage. Given that other DNA damage response–inducing agents cause hematopoietic and gastrointestinal toxicity, the group concluded that the effect of USP7 inhibition on this pathway was most likely responsible for the hematologic and gastrointestinal toxicity observed. A triplet combination that promotes PRC derepression, cell cycle arrest, and inhibition of PI3K maintained tumor growth inhibition efficacy seen with combined USP7 and PI3K inhibition, and improved tolerability. This work shows how delineating the effects of DUB inhibition can reveal vulnerabilities in tumor cells that can be leveraged to design effective, tolerable treatment combinations.

Molecular glues bring new substrates for E3 ubiquitin ligases

Immunomodulatory imide drugs (IMiDs), including lenalidomide and pomalidomide, are common agents for treating hematological malignancies. The primary target of IMiDs is CRBN, a substrate receptor of the CUL4 CRL. When bound to CRBN, IMiDs recruit new substrates, such as Ikaros/Aiolos and CK1α, to the complex, thus mediating their ubiquitination and subsequent degradation.[110]

Nicolas H. Thomä from the Friedrich Miescher Institute discussed how small molecule IMiDs serve as molecular glues to target novel substrates to the E3 ligase for ubiquitination and degradation. The structure of CRBN bound to lenalidomide and CK1α, solved in Thomä's group, shows that lenalidomide sits at the interface of CRBN and a β-hairpin loop within CK1α. This positions CK1α in such a manner so that it can be ubiquitinated by an E2 ligase attached to the CUL4–CRBN complex.[111]

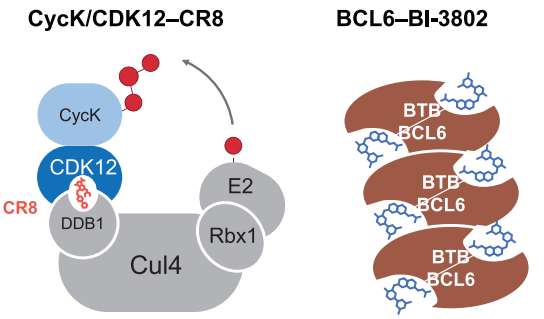

Figure 5. (*Left*) **Binding of kinase inhibitor CR8 to CDK12.** (*Right*) **Inhibitor BI-3802 induces BCL6-containing foci.**

Lenalidomide is also able to recruit Ikaros/Aiolos via a β-hairpin loop in the zinc-finger (ZFN) motif. Interestingly, there is no obvious sequence similarity between the β-hairpin loops in Ikaros/Aiolos and CK1α. Thomä's laboratory is interested in understanding the rules that govern specificity for IMiD-mediated recruitment to CRBN. They conducted a high-throughput ZFN library screen to identify new targets of IMiD-mediated CRBN degradation. While many ZFNs bind to CRBN upon addition of an IMiD, only a few are degraded. Altering the IMiD changed the specificity of the system. Thomä is working to understand the rules that govern IMiD-mediated binding and degradation with the goal of developing a system that can selectively degrade different ZFN-containing proteins.[112]

Thomä and **Mikołaj Słabicki** from Benjamin Ebert's group at Dana Farber Cancer Institute described a joint project to understand whether other drugs mediate protein degradation via similar molecular glue effects. Słabicki identified a kinase inhibitor, CR8, whose cytotoxicity correlates with the expression of DDB1, a component of the CUL4 ubiquitin ligase. Whole proteome quantification following CR8 treatment allowed the identification of CycK as the substrate for CR8-mediated degradation (Fig. 5, *left*). Słabicki described a cell sorting-based CRISPR screen approach to identify the E3 ligase machinery involved. In short, the protein of interest (CycK) is fused to an eGFP protein in a stability reporter and a pooled CRISPR library is used to knock out genes. Cells in which CycK degradation is attenuated are sorted and sequenced to identify the components whose deletion abrogated degradation. Using this strategy, Słabicki identified DDB1 and CDK12 required for CycK

degradation. Thomä's group solved the structure of the system and showed that, unlike the IMiD example, CR8-mediated degradation of CycK does not require a substrate receptor. Instead, CR8 induced binding between CRL and CDK12, which positions the CDK12 binding partner CycK in close proximity to E2, thus facilitating its ubiquitination and degradation.[113–115]

Słabicki also described a collaborative project with Eric Fischer's laboratory to understand how the small molecule BI-3802 induces degradation of BCL6. Treatment with BI-3802 induces BCL6-containing foci (Fig. 5, *right*). Słabicki conducted an alanine screen to identify restudies in BCL6 important for foci formation and degradation. Using a similar flow-based reporter assay as described above, Słabicki identified that BCL6 mutants E42 and Y58 are required for BI-3802–mediated degradation. The cryo-EM structured of BI-3802–induced BCL6 helical filaments of BCL6 confirms that these residues are critical for BCL6 polymerization and subsequent degradation.[116] Słabicki stressed that understanding the mechanisms by which drugs induce protein degradation via acting as molecular glues can expand the repertoire of druggable targets.

Induced proximity in drug development

Induced proximity agents, including PROTACs and LYTACs, are small molecules that serve as molecular bridges to link effector and target molecules. In terms of protein degradation, induced proximity agents, dubbed PROTACs, can link a protein target to an E3 ligase to facilitate its ubiquitination and degradation by the proteasome. This approach has the potential to target previously undruggable targets. Several PROTACs have been shown to mediate selective protein degradation in preclinical models, and the first in-human trials of such a compound, sponsored by Arvinas, were initiated in 2019.[117–119]

Induced proximity to target previously undruggable targets

Raymond J. Deshaies from Amgen described the company's approach to using induced proximity in drug discovery. One example of induced proximity that Amgen is currently working on is the development of bispecific T cell engagers that bind to T cells and effector cells, thus enabling T cells to selectively destroy diseased cells. Deshaies presented unpublished work on how induced proximity can mediate cancer cell death in SMARCA4-mutated lung cancer. Approximately 5% of patients with lung cancer have mutations in SMARCA4. SMARCA4 and its paralog SMARCA2 mediate chromatin remodeling and are essential for cell survival. Loss of SMARCA4 in cancer cells makes cells reliant on SMARCA2 activity for survival.[120] Therefore, inhibiting SMARCA2 should induce synthetic lethality in cancer cells while having limited effects on cells with intact SMARCA4. Designing a small molecule drug that selectively inhibits SMARCA2 has been difficult due to the similarities between the two homologs. Amgen is working to develop a PROTAC that mediates targeted degradation of SMARCA2 to selectively kill SMARCA4-mutated cancer cells.

Deshaies believes that targeting protein degradation is the tip of the iceberg for induced proximity strategies. For example, protein stabilization could be mediated by inducing proximity of a protein target to a DUB. TFs could be inactivated by linking them to cytosolic proteins. Enzyme activity can be mediated by inducing proximity of an enzyme–substrate pair. Deshaies focused on non-protein applications of induced proximity as well, specifically mediating RNA degradation via multispecific RIBOTACs. RNA degradation can mediate the inactivation of truly undruggable targets. For example, many TFs are unstructured in the absence of DNA and have no functional binding sites for small molecules or functional pockets for PROTAC binding. Targeted RNA degradation by bringing together a target mRNA and an RNA-degrading enzyme can prevent the protein from being made. Deshaies showed preliminary work on developing screens to identify RIBOTACs.

Deshaies believes that induced proximity, of which targeted degradation is a part, can usher in a new paradigm in drug development centered around multispecificity that can achieve profound effects on cells and effect previously undruggable targets.[117]

Targeting a SARS-CoV-2 protease to block infection and increase the immune response

Ivan Dikic from Goethe University discussed efforts to target the ubiquitin pathway in infectious diseases. While bacteria do not have ubiquitin or E1 or E2 enzymes, they do contain E3 ligases and

DUBs that are injected into the cytosol of host cells to hijack the host ubiquitin system. At the same time, the host ubiquitin system plays a role in innate immunity. For example, Dikic's group showed that *Salmonella typhimurium* couples with linear ubiquitin chains in the cytoplasm, which leads to the secretion of proinflammatory cytokines via the NF-κB pathway and selective autophagy.[121–123] Viruses are also known to manipulate the host ubiquitin system to promote budding, enhance protein production, block host restriction factors, and block innate immunity.[124] Developing therapeutics that target the ubiquitin system in infection must, therefore, inhibit the pathogen's effect on the system while strengthening the host's defensive aspects of the system.

Dikic described work done in conjunction with ProxiDrugs, a regional network of academic and industry partners, to develop drugs that act through a proximity-based mechanism. They focused on efforts to develop a proximity-based drug that targets the SARS-CoV-2 papain-like protease (PLpro). SARS-CoV-2 contains two proteases, PLpro and C3-like protease, that cleave the viral polypeptide to form the replicase complex necessary for viral replication. The proteases have been the subject of several structural studies in the effort to develop protease inhibitors.[125–130] Dikic's group has solved the crystal structure of PLpro in complex with ISG15, a ubiquitin-like protein that mediates an interferon (IFN)-induced immune response. Cleavage of mISG15 by PLpro inhibits the host innate immune response in addition to promoting viral replication. Interestingly, SARS-CoV-1 contains a similar protease that preferentially cleaves ubiquitin over ISG15; this activity inhibits NF-κB–mediated host immunity.[128] A small molecule inhibitor of SARS PLpro developed in Andrew Mesecar's group[131] blocked viral infection and reduced SARS-CoV-2 replication and release, while increasing the antiviral IFN response in infected cells.[128] Dikic's group is working to develop a proximity-based drug that mediates targeted degradation of PLpro.

LYTACS: targeting transmembrane and extracellular protein degradation

Carolyn R. Bertozzi from Stanford University discussed efforts to target protein degradation through the endosome/lysosome pathway. PROTACs, which generally promote target protein ubiquitination and subsequent proteasomal degradation, can only affect cytosolic proteins. Transmembrane and extracellular proteins, which make up a significant portion of the proteome, are typically degraded by the endosome/lysosome system. Many of these proteins, including cytokines, aggregates, adhesion molecules, and receptor tyrosine kinases, are attractive targets for therapeutics.

Bertozzi's group is working to develop bifunctional molecules—lysosomal-targeting chimeras (LYTAC)—that target proteins to the lysosome for degradation. They focused on the membrane receptor M6PR, which recognizes mannose-6-phosphate moieties on target proteins and traffics them to the lysosome for degradation.[132] Bertozzi showed several proof-of-principle studies in which a LYTAC composed of a mannose-6-phosphate glyco-polypeptide moiety chemically conjugated to an antibody can lead to target protein degradation. For example, conjugation of cetuximab, a monoclonal antibody that targets EGFR and is approved for several types of cancer, to a mannose-6-phosphate moiety induces EGFR internalization and degradation in cells. A similar LYTAC using the FDA-approved anti-PD-L1 antibody atezolizumab targets PD-L1 for lysosomal degradation, overcoming the endogenous endosomal recycling pathway that typically maintains high membrane PD-L1 levels.[133,134]

Bertozzi's group is working on leveraging other lysosomal trafficking receptors, such as the asialoglycoprotein receptor (ASGPR), which specifically targets liver proteins for degradation. An ASGPR LYTAC targeted to HER2 was shown to degrade HER2 in a xenograft mouse model.[135] The group is also working on developing LYTACs for targets that have yet been intractable to traditional antibodies and looking beyond proteins to target factors, such as glycans, lipids, nucleic acids, aggregates, and viruses.

Competing interests

The authors declare no competing interests.

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