Instituto Juan March
de Estudios e Investigaciones

145 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# The Ubiquitin-Proteasome System

Organized by

A. Ciechanover, D. Finley, T. Sommer and C. Mezquita

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# Introduction
Daniel Finley

Protein degradation, originally thought to function primarily in the elimination of defective proteins, is now understood to be a central regulatory mechanism in eukaryotes. In targeting proteins for degradation by the proteasome, ubiquitination controls the activity of regulatory proteins in response to extracellular signals, stress, developmental transitions, checkpoint activation, and cell cycle transitions. The same modification can also confer nonproteolytic fates on its target proteins. Among these alternative functions of ubiquitination, the best understood—and most extensively covered in the workshop—are in the sorting of membrane proteins. Ubiquitination can signal endocytic events, sorting of proteins into the interior vesicles of the multivesicular body (MVB), sorting of proteins exiting the golgi body, and the budding of many viruses. Within the nucleus, ubiquitination plays nonproteolytic roles in DNA repair and transcription. This type of function was best illustrated in the workshop by studies linking ubiquitination of the DNA polymerase-associated processivity factor PCNA to DNA repair.

It is largely unclear how the same protein modification can have such a variety of functions. At least four principles that may underlie the triaging of ubiquitin-conjugates to alternative fates were discussed. First, ubiquitin is often added to proteins in the form of a multiubiquitin chain, and such chains are thought to be required for recognition of substrates of the proteasome. Other functions of ubiquitination, such as in endocytosis and sorting into the MVB, are driven by monoubiquitination. Second, the multiubiquitin chain can be polymerized using alternative lysine residues in ubiquitin, to form topologically distinct chain configurations. For example, lysine-48-linked chains are critical for targeting proteins to the proteasome, while lysine-63-linked chains serve nonproteolytic roles in DNA repair, protein kinase activation, and translational control. Third, conjugated ubiquitin can be recognized together with additional signals, such as specific phosphoinositides. Finally, it is likely that the localization of proteins, especially whether they are soluble or membrane-bound, helps to determine the way in which ubiquitination affects their fates. This principle has emerged most clearly from studies of the ERAD pathway for the degradation of endoplasmic reticulum proteins, as discussed by several speakers.

The scope of the ubiquitin system is still poorly defined, however, mammalian genomes contain over 600 genes encoding probable components of the ubiquitin-proteasome pathway. Most of these genes encode putative ubiquitin-protein ligases, or E3 enzymes, the primary specificity factors of the system, but a surprising number encode deubiquitinating enzymes. Obviously, the bulk of enzymes in this system remain completely uncharacterized.

Recent studies have also described a family of ubiquitin-like molecules. Although only partially characterized as a group, these molecules have so far been implicated in the regulation of nucleo-cytoplasmic transport, DNA repair, photomorphogenesis, the cytoskeleton, topoisomerase activity, centriole duplication, cell cycle checkpoint control, inflammation, autophagy, and other processes. These aspects of the ubiquitin family were best represented at the workshop by the elegant recent work on autophagy. Ubiquitin-like proteins also serve as positive and negative regulators of the ubiquitin pathway itself. Negative regulation is achieved by competing with ubiquitin for modification of specific sites within substrate proteins, while positive regulation is achieved either by covalent modification of ubiquitin-ligase subunits or by noncovalent association with the proteasome.

If the success of a meeting is best gauged by its open discussions, this was a splendid few days. The intimate size of the workshop, its schedule, perhaps even the unique venue elicited friendly interaction and constructive thinking. It was the most skilfully but also the most creatively organized conference in my experience, and we are most grateful to the people at the Juan March Foundation for hosting us.

Daniel Finley

# Session 1: Ubiquitination, the cell cycle and signal transduction
## Chair: Stefan Jentsch

# The ubiquitin system for protein degradation and some of its roles in cell cycle control

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The programmed, ubiquitin-mediated degradation of positive and negative regulators of the cell division cycle is responsible for several cell cycle transitions. We have been studying two ubiquitin ligase complexes that have important roles in different aspects of cell cycle regulation. One is the cyclosome, or Anaphase-Promoting Complex (APC), which acts on mitotic cyclins and some other regulators in exit from mitosis. The cyclosome is activated at the end of mitosis by phosphorylation, a process that allows its further activation by the ancillary protein Fizzy/Cdc20. A different complex, which belongs to the SCF (Skp1-Cullin-F-box protein) family of ubiquitin ligases, is involved in the degradation of p27, a mammalian G1 Cdk inhibitor, following mitogenic stimulation. Its action is triggered by Cdk2-dependent phosphorylation of p27, as well as by the increase in levels of a specific F-box protein, Skp2, that takes place in the G1 to S-phase transition. The binding of phosphorylated p27 to the $SCF^{Skp2}$ complex requires Cks1 (cyclin-dependent kinase subunit 1), a small cell cycle regulatory protein that has high-affinity binding sites for Skp2, cyclin-dependent kinases and phosphorylated proteins. All three binding sites of Cks1 are required for its action to facilitate p27-ubiquitin ligation by the $SCF^{Skp2}$ complex.

## Discovery of a new family of isopeptidase enzymes based on the 'JAMM' motif

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COP9 Signalosome (CSN) cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF ubiquitin ligases. The Jab1/MPN domain metalloenzyme (JAMM) motif in the Jab1/Csn5 subunit was found to underlie CSN's Nedd8 isopeptidase activity. JAMM is found in proteins from archaea, bacteria and eukaryotes, including the Rpn11 subunit of the 26S proteasome. Metal chelators and point mutations within JAMM abolished CSN-dependent cleavage of Nedd8 from Cul1, yet had little effect on CSN complex assembly. Optimal SCF ubiquitin ligase activity in yeast and both viability and proper photoreceptor cell (R cell) development in *Drosophila melanogaster* required an intact Csn5 JAMM domain. We propose that JAMM isopeptidases play important roles in a variety of physiological pathways.

Among the proteins that contain a JAMM motif is the Rpn11 subunit of the 26S proteasome. Although ubiquitin is recycled from proteasome substrates, the molecular basis of deubiquitination at the proteasome and its relationship to substrate degradation remain unknown. Mutation of the predicted active site histidines in the JAMM domain of Rpn11 to alanines (*rpn11AXA*) was lethal and stabilized ubiquitin pathway substrates in yeast. $Rpn11^{AXA}$ mutant proteasomes assembled normally, but failed to either deubiquitinate or degrade ubiquitinated Sic1 *in vitro*. Our findings reveal an unexpected coupling between substrate deubiquitination and degradation, and suggest a unifying rationale for the presence of the lid in eukaryotic proteasomes.

Given that Csn5 activity is important for SCF ubiquitin ligase function, and that Rpn11 activity is essential for the function of the proteasome, JAMM-dependent isopeptidases are attractive targets for anti-cancer drugs. Screening of a compound library (in collaboration with Randy King, Harvard Medical School) has revealed small molecules that disrupt Rpn11-dependent deubiquitination with a sub-micromolar Ki. Small molecule inhibitors of Rpn11 and Csn5 may point the way to a new generation of anti-cancer therapeutics.

# The N-terminal residue of the protein substrate as a novel ubiquitination target

Aaron Ciechanover, Sigal Aviel, Eyal Reinstein, Limor Dori, Ronen Ben-Saadon, and Kristin Breitschopf

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Degradation of a protein via the ubiquitin pathway involves two steps: (i) generation of a substrate-anchored polyubiquitin chain, and (ii) proteolysis of the tagged substrate by the 26S proteasome. In most cases, the first ubiquitin moiety is attached to an internal Lys residue of the target protein. In some cases however, that of transcription factor MyoD (1), the Epstein Barr Virus (EBV) proteins LMP1 (2) and LMP2A (3), and the Human Papillomavirus (HPV) oncoprotein E7 type 16 (4), for example, it is conjugated to the free N-terminal residue and generates a fusion chimera between ubiquitin and the substrate. The polyubiquitin chain is then synthesized on an internal Lys residue of the N-terminally fused ubiquitin. Substitution of all the Lys residues of these proteins with Arg or Ala did not affect their conjugation to ubiquitin or degradation. In contrast, blocking of the N-terminal residue by chemical modification inhibited significantly both conjugation and degradation of these proteins. While all these experiments strongly support the notion that ubiquitination occurs indeed on the N-terminal residue, a direct evidence has been missing. Recently, we have been able to demonstrate directly that this is indeed the case. Insertion of a specific proteolytic site (that of the Tobacco Etch Virus, TEV; contains 7 amino acids that are cleaved at their C-terminus by the TEV protease) at the N-terminal domain, generated, following digestion with the TEV protease, a ubiquitin derivative that contains in its C-terminal domain the TEV site. Additional studies led to better mechanistic understanding of this novel type of modification. Deletion of a short N-terminal segment from these proteins or attachment of a tag (6 x myc) to their N-terminal, but not to their C-terminal residue, stabilized them. Fusion of the N-terminal segment of these proteins to the N-terminal residue of GFP, an otherwise stable protein, rendered the fused proteins susceptible to degradation. Thus, it appears that the N-terminal domain serves as an important determinant directing this novel mode of ubiquitination. Mechanistically, the N-terminal domain can serve as a binding site to a specific E3. Additional related studies aimed at elucidating the processes that led to the evolution of this unique tagging mechanism, revealed that many proteins do not contain a single Lys residue. An obvious and immediate problem that arises is whether degradation of these proteins traverses the ubiquitin pathway, and if they are targeted by the system, what are the underlying mechanisms involved. We have recently shown that naturally occurring lysine-less proteins such as the CDK inhibitor p16 and the HPV oncoprotein E7 type 58 are degraded by the ubiquitin system following their ubiquitination, most probably at the N-terminal residue. Thus, N-terminal ubiquitination appears to be a novel form of modification characteristic to a broad subset of proteins that cannot be ubiquitinated on internal lysine residues or that do not contain internal Lys residue. For proteins that contain internal Lys residues, it is possible that they are not localized to appropriate ubiquitin ligase recognition motifs, and are therefore not accessible to these enzymes.

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## Regulation of proteolysis during the fission yeast meiosis by APC activators

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Meiosis is the developmental program by which sexually reproducing diploid organisms generate haploid gametes. In yeast, meiosis is followed by spore morphogenesis. These two events are normally coordinated in such a way that spore formation is dependent upon completion of the meiotic nuclear divisions. We have looked at the regulation of the Anaphase Promoting Complex (APC) during meiosis and sporulation in fission yeast by studying the function of the APC regulators Ste9p, Mfr1p, Mfr2p and Mfr3p. Ste9p is present in both mitotic and meiotic cells but it is not necessary to complete the meiotic nuclear divisions or for sporulation. Ste9p, however is required for spore viability. Mfr1p is a meiotic specific protein which is necessary for the rapid degradation of the Cdc13 cyclin at the end of meiosis II. A *mfr1* null mutant completes meiosis II but retains high levels of cdc13 and cdc2 kinase activity and is considerably delayed for spore formation. By analogy with the mitotic cell cycle, where proteolysis of cyclins and inactivation of cdc2 kinase are necessary to trigger mitotic exit and cytokinesis, we propose that at the end of meiosis a rapid and timely proteolysis of cyclins is required to switch on the differentiation program that eventually leads to the formation of haploid gametes. Mfr2p and Mfr3p are proteins related to Mfr1p that are also induced during meiosis. Cells lacking Mfr2p cannot complete meiosis.

## Session 2: Membrane Trafficking
## Chair: Thomas Sommer

## Monoubiquitin-binding proteins in the endocytic pathway

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Monoubiquitin serves as a sorting signal at different steps of the endocytic pathway and regulates the activity of the endocytic machinery. Monoubiquitin signals are likely to be recognized by ubiquitin-binding proteins that transmit the regulatory information conferred by monoubiquitination. We have identified and characterized endocytic proteins carrying monoubiquitin-binding domains. These domains include ubiquitin-interacting motifs (UIMs) and ubiquitin-associated (UBA) domains. Epsins and Ede1/Eps15 carry UIMs or a UBA domain and function at the internalization step of endocytosis. Vps27/Hrs carries UIMs that are important for receptor sorting at an endosome. We have also identified a new ubiquitin-binding motif, the CUE domain, that is found in the Vps9 Rab nucleotide exchange factor required in the endocytic pathway and in mammalian Tollip, a protein required for downregulation of interleukin-1 receptor signaling. We demonstrate that a subset of CUE domains, CUE-Ub domains, bind directly to monoubiquitin and we have defined the interaction surfaces on both binding partners. The CUE-Ub domain of Vps9 is required to promote monoubiquitination of Vps9 itself. We conclude that the CUE-Ub motif is an evolutionarily conserved monoubiquitin-binding domain that mediates intramolecular monoubiquitination. The number and variety of ubiquitin-binding proteins involved in endocytic traffic highlights the diverse roles ubiquitin plays in regulating protein and membrane transport.

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# Ubiquitin- and phosphoinositide-dependent receptor sorting in the endosomal system

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Phosphoinositides play critical roles in the regulation of membrane trafficking in addition to their classical roles as second messengers in signal transduction pathways at the cell surface. In yeast, a large collection of *vps* (vacuolar protein sorting) mutants have been isolated that missort and secrete vacuolar/lysosomal hydrolases. The *vps15* and *vps34* mutants share a common set of growth, morphology and protein sorting defects. We found that the *VPS15* gene encodes a membrane-associated Ser/Thr protein kinase that is myristoylated at its N terminus. The VPS34 gene encodes a PI 3-kinase that is homologous to the catalytic subunit (p110) of mammalian PI 3-kinases. Activation of the Vps34 PI 3-kinase by the Vps15 protein kinase results in production of the membrane-restricted lipid second messenger PI3P which triggers the recruitment/activation of effector proteins. These effector molecules contain either the cysteine-rich RING FYVE domain or the Phox homology PX domain. Two FYVE domain-containing proteins, Vps19 and Vps27, are required for the docking/fusion of vesicles with the endosome (Vps19) and for protein sorting at the late endosome (Vps27).

Vps27 and its mammalian homolog HRS are required for the formation of late endosomal compartments called multivesicular bodies (MVBs). The MVB sorting pathway is responsible for both the biosynthetic delivery of hyrolases to the lysosome and the down-regulation of numerous activated cell surface receptors (e.g., EGFR) which are degraded in the lysosome. We have found that ubiquitination of both biosynthetic and endocytic cargo serves as a signal for sorting into the MVB pathway. By contrast to proteasome degradation which generally requires a polyubiquitin chain, internalization and sorting of endocytic cargo at late endosomes is mediated by monoubiquitination. We have identified a peptide signal that is required for ubiquitination of the cytoplasmic tail of one of the MVB pathway cargo proteins. Mutations that block ubiquitination prevent sorting of the cargo into the MVB pathway. We also identified a 350kD complex referred to as ESCRT-I (**E**ndosomal **S**orting **C**omplex **R**equired for **T**ransport I) which is composed of the Vps proteins Vps23, Vps28 and Vps37. This complex has been found to recognize ubiquitinated MVB cargo and is required for cargo sorting into the MVB pathway. The Vps27 protein interacts with the ESCRT-I complex and Vps27 is required for recruitment of the ESCRT-I complex to the endosome. Vps27 and HRS also can bind ubiquitin via the ubiquitin-interacting motifs (UIMs) present in these proteins. We have proposed that monoubiquitin regulates receptor internalization and endosomal sorting by interacting with modular ubiquitin-binding domains in core components of the protein transport machinery. Ubiquitin-binding domains are found in a broad spectrum of proteins, consistent with a role for monoubiquitin as a regulatory signal in the control of diverse biological systems.

Recently, we have identified two new ESCRT complexes, ESCRT-II and –III. Proper endosomal localization of ESCRT-III requires ESCRT-II function and our data suggests that ESCRT-III is crucial for the sorting of membrane protein cargoes into MVBs subsequent to their recognition. An additional function of the ESCRT-III complex appears to be the

recruitment of accessory factors that (1) catalyze the removal of ubiquitin from MVB cargoes prior to their sequestration within MVB vesicles, and (2) catalyze the release of the ESCRT complexes from the endosomal membrane when MVB sorting is complete.

The mammalian tumor susceptibility gene *tsg101* encodes a homolog of Vps23, a subunit of the yeast ESCRT-I complex. We found that the TSG101 protein also assembles into a large (~350 kD, ESCRT-I) cytosolic protein complex. *tsg101* mutant fibroblasts, like yeast *vps23* mutants, exhibit defects in sorting and proteolytic maturation of the lysosomal hydrolase cathepsin D. Unexpectedly, *tsg101* mutant cells recently, have also been shown to exhibit defects in the budding and release of HIV-1 virus, a budding reaction that is topologically similar to the invagination of vesicles into the MVB. In addition, endocytosed EGF receptors that are normally sorted to the lysosome for down-regulation are instead rapidly recycled back to the cell surface in *tsg101* mutant cells. We proposed that similar to yeast ESCRT-I mutants, *tsg101* mutant cells are defective in MVB sorting. One consequence of this endosomal trafficking defect is the delayed down-regulation/degradation of activated cell surface receptors, resulting in prolonged cell signaling. This may contribute to the tumorigenic phenotype exhibited by the *tsg101* mutant fibroblasts. Our observations indicate that both PI kinase signaling and monoubiquitination function in the regulation of endosomal transport and receptor signaling pathways.

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## Ubiquitylation in the intracellular traffic of yeast transporters

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The yeast uracil and uridine permeases, encoded by the homologous *FUR4* and the *FUI1* genes, respectively, are phosphoproteins (1). They belong to the long list of plasma membrane proteins that display ubiquitin-dependent endocytosis. Phosphorylation of Fur4p occurs at the plasma membrane, on a PEST sequence, located in ther N-terminus of the protein. This modification is essential for the protein to be modified by ubiquitin (2). The cell surface ubiquitylation of Fur4p (3) and Fui1p is mediated by the essential Rsp5p ubiquitin protein ligase, involved in ubiquitin-dependent endocytosis of many transporters and receptors. This enzyme is the unique member in yeast of the Nedd4 protein family of ubiquitin ligases, characterized by a C2 domain, several WW domains and a catalytic HECT domain. In the case of Fur4p, plasma membrane ubiquitylation occurs on two target lysines (K38, 41), preceding the PEST sequence (4). Each of these Ks receives two ubiquitin moieties linked through ubiquitin lys 63 (5), a modification also displayed by the general amino acid permease (Gap1p) (6). After Rsp5-dependent ubiquitylation, Fur4p and Fui1p are internalized and targeted to endosomal compartments. In late endosomes, they undergo a deubiquitylation step, catalyzed by the Doa4p ubiquitin isopeptidase (7). This deubiquitylation occurs before the sorting of these permeases into inward budding internal vesicles that generate the multivesicular bodies (MVBs). After fusion of the MVBs with the vacuole, Fur4p and Fui1p are degraded by vacuolar proteases.

The steady state level of several plasma membrane transporters was described to be regulated upon specific physiological conditions. Steady state amount of plasma membrane Gap1p was described to be regulated by amonium at two different intracellular trafficking steps : ammonium promotes rapid ubiquitylation and subsequent internalization of plasma membrane Gap1p (8). In addition, it promotes sorting of newly synthesized Gap1p from the Golgi to the vacuole for degradation (9,10). The tryptophan permease undergoes equivalent double regulation upon nutrient starvation (11). Similarly, we observed a dual regulation of intracellular trafficking of Fur4p and Fui1p upon availibility of their respective substrates. Addition of their substrates in the medium triggers Rsp5p-dependent ubiquitylation and subsequent internalization of plasma membrane located permeases, as previously reported in the case of Fur4p (12). In the presence of externally added substrates, newly synthesized Fur4p and Fui1p also display direct Golgi to vacuole targeting, in addition to plasma membrane delivery.

We analyzed the molecular requirements for direct sorting to the vacuolar pathway, and sorting into MVB, as compared to that at the plasma membrane. Analysis was performed using GFP-tagged versions of Fur4p and Fui1p, both normally targeted to the plasma membrane in active form. Uracil-induced sorting of Fur4-GFP to the vacuolar pathway, and subsequently to the vacuolar lumen does not require prior phosphorylation of the transporter in the PEST sequence. In cells having strongly reduced amount of Rsp5p (mutant *npi1*), Fur4-GFP displays normal uracil-induced direct trafficking to the vacuolar pathway, but reduced sorting into MVB, and partly remains at the vacuolar membrane, instead of the vacuolar lumen. Mutation of the two Ks that are critical for cell-surface ubiquitylation

(mutant K3R-Fur4) further reduces internalization into MVB in *npi1* mutant cells. This result indicates that sorting of Fur4p into invaginating regions of the MVB involves Rsp5-dependent ubiquitylation, and that Fur4 K38, 41 are important sites for this sorting. However, diverted K3R-Fur4-GFP was correctly delivered to the vacuolar lumen in wild type cells, suggesting that other lysines are also target for ubiquitylation. Strikingly, Fur4-GFP sorting to the vacuolar pathway, and to MVB does not involve the Tul1p ubiquitin ligase, responsible for ubiquitylation of the carboxypeptidase S (CPS) during its Golgi to vacuolar sorting (13).

To further analyze the role of ubiquitin at several steps of permeases trafficking, we fused at the N-terminus of either Fui1p or Fur4p a non ubiquitylable, non removable version of ubiquitin (Ub6KR, G76V). Most of the newly synthesized Ub-permease chimera are still able to reach the plasma membrane in the absence of uracil/uridine, and only a modest part is diverted to the vacuolar pathway. Thus a single fused ubiquitin is not sufficient to achieve an efficient sorting of the permeases to the vacuole. In contrast, an almost complete sorting is promoted by the availibility of the substrate. Whether the substrate acts by a conformational change, or/or by triggering additional ubiquitylation remains to be determined.

In order to check the level of ubiquitylation required for efficient Fur4-GFP endocytosis, and sorting to invaginated regions of the MVBs, we compared the fate of Fur4-GFP and K3R-Fur4-GFP with or without in frame ubiquitin, lacking or not Fur4p K38,41. K3R-Fur4-GFP displays no uracil-induced internalization, and N-terminally fused Ub restores significant internalization. Thus, monoubiquitylation is sufficient for endocytosis and subsequent vacuolar delivery. However, Ub-Fur4-GFP was internalized 5 fold more rapidly than Ub-K3R-Fur4-GFP, indicating that additional ubiquitylation is required for efficient internalization. Similarly, a part of Ub-K3R-Fur4-GFP remains at the vacuolar membrane under conditions leading to correct delivery of Ub-Fur4-GFP into the vacuolar lumen. Hence, a single ubiquitin is not sufficient for complete MVB sorting of this multispanning protein.

There is thus an expanding list of plasma membrane transporters that undergo nutritional and/or substrate regulation of intracellular sorting at least at two steps of intracellular trafficking, internalization at the plasma membrane, and direct targeting for vacuolar degradation. The molecular mechanisms underlying the sorting decision for latter pathway, and the role played by ubiquitylation events at this step remain to be precised. In the case of Fur4p and Fui1p, it remains to be described whether this decision occurs at the TGN and/or endosomes. A decision at the endosome would fit with the recent description that some proteins undergo endosome to plasma membrane trafficking, wheras others display the usual TGN to plasma membrane trafficking (14). A sorting decision at the endosome, accompanied or followed by Rsp5-dependent ubiquitylation would also fit with the localization of Rsp5p at late endosomes, in addition to its localization close to plasma membrane (15). There is clear evidence from our data that Rsp5p-dependent permeases ubiquitylation plays a crucial role for their internalization at the plasma membrane, and their sorting at the MVB, and that these two events involve both the same and distinct permeases signals.

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## Polyubiquitin-dependent membrane protein sorting in the secretory and endocytic pathway

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Gap1p, the general amino acid permease of *S. cerevisiae*, is regulated by intracellular sorting decisions that occur in either Golgi or endosomal compartments. Depending on nitrogen source, Gap1p is transported to the plasma membrane where it functions for amino acid uptake or to the vacuole where it is degraded. We demonstrate a role for Bul1p and Bul2p, two nonessential components of the Rsp5p E3 ubiquitin ligase complex, in Gap1ppolyubiquitination and intracellular trafficking.

Overexpression of Bul1p or Bul2p causes Gap1p to be sorted to the vacuole regardless of nitrogen source, and a bul1? bul2? strain has the inverse phenotype, causing Gap1p to be delivered to the plasma membrane more efficiently than in wild type cells. bul1? bul2? can reverse the effect of lst4?, a mutation that normally prevents Gap1p from reaching the plasma membrane. In wild type, Gap1p is polyubiquitinated, but these polyubiquinated forms of Gap1p are greatly diminished in a bul1? bul2? mutant. A rsp5-1 mutant, a double lysine to arginine mutant of Gap1p (Gap1p-K9R K16R), and a C-terminal truncation of Gap1p behave as bul1? bul2?; causing constitutive delivery of Gap1p to the plasma membrane (even in lst4?) and decreasing Gap1p polyubiquitination. These results indicate that Bul1p and Bul2p, together with Rsp5p, generate a polyubiquitin signal on Gap1p that specifies its intracellular targeting to the vacuole.

# Two ubiquitin-like conjugation systems essential for autophagy

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Since the lysosome was discovered, it has been thought to be a central organelle for intracellular degradation. Soon after that autophagy was evidenced as a major pathway of lysosomal degradation. However, the molecular mechanism of autophagy has not been understood for a long time. In the past decade, the genetic approach using the yeast *Saccharomyces cerevisiae* was introduced in this research field. We discovered two ubiquitin-like conjugation systems, the Apg12 and Aut7/Apg8 systems. Both systems are essential for membrane dynamics during autophagy.

The Apg12 system is one of the ubiquitin-like protein conjugation systems conserved in eukaryotes. Covalent attachment of Apg12 to Apg5 is requisite for autophagy. Furthermore we found reversible conjugate formation of Apg8 to membrane phospholipids, phosphatidylethanolamine (PE) is also necessary for autophagosome formation. These two conjugation reactions are closely related. Two UBls, Apg12 and Apg8, are activated by common activating enzyme Apg7, and transferred to specific E2 molecule, Apg10 and Apg3, respectively. These two conjugate act on the intermediate structure of autophagosome concertedly. Apg12-5 conjugate is necessary for the maintenance of Apg8-PE level. Studies using mammalian cells further revealed the function of these two systems. Discoveries of these systems have facilitated our understanding of the molecular mechanism of autophagosome formation. Significance of this discovery is not only it brought new insights into the mechanism of autophagy but also it extended our understanding of ubiquitin-like molecules.

## Session 3: The proteasome
## Chair: Avram Hershko

# Molecular machines for protein degradation

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Within cells or subcellular compartments misfolded and/or short-lived regulatory proteins are degraded by protease machines, cage-forming multi-subunit assemblages. Their proteolytic active sites are sequestered within the particles and located on the inner walls. Access of protein substrates is regulated by protein subcomplexes or protein domains which may assist in substrate unfolding dependent of ATP. Four protease machines will be described displaying different subunit structures, oligomeric states, enzymatic mechanisms, and regulatory properties.

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**DegP(HtrA)**

**Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T.** (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. Nature **416**, 455-459.

# Recognition of polyubiquitin chain signals

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Self-compartmentalized proteases such as the 26S proteasome are gated, such that folded substrates must be unfolded in order to reach the sequestered active site chamber. The 26S proteasome is unique because unfolding and proteolysis are coupled to the recognition of a covalent signal—a lysine 48-linked polyubiquitin chain—that is linked to the substrate by upstream conjugating factors (E3s and E2s) which govern proteolytic specificity.

We are interested in the molecular interactions and mechanisms by which recognition of the polyubiquitin proteolytic signal is transduced into proteolysis. At the level of the signal, we have defined tetraubiquitin as the minimum signal for efficient proteasomal targeting; we have also identified several molecular determinants that are critical for tetraubiquitin recognition by 26S proteasomes (1). At the level of the proteasome, we have shown that the S6'/Rpt5 ATPase, in the base of the 19S complex, contacts the bound chain and is likely to serve as a chain receptor (2). Consistent with this model, interaction of the chain with S6'/Rpt5—like assembly of the 26S proteasome and substrate unfolding—is coupled to ATP hydrolysis (2).

Recent findings suggest that additional factors may assist in the targeting of polyubiquitin-tagged substrates to proteasomes, or in the unfolding of such substrates once they are bound to proteasomes. Rad23/Rhp23 and Dsk2/Dph1, which possess N-terminal ubiquitin-like (UbL) domains and distally positioned ubiquitin-associated (Uba) domains, are required for the turnover of certain proteasome substrates in yeast cells (3-5). UbLs bind to the 19S complex (6, 7), while Ubas bind polyubiquitin chains (4). These biochemical properties suggest that polyubiquitinated substrates could be targeted to proteasomes via UbL-Uba proteins (4, 5). Biochemical studies to address this model have revealed new properties of UbL-Uba proteins that should help to understand their cellular functions.

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## ATP-dependent proteases unfold their substrates by sequentially unraveling them from the degradation signal

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Protein unfolding is a key step in several cellular processes, including protein translocation across some membranes and protein degradation by ATP-dependent proteases. The proteasome and Clp AP protease can actively unfold proteins in a process that hydrolyses ATP. We found that these proteases, as well as ClpXP and Lon, catalyze unfolding by processively unraveling their substrates from the attachment point of the degradation signal. As a consequence, the ability of a protein to be degraded depends on its local structure adjacent to the degradation tag, as well as its stability. In multi-domain proteins, independently stable domains are unfolded sequentially. A degradation-resistant domain protects the following part of the substrate from proteolysis. We show that these results explain the limited degradation by the proteasome that occurs in the processing of the precursor of the transcription factor NF-kappaB.

## The proteasome and its associated proteins

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We have identified proteins that are abundant in affinity-purified proteasomes, but absent from proteasomes as previously defined because elevated salt concentrations dissociate them during purification. The major components are a deubiquitinating enzyme (Ubp6), a ubiquitin-ligase (Hul5), and an uncharacterized protein (Ecm29). Ecm29 helps to tether the proteasome core particle to the regulatory particle. Proteasome binding activates Ubp6 300-fold and is mediated by the ubiquitin-like domain of Ubp6, which is required for function *in vivo*. Ubp6 recognizes the proteasome base and its subunit Rpn1, suggesting that proteasome binding positions Ubp6 proximally to the substrate translocation channel. *ubp6Δ* mutants exhibit accelerated turnover of ubiquitin, indicating that deubiquitination events catalyzed by Ubp6 prevent translocation of ubiquitin into the proteolytic core particle.

Another ubiquitin-like protein, RAD23, binds proteasomes as well, and as with Ubp6, its UBL domain mediates binding. Previous work has suggested that Rad23 binding to the proteasome promotes both DNA repair and protein degradation, possibly by delivering ubiquitinated cargo to proteasomes. We find that Rad23 binds proteasomes through a direct interaction with the base, and that Rpn1 specifically recognizes $Rad23^{UBL}$. The ability of Rpn1 to recognize Rad23 was mapped to its leucine-rich-repeat-like (LRR-like) domain. Another ubiquitin-like protein, Dsk2, competed with Rad23 for proteasome binding, indicating (together with the Ubp6 data) that Rpn1 participates in the recognition of multiple functionally distinct UBL proteins. Contrary to expectation, subunit Rpn10 does not mediate proteasome binding by ubiquitin-like proteins in yeast, although it can contribute to the binding of ubiquitin chains by intact proteasomes.

# Roles of 26s proteasomes in protein breakdown and antigen presentation: some unfolding stories

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The great majority of proteins in mammalian cells are degraded by 26S proteasomes, and this ATP-dependent proteolytic complex is the source of most antigenic peptides presented to the immune system on MHC class I molecules. Its 19S regulatory particle contains six homologous ATPases, which are believed to bind, unfold, and translocate substrates into the 20S particle for degradation. To elucidate these processes, we studied the homologous ATPase complex, PAN, from archaea. This hexameric ring catalyzes the ATP-dependent unfolding of the model globular protein, GFP-SsrA, and stimulates its degradation by 20s proteasomes. By derivatizing this and other proteins with moieties that prevent their passage through PAN, we showed that 1) translocation into the proteasome requires threading through the ATPase ring in a specific direction (C->N or N->C, depending on the substrate); 2) translocation does not cause, but follows, ATP-dependent unfolding of the substrate on the surface of the ATPase; 3) ATP hydrolysis is activated by binding of protein substrates and is independent of their folding states; 4) ATP hydrolysis also triggers opening of the gate into the 20S; 5) in eukaryotes, one ATPase subunit (Rpn2) controls the opening of the entry channel in the $\alpha$-ring of the 20S particle.

Proteasomes degrade proteins to peptides, 3-23 residues long. Product length is determined by a kinetic competition between further cleavages by the 20S's active sites and the ability of peptides to diffuse out of the gate in the $\alpha$-ring. Since most proteasome products are too small to bind to MHC-molecules, opening this gate may enhance the yield of peptides sufficiently long for antigen presentation. Accordingly, yeast mutants ($\alpha 3\delta N$) with an open gate generate peptides of longer mean-size. PA28 is a heptameric ring complex induced by interferon-$\gamma$ that causes gate opening. We have recently characterized hybrid 26S proteasomes, which contain a 20S capped at one end by a 19S complex and at the other by a PA28 ring, through which products emerge. These asymmetric complexes generate different peptide products than 26S proteasomes, which presumably accounts for the ability of PA28 to enhance antigen presentation. We have shown that purified 26S proteasomes generate the antigenic peptide, SIINFEKL, or a larger precursor only occasionally when they degrade ovalbumin. Surprisingly, proteasomes produce mainly N-extended versions of the MHC presented 8-mer, and immunoproteasomes, the forms induced by interferon-$\gamma$, have an enhanced capacity specifically to generate these longer versions, which must be trimmed by aminopeptides before presentation on MHC class I molecules.

MHC class I-presented peptides with extra N-terminal residues can be efficiently trimmed to mature epitopes in the endoplasmic reticulum (ER). We purified from liver microsomes a lumenal, soluble aminopeptidase that removes $NH_2$-terminal residues from many antigenic precursors. Because we localized this metallopeptidase to the ER, we propose it be renamed ER-aminopeptidase 1 (ERAP1). ERAP1 is inhibited by agents that block precursor trimming in ER vesicles and although it trimmed $NH_2$-extended precursors, it spared presented peptides of 8 amino acids and less. Like other proteins involved in antigen

presentation, ERAP1 is induced by interferon-γ. When overexpressed *in vivo*, ERAP1 stimulates the processing and presentation of an antigenic precursor targeted to the ER. Also, RNAi for ERAP1 reduces the stimulation of antigen presentation by interferon-γ.

## Session 4: Conjugation of ubiquitin to proteins
## Chair: Aaron Ciechanover

# The role of the ubiquitin-proteasome pathway in the protein quality control of the cell

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There are growing lines of evidence addressing a potential link between failure of the protein quality control and neurodegeneration. Hence, it is of importance to know the quality control mechanism of the cell for understanding to the molecular basis underlying neurodegenerative diseases. To date, it is notable that the ubiquitin-proteasome system plays a central role for maintenance of protein homeostasis by catalyzing the immediate destruction of misfolded or impaired proteins generated in cells. However, how this proteolytic machinery recognizes abnormality of cellular proteins for selective elimination remains largely elusive. Recently, we reported that CHIP functions as a chaperone-dependent E3 ligase that ubiquitylates unfolded protein, indicating CHIP can be regarded as "a quality-control E3" that selectively ubiquitylates unfolded protein(s) by collaborating with molecular chaperones Hsp90 and Hsc70 (1). We also found a unique E3 ligase, termed $SCF^{Fbx2}$, which recognizes sugar chains for ubiquitylation of N-glycosylated proteins. $SCF^{Fbx2}$ is a new type of E3 linked to ERAD (endoplasmic reticulum associated degradation) caused by stresses in the ER of eukaryotic cells (2). Furthermore, we found that a RING-finger type "Dorfin" ubiquitylates mutant Cu/Zn-superoxide dismutase 1 (SOD1) responsible for familial Amyotrophic lateral sclerosis (ALS), but not its wild-type enzyme. Based on these findings, I discuss the role of ubiquitin-proteasome system in the protein quality control of the cell.

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# Ubiquitin/SUMO and DNA repair

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The *RAD6* pathway is central for post-replicative DNA repair in eukaryotic cells; however, the machinery and its regulation remain poorly understood. Two principle elements of this pathway are two ubiquitin-conjugating enzymes, RAD6 and the MMS2/UBC13 heterodimer, which are recruited to chromatin by the RING-finger proteins, RAD18 and RAD5, respectively. Recently, we discovered that also the SUMO-conjugating enzyme UBC9 is affiliated with the pathway and that proliferating cell nuclear antigen (PCNA), a DNA-polymerase sliding clamp involved in DNA synthesis and repair, is a substrate. PCNA is monoubiquitylated via RAD6/RAD18, modified by lysine 63-linked multiubiquitylation, which additionally requires MMS2/UBC13/RAD5, and SUMOylated by UBC9. All three modifications affect the same lysine residue of PCNA, suggesting that they label PCNA for alternative functions. We demonstrate that these modifications indeed differentially affect DNA damage resistance, and that damage-induced PCNA ubiquitylation is elementary for DNA repair and occurs at the same conserved residue in yeast and humans.

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Hoege *et al.*, *Nature* **419**, 135-141, 2002
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# Potential regulators of variant (K63) polyubiquitination

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The past two years have seen the confirmation of ubiquitination as an immensely versatile postranslational modification of proteins, matching or even surpassing phosphorylation in the possibilities it provides for the modulation of numerous biochemical patwhays and cellular processes. The major steps and components involved in the most conspicuous function of ubiquitination, the targeting of proteins for degradation by the proteasome, have been described in great detail, enzymatically and structurally, and, although important basic questions still remain to be answered, much of the present work in this area is centered in the generation of a catalogue of specific biochemical pathways that are regulated by ubiquitin-prompted proteolysis. This also includes identifying modes of reversible regulation of ubiquitin ligases and hydrolases that, in turn, control the abundance of specific proteins in response to intrinsic or extrinsic stimuli. A more recent area of progress inolves the study of protein modification by ubiquitin-like polypeptides. Substrate modification by each of these molecules requires specific components, and many of the mechanistic details of these processes, and the consequences of this modality of protein tagging, are being actively unveiled. A third, relatively little explored, theme in ubiquitination concerns the regulation of the modification of proteins by non-canonical polyubiquitination. This refers to the use of lysine residues on ubiquitin other than the one at position 48 for the formation of isopeptide bonds with the carboxy-terminal glycine of another ubiquitin polypeptide. For several of these modes of ubiquitination, it has been shown that protein modification by a single ubiquitin moiety can lead to consequences on the substrate protein quite different from those produced by the addition of a polyubiquitin chain containing two or more ubiquitin moieties. Finally, a further major theme regards the evidences for the existence of direct connectivities among the different modes of ubiquitination, and of ubiquitination with other types of protein modification such as acetylation. Therefore, it is becoming increasingly evident that the combinatorial possibilities provided by the covalent addition of ubiquitin and ubiquitin-like polypeptides onto substrate proteins are innumerable. These modifications direct many biochemical processes in different ways depending on the mode employed in a given context, and the different modes of ubiquitination are orchestrated by intricate and exquisite control mechanisms in response to a wide variety of stimuli.

In our laboratory, we are studying several aspects regarding the ubiquitination mode that uses lysine 63 of ubiquitin (hereafter denoted K63 variant polyubiquitination, or K63VPU). Of the seven lysine residues in ubiquitin, those at positions 29, 48 and 63 have been shown to be used *in vivo* for the formation of polyubiquitin chains (1). The use of different lysines on ubiquitin to chain up ubiquitin moieties produces conformationally distinct modifiers, which determine the fate of the modified proteins. While polyubiquitin chains that use lysine 48 tether the substrate proteins to the proteasome, where they are specifically recognized and proteolyzed, chains making use of lysine 63 do not necessarily follow the same fate *in vivo* (2, 3). So far, only one ubiquitin conjugating enzyme, Ubc13, has been shown to mediate K63VPU in yeast or in humans (2, 3). To exert its function, Ubc13 needs to form a high affinity heterodimer with the structurally similar protein UEV (2, 4, 5). In this association, Ubc13 acts as the subunit bearing a catalytically active center with the

requisite Cys residue common to all E2 enzymes, while UEV acts as a regulatory subunit devoid of intrinsic catalytic activity (4, 5). This two-molecule complex is structured such that it positions the entering ubiquitin polypeptides for the formation of isopeptide bonds between lysine 63, and not lysine 48, on one ubiquitin molecule and glycine 76 on a second ubiquitin molecule (4). To date, two biochemical pathways have been shown to be regulated by K63VPU. In *Saccharomyces cerevisiae*, the RAD6-modulated error-free DNA repair requires Ubc13 and the yeast UEV protein MMS2 (2, 6), in a process that involves K63VPU of PCNA upon DNA damage (7). In mammalian cells, K63VPU by Ubc13-UEV controls the triggering by cytokines such as TNFα and IL-1 of a kinase cascade that leads to the phosphorylation of IκBα and the subsequent release and nuclear translocation of NFκB and activation of specific gene transcription (3, 8). The transmission of the signal from the membrane receptors to activate this cascade requires K63VPU of the cytoplasmic adaptor proteins TRAF6 or TRAF2, a modification that ensues their oligomerization (3).

Our laboratory analyzing pathways potentially regulated by K63VPU, by using yeast-two hybrid as a starting screening technique. Using human Ubc13 as the bait, we have identified a number of specific interaction partners in screenings. All of the interacting proteins contain RING finger domains, which are essential for their association with Ubc13. Therefore, these proteins are potential E3 proteins or ligases for Ubc13-UEV-conducted K63VPU. We have designated these proteins UURF1 through UURF5, for Ubc13 and UEV-interacting RING finger. In addition to their RING finger domains, these proteins bear domains with the potential to mediate specific protein-protein interactions, which could tether candidate substrates for VPU by the UURF proteins. UURF1 contains a FHA domain for the recognition of phosphopeptides in specific sequence contexts, and a coiled-coiled region. The domain composition of UURF1 is similar to that of the mitotic checkpoint regulator CHFR. UURF2 is a large protein that contains also a coiled-coiled region, and several tetratricopeptide motifs presumably involved in protein-protein interactions. A close homolog to this protein is another large protein, known as TTC3 or TPRD, of unknown function, coded for by a gene located in the Down syndrome critical region. UURF2 and TPRD define a distinct and novel protein family. UURF4 is a small protein that, in addition to its RING finger, contains PPY motifs, known to interact with one of the WW domains of Nedd4, a ubiquitin ligase with a number of known substrates and well-studied biochemical functions. Finally, UURF5 is identical to a protein designated in databases as KF-1 or Zfp103, for which the function is not known. The specificity of the interaction of Ubc13 with these RING finger domains was shown by comparison with five other domains with similar sequence. In order to identify candidate substrates for VPU by some of these UURF proteins, we have performed yeast-two-hybrid screenings with these proteins as baits. Using UURF1 as a bait, we have identified futher interaction partners. These proteins interact with the FHA domain of UURF1, and could thus form complexes with UURF1, Ubc13 and UEV. A number of these UURF1 interaction partners have known biochemical and biological functions. Specific effects of some of these interactions on cellular processes will be presented.

A detailed analysis of the genomic region harboring the gene for human UEV has revealed an interesting modality of gene evolution. In addition, it has revealed a potential novel regulatory mechanism affecting the UEV protein (9). Our analysis has shown that human UEV can be present in at least two distinct forms, generated by alternative splicing. One of the proteins is UEV, while the second protein bears a domain at the amino terminus of UEV that can be expressed also as an independent transcriptional unit and protein, called Kua. Therefore, the human UEV gene locus can express three proteins, UEV, Kua, and a hybrid

Kua-UEV protein. Kua is a protein that appears to be associated with cytoplasmic organelles, as is the hybrid Kua-UEV protein, but is never found in the nucleus. In contrast, UEV is frequently localized in the nucleus. The possible implications for this arrangement and the expression of these distinct proteins from the UEV locus will be discussed.

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# Molecular pathogenesis of the von Hippel-Lindau hereditary cancer syndrome

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von Hippel-Lindau Disease is caused by germline mutation of the von Hippel-Lindau (VHL) tumor suppressor gene and is characterized by the development of blood vessel tumors of the retinal and central nervous system (hemangioblastomas) and, in some families, renal carcinomas and pheochromocytomas. Clinically VHL disease behaves in an autosomal dominant manner but at the molecular level the disease is recessive, with tumor development linked to somatic inactivation of the remaining wild-type VHL allele in a susceptible cell. In keeping with the Knudson 2-hit model of carcinogenesis, VHL inactivation is also common in sporadic (non-hereditary) renal cancer and hemangioblastoma. The VHL gene product, pVHL, is the substrate recognition module of a multicomponent E3 ubiquitin ligase that also contains elongin B, elongin C, Cul2, and Rbx1 (also called ROC1 or Hrt1). These complexes resemble SCF (Skp1/Cdc53/F-box protein) ubiquitin ligases, with pVHL subserving the same role as an F-box protein. The best understood targets of the pVHL complex are the alpha subunits of the heterodimeric transcription factor called HIF (hypoxia-inducible factor). In the presence of oxygen, conserved prolyl residues in the HIF alpha subunits are enzymatically hydroxylated by members of the EGLN family in a biochemical reaction that also requires iron, vitamin C, and 2-oxoglutartate. This hydroxylation serves as a signal for pVHL to bind to, and polyubiqutinate, the HIF alpha subunits. In the absence of oxygen, or the absence of pVHL, HIF alpha subunits accumulate, heterodimerize with ARNT, and transcriptionally activate genes involved in adapatation to acute and chronic hypoxia such as VEGF, PDGF B and TGFalpha. VEGF and PDGF B liklely contribute to the hypervascular nature of pVHL-associated neoplasms whereas TGFalpha, and its receptor, EGFR, are suspected of establishing an autocrine loop in these tumors. We recently showed that downregulation of HIF target genes is necessary for tumor suppression by pVHL and our preliminary data suggest that it is likewise sufficient. Accordingly, we and others have begun treating pVHL-associated tumors with small molecules that block signal transduction by these growth factors and have accumulated anectodal data in support of this concept. In addition, we have identified small molecules that block EGLN, leading to the accumulation of transcriptionally active HIF. Preclinical data suggest that such agents, administered acutely, might be useful in diseases characterized by tissue ischemia such as myocardial infarction and stroke.

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## MuRF1 and MAFbx are ubiquitin ligases required for skeletal muscle atrophy

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Skeletal muscle atrophy occurs during pathological conditons such as cancer and AIDS, and as a reaction to decreases in activity and load. Two genes which have been demonstrated to be involved in the atrophy process are the E3 ubiquitin ligases: Muscle RING Finger 1 (MuRF1) and a gene we designated Muscle Atrophy F-box (MAFbx, also known as Atrogin), the latter being a member of the "SCF" family of E3 ubiquitin ligases. Mice lacking either MuRF1 or MAFbx were produced; skeletal muscle was spared during atrophy-inducing conditions, as measured by maintenance of skeletal muscle mass. These mice have now been analyzed further: for changes during multiple models of atrophy, including denervation and immobilization; for changes in muscle function; and for differences in gene regulation. These analyses demonstrate key differences between MuRF1 and MAFbx pathways, which will be discussed.

## Session 5: Endoplasmic reticulum-associated degradation
## Chair: Daniel Finley

## Regulated degradation of HMG-CoA reductase

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Protein degradation pathways function both in the removal of mis- or un-folded proteins, and in the destruction of normally functioning proteins for physiological regulation. In physiological regulation, features of a target must be uniquely identified to distinguish it from all other proteins, whereas in quality control targets must be recognized by hallmarks common to a large number of substrates that transcend specific protein sequence. The degree to which regulation and quality control are mechanistically distinct is an open question. A telling case is found in HMG-CoA reductase (HMGR), a key enzyme of cholesterol synthesis. In yeast, HMGR undergoes feedback-regulated ER degradation through regulated ubiquitination at the ER surface. Regulated ubiquitination of Hmg2p is mediated by the HRD or DER complex, in conjunction with E2s Ubc7p and Ubc1p, and downstream components that facilitate proteasome delivery. Entry of Hmg2p into the HRD pathway is stringently dependent on both specific sequence features of the protein, and on levels of cholesterol pathway signals that herald degradation. In this sense, Hmg2p degradation is reminiscent of many examples of regulated protesolysis. However, the HRD pathway also mediates ER-associated degradation (ERAD) of a large number of misfolded proteins with no obvious sequence similarity. Thus, Hmg2p undergoes physiologically regulated degradation mediated by a quality control pathway. In order to understand this juxtaposition of the two branches of degradation, we are directly studying the Hmg2p molecule *in vivo* and *in vitro* to evaluate the structural features that contribute to regulated quality control. The Hmg2p molecule behaves in a manner consistent with a regulated transition to a quality control. The structural alterations of Hmg2p are consitent with the physiological regulation, in cis and in trans genetics of the process as we understand it *in vivo*. This novel behavior of Hmg2p has utility in understanding the cell biology of sterol regulation and in the design of new strategies for altering protein levels with small molecules.

# Protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome-chaperone connection

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The endoplasmic reticulum (ER) contains a highly effective protein quality control system which guarantees delivery of only properly folded proteins to their site of action. Proteins that pass through the folding process but are unable to acquire their proper conformation or cannot assemble with a respective binding partner are rapidly degraded. Recent studies have uncovered that degradation of misfolded or unassembled secretory proteins requires retrograde transport from the ER into the cytosol via the Sec61 translocon followed by degradation by the ubiquitin-proteasome system. The high conservation of this process during evolution and the involvement of ER-associated degradation (ERAD) in the generation of a variety of severe human diseases emphasizes its basic cellular importance (1,2). The yeast *Saccharomyces cerevisiae* has turned out to be an excellent model organism to study ER-degradation. A mutated and by this malfolded soluble vacuolar peptidase, CPY*, is retained in the ER, retro-translocated into the cytoplasm, polyubiquitinated by an ER-membrane located machinery and degraded via the cytoplasmic proteasome. Degradation of soluble CPY* is dependent on the ER lumenal Hsp70 chaperone Kar2p (BiP) and Der1p, an ER membrane protein of as yet unknown function. To learn more about the different requirements of soluble and ER-membrane proteins for ERAD we have constructed two integral membrane proteins, both containing CPY* as ER-lumenal, malfolded recognition domain. One, having soleley a transmembrane domain, (CT*), does not require Kar2p (BiP) and Der1p for degradation. The other, which in addition to the transmembrane domain contains the tightly folded GFP domain in the cytoplasm (CTG*), also does not depend on Kar2p (BiP) and Der1p for degradation but in contrast to CPY* and CT* requires a cytoplasmic Hsp70-Hsp40-Hsp104 chaperone complex for ERAD. All three topologically different proteins depend on the AAA-ATPase complex Cdc48-Ufd1-Npl4 for elimination.

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# Functions of a conserved transmembrane ubiquitin ligase of the nuclear envelope/endoplasmic reticulum

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The budding yeast *Saccharoymces cerevisiae* has three different cell types: two haploid forms, **a** and α, and an **a**/α diploid, produced by mating of haploid cells of opposite cell type. In homothallic strains, the *MAT* allele is unstable because unexpressed **a** or α information at either of two other genomic sites is copied into the *MAT* locus during a mating-type switch. The change in cellular phenotype that results from this gene conversion event is apparent within a single cell cycle, suggesting that the transcriptional regulators encoded by the *MAT* locus are metabolically unstable. Indeed, all of these factors are now known to have half-lives of 5 min or less, and their degradation is mediated by the ubiquitin-proteasome pathway. We recently identified a ubiquitin-protein ligase (E3) required for ubiquitination of Matα2, a transcriptional repressor expressed specifically in α and **a**/α cells. This E3 protein, called Doa10/Ssm4, is a polytopic membrane protein of the ER/nuclear envelope that functions with the Ubc6 and Ubc7 ubiquitin-conjugating enzymes. Besides Matα2, Doa10 also participates in ER quality control, with a set of substrates distinct from those acted upon by Hrd1, the other major ER ubiquitin ligase. Doa10 contains a noncanonical RING motif that is related to the PHD/LAP domain of certain viral proteins important for evading MHC class I antigen presentation.

An important characteristic of Matα2 degradation is its cell type-specific inhibition by associated transcriptional corepressors. Previously, we found that Matα2 becomes much more long-lived in **a**/α diploid cells due to its association with Mata1. More recently, we have discovered that binding of Matα2 to the corepressor complex Ssn6-Tup1 inhibits Matα2 degradatation. Ssn6 and Tup1 bind to distinct regions of Matα2, and binding of each corepressor protein specifically blocks degradation signal access to one of the two major ubiquitination pathways that act on Matα2. Thus, binding of both Ssn6 and Tup1 is required for strong inhibition of degradation. Because Ssn6-Tup1-bound Matα2 is the repressor-active form, these results imply that DNA operator-bound Matα2 is resistant to degradation, a prediction we are currently testing directly.

Inactivation of both major Matα2 ubiquitination pathways, one involving Doa10, Ubc6, and Ubc7 and the other Ubc4 and Ubc5, greatly impairs the switch from α to **a** cell type. Additional experiments indicate that reduced degradation of Matα2 is the major component of this defect in cell-switching dynamics. Thus, proteolysis by the ubiquitin-proteasome pathway underlies this classical example of cellular differentiation in a stem-cell lineage.

## Protein degradation at the Endoplasmic Reticulum

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The secretory pathway of eukaryotic cells harbors an elaborate protein quality control system, which prevents the deployment to the secretory pathway of misfolded or unassembled proteins. This system is localized in the Endoplasmic Reticulum (ER). ER associated protein degradation (ERAD) is an important component of this quality assurance system and directs misfolded proteins for destruction by the cytoplasmic ubiquitin-proteasome pathway.

ERAD can be divided mechanistically into distinct steps: First, misfolded proteins are detected in the ER-lumen. Second, the proteolytic substrates are targeted to and inserted into an aqueous transport channel that includes the multispanning membrane protein Sec61p. Third, the substrates are transported back into the cytosol (dislocation). Fourth, dislocated substrates are marked with the polypeptide ubiquitin by membrane-bound components of the ubiquitin system. These include the ubiquitin-conjugating enzymes Ubc1p, Ubc6p and Cue1p assembled Ubc7 and the ubiquitin ligase Hrd1p. Fifth, the ubiquitin-conjugated and dislocated molecules, which are still attached to the cytosolic surface of the ER-membrane are mobilized by the Cdc48p/Ufd1p/Npl4p ATPase complex. Finally, the cytosolic 26S-proteasome complex digests the misfolded proteins.

# Protein transport in and out of the ER

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Protein transport across the ER membrane in eukaryotes and across the cytoplasmic membranes in prokaryotes occurs through a protein-conducting channel formed from the heterotrimeric Sec61p/SecYEG complex[1]. The channel itself is passive; it needs to associate with partners that provide the driving force for translocation and determine directionality. We know of four different ways of how the channel can be put to work: 1. Cotranslational translocation in which the ribosome is the major partner; 2. Posttranslational translocation in eukaryotes in which the Sec62/63p membrane protein complex and lumenal BiP (Kar2p) are the partners; 3. Posttranslational translocation in prokaryotes in which SecA is the partner; and 4. Retrograde translocation across the ER membrane, a pathway used normally to dispose of misfolded proteins in the ER. Recent structural studies have given us a better view of the protein-conducting channel and how it connects with the ribosome during co-translational translocation.

In contrast to "forward translocation", little is known about retro-translocation. To study the first steps in retro-translocation we have used cholera toxin as a model substrate[2]. The toxin is assembled from two subunits in the periplasm of *Vibrio cholerae* and disassembled in the analogous compartment of target cells, the lumen of the ER, before a fragment of it, the A1-chain, is transported into the cytosol. We have found that protein disulfide isomerase (PDI) in the ER lumen functions to disassemble and unfold the toxin once its A-chain has been cleaved. PDI acts as a redox-driven chaperone: in the reduced state, it binds to the A-chain and in the oxidized state it releases it. Our results also suggest a role for PDI in initiating the retrograde transport of proteins into the cytosol. We have also started to look at the last step in retro-translocation, the release of proteins from the ER membrane into the cytosol. Our results suggest a role for a AAA ATPase family member, Cdc48p/p97, and its partner proteins Ufd1 and Npl4p, in extracting proteins out of the membrane.[3]

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# POSTERS

## Proteasome dynamics during cell cycle in rat Schwann cells

Lafarga, M., Fernández, R., Mayo, I., Berciano, M.T. and Castaño, J.G.

Proteasome is responsible of most part of protein degradation in the cytoplasm and nucleus. We have studied proteasome dynamics during cell cycle in rat Schwann cells using immunofluorescence and electron microscopy. Proteasome is present in the nucleus and cytoplasm, and a part of cytoplasmic proteasomes always localize in the centrosome both in interphase and mitotic cells and only associate with microtubules during mitosis. Proteasome exits from the nucleus during prophase. In anaphase, the proteasome becomes prominent in the region between the two sets of migrating chromosomes and in association with interzonal microtubules and stem bodies. In telophase, proteasome starts to re-enter the nucleus, and is prominent in the midbody region until the end of cytokinesis.

Proteasome does not colocalize with actin or vimentin during mitosis, except for colocalization with actin in the sheet-like lamellipodia that serves as substrate attachments for the cell during mitosis. During S phase, nuclear proteasomes colocalize with foci of BrdU incorporation but this association changes with time, being maximal at early S phase and declining as S phase progress to its end. All these results are discussed in relation to the biochemical pathways involved in cell cycle progression.

## Ubiquitin modification of serum and glucocorticoid-induced protein kinase-1 (SGK-1)

Deanna R. Brickley, Christina A. Mikosz, Christy R. Hagan, and Suzanne D. Conzen

The serum and glucocorticoid-induced protein kinase gene (sgk-1) encodes amulti-functional kinase that can be phosphorylated and activated through a PI-3 kinase-dependent signaling pathway. In many cell types, endogenous SGK-1 steady-state protein levels are very low but can be acutely upregulated following glucocorticoid receptor-mediated transcriptional activation; in breast epithelial and cancer cell lines, this upregulation is associated with promotion of cell survival. We and others have noted that ectopically-introduced full-length SGK-1 is poorly expressed, although SGK-1 lacking the first 60 amino acids (D60SGK-1) is expressed at much higher-fold protein levels than wild type SGK-1 in both human embryonic kidney 293T and MCF10A mammary epithelial cells. In this report, we demonstrate for the first time that SGK-1's low steady-state expression level is due to polyubiquitination and subsequent degradation by the 26S proteasome. Deletion of the N-terminal 60 amino acids of SGK-1 results in a mutant SGK-1 protein that is neither efficiently polyubiquitinated nor degraded by the 26S proteasome, accounting for the truncated protein's higher steady-state levels. We also demonstrate that a subset of SGK-1 localizes to the plasma membrane and that the polyubiquitin-modified SGK-1 localizes to a membrane-associated fraction of the cell. Taken together, these data suggest that a significant fraction of SGK-1 is membrane-associated and ubiquitinated. These findings are consistent with the recently described role of SGK-1 in phosphorylating the integral membrane proteins Nedd4-2 and the $Na^+/H^+$ exchanger isoform 3 (NHE3) and suggest a novel mechanism of regulation of SGK-1. Current efforts are focused on determining the relationship between ubiquitin modification of SGK-1 and its acivity as a survival kinase.

## The ubiquitin-protein ligase Hul5 associates with the proteasome

Bernat Crosas, David S. Leggett, John Hanna and Daniel Finley

Affinity-purified proteasomes contain components not detected previously because elevated salt concentrations dissociate them during purification.

The major additional components are Ecm29, Ubp6, and Hul5. Recently, we have reported the role of Ecm29, a 210 kDa protein, and Ubp6, a deubiquitinating enzyme, in the regulation of the structure and function of the 26S proteasome (1). Hul5 is a ubiquitin-protein ligase from the HECT family of ligases. The HECT ubiquitin ligases show a conserved catalytically active C-terminal domain that binds E2 ubiquitin conjugating enzymes. The N-terminal domain is thought to mediate substrate binding (2,3). Although substoichiometric, Hul5 is a very abundant component of the affinity-purified proteasomes, and cofractionates with the 26S complex in Superose 6 gel filtration. Binding assays indicate that Hul5 interacts with the base of the regulatory particle. KIAA10, the human Hul5 ortholog, also exhibits association with the proteasome (4), suggesting a conserved function of this E3 component as a proteasome-associated protein.

Functional analysis of Hul5 will involve characterization of its binding to the 26S proteasome subunits, identification of its protein substrates, and characterization of its ligase activity, physically associated to the proteasome.

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# AtE2F2 is regulated by ubiquitin-mediated degradation in dividing cells and in response to light

Carlos del Pozo, Beatrice Boniotti and Crisanto Gutierrez

Selective ubiquitin-mediated proteolysis through the cell cycle controls the availability, and therefore the activity, of several proliferating factors. E2F transcription factors regulate the expression of cell cycle and differentiation genes depending on their interaction with RB proteins.

The components of RB/E2F pathway, as well as other cell cycle regulators, have been identified in plants. In this work, we will present data that support that *Arabidopsis* E2F2 is regulated by the ubiquitin-proteasome pathway, implicating the function of the E3 ubiquitin-ligase SCFAtSKP2.

Using the GUS reporter protein fused to the N-terminal of E2F2 we have found that this region is sufficient to drive the ubiquitin-mediated proteolysis of AtE2F2 in cycling cells and in light-stimulated seedlings. Furthermore, phosphorylation of AtE2F2 by an AtCDC2a/CycA complex is required for interaction with the F-box protein AtSKP2. Interestingly, the auxin response mutant axr1-12, in which the modification of CUL1 with RUB1 is impaired, shows increased AtE2F2 protein levels, suggesting a dysfunction in the control of AtE2F2 stability.

To understand the role of AtE2F2 we generated transgenic plants that express a truncated AtE2F2, lacking the regulatory N-terminal region.

Preliminary results show that both the cell shape and cell length are affected in these transgenic plants. We also found that some cell cycle genes containing E2F-site in their promoter are down-regulated. *In vitro* assays showed that AtE2F2 directly interacts with a plant RB, suggesting a repressor role for AtE2F2. Taken together, these data suggest that AtE2F2 functions as a repressor of gene transcription cycle genes and its availability is regulated by ubiquitin-mediated proteolysis.

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# Sequential processing by metallopeptidases and proteasomes generates several N-terminally-extended peptidic species from an HIV-1 ENV epitope for endogenous MHC class I antigen presentation

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Antigenic peptides derived from viral proteins by multiple proteolytic cleavages are bound by major histocompatibility complex (MHC) class I molecules and recognized by cytotoxic T lymphoytes, CTL. Processing predominantly takes place in the cytosol of infected cells by the action of proteasomes. To identify other proteases involved in the endogenous generation of viral epitopes, specifically those derived from proteins routed to the secretory pathway, we investigated presentation of the HIV-1 ENV protein p18 10mer 318RGPGRAFVTI327 epitope to specific CTL in the presence of diverse protease inhibitors. Both metalloproteinase and proteasome inhibitors decreased CTL recognition of the p18 epitope expressed from either native gp160 or from a chimera based on the hepatitis B virus secretory core protein (HBe) as carrier protein. Thus, processing of this epitope from both native ENV and the HBe chimeric protein appeared to proceed by a TAP-dependent pathway that involved sequential cleavage by proteasomes and metallo-endopeptidases.

Experiments aimed at identifying the natural peptides produced in infected cells indicated the presence of, at least, two peptidic species of different length and sharing the same antigenic core, which are associated with the presenting molecule Dd. One species is a nonamer, while the other is, at least, probably the optimal synthetic decapeptide. The generation of various MHC/peptide complexes that can induce cytotoxicity by lymphocytes against the same gp160 region may increase the diversity of the TCR repertoire in the population of activated T lymphocytes, and help raise the effectiveness of the immune response against the viral infection. These data have implications for HIV vaccine development.

## JunB protein degradation in mitosis

Rosa Farras and Marc Piechaczyk

Jun B belongs to the AP-1 transcription factor family, which consists of homo- and heterodimers of variety of bZip proteins. The AP-1 transcription factor is essential for cells to integrate a wide array of stimuli and particularly mitogenic growth factor stimulation. AP-1 proteins, most of which are metabolically unstable, are also essential effectors of neoplasic transformation in numerous situations. JunB can physiologically exert a negative effect on cell division and has recently been shown to be a tumour suppressor in a mouse model. Because of the deleterious effects of jun B gene dysfunction, it is crucial to elucidate how its expression is physiologically regulated. Thus, Jun B is an intrinsically unstable protein whose degradation is accelerated in mitosis most probably in response to phosphorylation by the cyclin B/cdc2 kinase complex (Bakiri et al., 2000. EMBO J. 19(9): 2056). Its destruction is necessary for optimum activity of c-Jun during the G1 phase and an impetus through G1 progression. However, the mechanisms controlling the variation in JunB abundance during the cell cycle and the mechanisms whereby Jun B protein is degraded in a timely controlled manner are still ill defined. We have formally established that JunB protein accumulates in S phase and its abundance decline at the onset of M phase due to accelerated degradation.

JunB is ubiquitinylated in early mitosis and stabilised in mitotic cells treated with the proteasome inhibitor MG132 suggesting a proteasomal degradation of the protein. We are currently aiming at identifying the E3 enzyme(s) responsible for accelerated degradation of JunB in mitosis and the JunB determinants involved in this process in relation with intracellular signalling.

# The PARKIN-related protein ARIADNE alters neurotransmitter release in *Drosophila* neurons

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The *Drosophila* gene *ariadne (ari)* encodes a novel type of protein characterized by the motif string: RING-finger/B-box/RING finger (RBR). This is a very ancient protein family to which the Parkinson disease causing Parkin belongs to. Mutations in *ari* result in lethality at metamorphosis, and the occasional adult escapers exhibit aberrant axonal projections, tremor, movement uncoordination and short life span. ARI interacts specifically with an E2-type enzyme, UbcD10. The interaction is mediated by the amino-terminal R motif, but not by the amino-terminal one. The corresponding murine homologues also sustain the interaction with their *Drosophila* counterparts. In an attempt to explain the axonal projection defects in the mutant we have studied the parameters of neurotransmitter release in larval motorneurons. The spontaneous release is severely reduced in all alleles. By contrast, evoked release is augmented in a mutant allele that inactivates the amino-terminal R motif but not in another that causes the same type of inactivation in the carboxy-terminal R motif. We interpret these observations as indicative that ARI is required for normal release, and the inactivation of the first R motif leaves intact another interaction through the second R motif that results in the apparent increase of induced release. Giving the $Ca^{2+}$-dependence of evoked, but not spontaneous release, we hypothesize that the second R motif might interact with a $Ca^{2+}$-binding protein that would be one the natural substrates of the presumed ubiquitinating complex. Current attempts to identify the protein that interacts with ARI through the second R motif allow, so far, to discard several candidates including all known septins and some cullins. Also, current efforts include the clarification if ARI is an E3 enzyme itself.

At the cellular level, ARI is required for membrane deposition in organelles and cell structures such as endoplasmic reticulum, mitochondria and rhabdomeres. In addition, synapses exhibit larger than normal active zones. These cellular features are fully compatible with the observed phenotypes in neurotransmitter release, axonal projection, phototransduction and oogenesis. Taken together, the available data compose a coherent picture of the mutant defects at various levels of organization, and point towards a novel protein complex involved in the ubiquitin system.

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## Mechanistic studies on the dilocation of a malfolded protein from the endoplasmatic reticulum

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Proteins that fail to fold properly as well as constitutive or regulated short-lived proteins of the endoplasmatic reticulum (ER) are subjected to proteolysis by cytosolic 26S proteasomes. This process is known as ER-associated protein degradation (ERAD). In order to become accessible to the proteasome, ERAD substrates must first be retrogradely transported from the ER into the cytosol. It seems that protein dislocation from the ER occurs via the Sec61 hetrotrimeric complex that is also necessary for import into this compartment.

However different associated protein complexes determine the direction in which a protein moves within this channel. It is believed that in addition to marking it for degradation, ubiquitination of a substrate provides the driving force for export.

Yeast mutants, defective in ubiquitin-conjugation at the ER membrane, accumulate ERAD-substrate molecules as transported intermediates that are associated with the ER. Our data indicates that these intermediates are associated with a membrane bound ubiquitin ligase Hrd1p, as well as the Sec61p channel protein, suggesting that a Hrd1p/Sec61p complex forms during dislocation of an ER malfolded protein. A detailed characterization of the Hrd1p/Sec61p complex is in progress.

# Identification and functional characterization of USP25 and USP28, two members of a new ubiquitin protease subfamily

Marfany G.,Valero R., Bosch A. and González-Duarte R.

After extensive gene search for genes located on the gene-poor region 21q11.2 and pressumptively involved in the Down Syndrome (DS) phenotype, we identified USP25. This gene encodes a novel ubiquitin specific protease enzyme, spans more than 150 kb and contains 25 exons. It is ubiquitely expressed, although in situ hybridizations on tissue sections of adult testis and developing brain show a very specific pattern of expression, correlating with actively proliferating regions or cells with a high protein turnover (1). Alternatively spliced transcripts have been detected in most tissues and one of the alternatively spliced in-frame exons is only expressed in skeletal muscle and heart, pointing to a specific function in muscle. Using in silico comparisons of the USP25 sequence against the human genome database, we have identified an homologous gene in11q23, named USP28, which shows 55.7% and 51.36% of nucleotide and amino acid identities, respectively. This new gene shares with USP25 the exon-intron organization and presents alternative transcripts for muscle, heart and brain (2).

We have shown that USP25 and USP28 when expressed in *E.coli* cleave ubiquitin from a recombinant substrate. This, together with the high sequence and structure homology suggest that the two genes derive from a common ancestor and belong to a new subfamily of UBPs (ubiquitin specific proteases). Several UBPs have been involved in gene-dosage imbalance pathogenic effects and/or associated to aneuploidy syndromes, such as Turner or Di George syndromes. Additionally, we have shown that USP25 is over-expressed in Down syndrome fetal brains although the putative contribution of the three USP25 allelic doses to the DS phenotype is yet to be determined. The search for pressumptive partners and substrates as well as the cellular effects of USP25 over-expression are currently investigated.

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# RINGO degradation is coupled to meiosis progression and absence of S phase during *Xenopus* oocyte maturation

Gustavo J. Gutiérrez, Peter Lenart, Jan Ellenberg and Angel R. Nebreda

During the process of meiotic maturation, G2 arrested *Xenopus* oocytes undergo two consecutive meiotic divisions without an intervening S phase.

The mechanisms responsible for S phase omission are still poorly understood, although this is a crucial step for the generation of haploid gametes. Here we show that RINGO, a Cdk1 and Cdk2 activator that accumulates early during oocyte maturation is then specifically proteolysed at the transition between meiosis I and meiosis II. We demonstrate that RINGO destruction is mediated by the ubiquitin/proteasome pathway. Activation of both MAP kinase and Cdc2/cyclin B at the onset of meiosis I ensures RINGO degradation just after the germinal vesicle breakdown (GVBD). Oocytes that are induced to enter the meiotic cell cycle by injection of recombinant RINGO are unable to reach the metaphase II arrest (do not complete meiotic maturation). These RINGO-overexpressing oocytes enter meiosis I normally but then undergo DNA replication and show abnormal spindle and chromosomal structures. Interestingly, incubation of maturing oocytes with drugs such as cycloheximide or induction of maturation by progesterone in the presence of the MAP kinase kinase inhibitor U0126, that are known to induce S phase entry after meiosis I, also results in RINGO stabilization. Our results indicate that RINGO degradation during meiosis I may be a key event to allow the completion of the meiotic cell cycle, including S phase omission and metaphase II arrest, in *Xenopus* oocytes.

## Functional p53 chimeras containing the Epstein-Barr virus Gly-Ala repeat are protected from Mdm2- and HPV-E6-induced proteolysis

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Functional inactivation of the tumor suppressor protein p53 by accelerated ubiquitin-proteasome-dependent proteolysis is a common event in tumor progression. Proteasomal degradation is inhibited by the Gly-Ala repeat (GAr) of the Epstein-Barr virus nuclear antigen (EBNA)-1, which acts as a transferable element on a variety of proteasomal substrates. Here we report on the generation of a set of functional p53-GAr chimeras containing GAr domains of different length and position within the protein. The chimeras are protected from proteolysis induced by the ubiquitin ligases Mdm2 and E6-AP, both in co-transfection assays and in human tumor cell lines that exhibit accelerated proteolysis of endogenous p53. The chimeras are efficiently ubiquitinated and interact with the S5a ubiquitin-binding subunit of the proteasome but retain the capacity to transactivate p53 target genes and induce cell cycle arrest and apoptosis.

The chimeras have improved growth inhibitory activity in tumor cells with impaired endogenous p53 activity, thus providing an attractive new tool for gene replacement therapy of a wide variety of human malignancies.

# Mib is an unique E3 ligase with three RING domains that mediates Notch activation

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Notch is a transmembrane receptor that interacts with Delta and Serrate, two alternative ligands. When *Notch* is activated, the signal is transduced through intracellular components, such as *Su(H)* and *deltex*, down to the target genes, such as *E(spl)*. Modulation by Fringe and processing by Presenilin are essential for the proper Notch activation. In addition to the nervous system, *Notch* and/or its ligands *Delta* and *Serrate* are expressed in a variety of different tissues.

Ubiquitination is an important posttranslational modification, regulating many cellular processes, including degradation, endocytosis and gene expression. Many of the Notch components are also subject to ubiquitination regulation. For example, Su(dx) and Sel-10 regulate NotchIC, LNX regulates Numb and Neuralized regulates Delta. In collaboration with Dr. Ajay Chitnis, we have positionally cloned *mind bomb* (*mib*) from zebrafish and showed that it encodes a RING E3 ligase, activating Notch via Delta ubiquitination-dependent endocytosis.

Here we would like to show our further analyses on Mib protein and three different zebrafish *mib* alleles. Mib is unstable and this instability is RING- and proteosome-dependent. Interestingly, the most C-terminal RING seems to be solely responsible for Mib degradation shown by *in vitro* ubiquitination assay. Morphological and molecular analysis demonstrated that the phenotypes of m132 and ta52b alleles (deleted all three RINGs and point-mutated in the most C-terminal RING, respectively) are more severe than tfi91 allele (null mutant). This observation suggests that the former ones are dominant-negative mutants as those RING-containing ubiquitin E3 ligases behave *in vitro* and *in vivo*.

## The ubiquitin-proteasome system in spermiogenesis

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We have demonstrated that, during the haploid phase of spermatogenesis termed "spermiogenesis", the structural changes that chromatin undergoes in chicken late spermatids are associated with three main events: 1) Histone hyperacetylation, 2) Ubiquitination of histones, and 3) Changes in DNA topology with transient DNA strand breaks produced by the activity of DNA topoisomerase II.

We first reported that ubiquitination of histone H2A reaches its highest level in chicken late spermatids before the replacement of histones by protamine, and proposed that histone ubiquitination could be instrumental in the loss of the nucleosomal organization of chromatin during spermiogenesis [1]. Similar observations have been reported during mammalian spermiogenesis [2].

Our studies showed that both, free ubiquitin and nuclear ubiquitin conjugates, are abundant in chicken spermatids [3]. All the members of the chicken ubiquitin family, polyubiquitin UbI and UbII, and the ubiquitin-fusion proteins Ub-t52 and Ub-t80, are highly expressed in mature chicken testis in relation to prepuberal testis and somatic tissues, giving rise to high levels of ubiquitin in chicken meiotic and postmeiotic testicular cells [4-7].

In mature testis, transcription of the heat shock inducible chicken polyubiquitin UbI gene starts in a site that is situated closer to the heat shock promoters in relation with the site used in somatic cells [4]. In addition, the testis polyubiquitin UbI transcript undergoes alternative splicing resulting in a longer 5'UTR [4]. These findings may provide the basis for the observed increase in the levels of UbI mRNA in meiotic and postmeiotic cells and could explain the stability of the message when transcription ceases in late spermatids.

A second chicken polyubiquitin gene, UbII, is preferentially expressed during spermatogenesis with most of the mRNA present in spermatids [5, 6]. Transcription of UbII also uses, in mature testis, different initiation sites than in somatic tissues, producing both longer and shorter transcripts. Distinct promoter sequences, present in each initiation site, may be responsible for the preferential gene expression observed in meiotic and postmeiotic cells and for the increased stability of the messages at the end of spermiogenesis.

All ubiquitin transcripts are highly polyadenylated upon heat shock in chicken mature testis but not in prepuberal testis [7]. Polyadenylation may increase the stability of the transcripts during heat shock and also may enhance the efficiency of translation and the export of the mRNAs from the nucleus [8, 9].

The presence of high levels of ubiquitin transcripts, free ubiquitin, ubiquitin conjugates and proteasomes in spermatids [10], together with high expression of ubiquitin conjugating enzymes [11, 12], suggest an important function of ubiquitin conjugation during spermiogenesis. Histone acetylation and ubiquitination may open the structure of chromatin in

late spermatids and destabilize the nucleosomes as a prerequisite of histone replacement by protamines [13-15]. Another possibility is that the removal of ubiquitinated histones in late spermatids is accomplished through the recruitment and activation of the proteasome.

Recently it has been proposed that a nuclear proteasome activator abundantly expressed in testis, PA200, may recruit proteasomes to double strand DNA breaks [16]. In late spermatids, DNA topoisomerase II transiently breaks both strands of the DNA molecule [17, 18]. Inhibition of the topoisomerase II by teniposide produces a maximal DNA cleavage in late spermatids, in comparison with previous stages of spermatogenesis, including spermatocytes undergoing meiotic recombination [17]. It has been reported that when topoisomerase II is inhibited, degradation of the molecule by the 26S proteasome becomes essential to repair the double strand breaks [19]. Taking in consideration the high topoisomerase II activity present in chicken late spermatids, and the possibility of inhibition of the enzyme under cell stress conditions [20], we may speculate that the ubiquitin-proteasome system, recruited on DNA double strand breaks, might contribute to restore the genetic integrity of late spermatids. A similar function may operate during genetic recombination in spermatocytes. An adequate balance between mutageneity and DNA repair during spermatogenesis could be necessary to generate genetic diversity among spermatozoa and for the faithful transmission of genetic information to the next generation [21]. Further work on the effect of proteasome inhibitors on the number of DNA strand breaks induced by teniposide in late spermatids will show whether or not the ubiquitin-proteasome system plays a role maintaining the integrity of the genome at the end of spermiogenesis.

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# CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein

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The ubiquitin-proteasome system catalyses the immediate destruction of misfolded or impaired proteins generated in cells, but how this proteolytic machinery recognizes abnormality of cellular proteins for selective elimination remains elusive. Herein, we report that CHIP with a U-box domain is an E3 ubiquitin-ligase collaborating with molecular chaperones Hsp90 and Hsc70. Thermally-denatured firefly luciferase was multiubiquitylated by CHIP in the presence of E1 and E2 (Ubc4 or UbcH5c) *in vitro* only when the unfolded substrate was captured by Hsp90 or by Hsc70 and Hsp40. No ubiquitylating activity was detected in CHIP lacking the U-box region. CHIP efficiently ubiquitylated denatured luciferase trapped by the C-terminal region of Hsp90, which contains a CHIP binding site. CHIP also showed self-ubiquitylating activity independent of target ubiquitylation. Our results indicate that CHIP can be regarded as a quality-control E3 that selectively ubiquitylates unfolded protein by collaborating with molecular chaperones.

## Beta-arrestin and C-Src dependent degradation of G-protein-coupled receptor kinase 2

Penela P, Elorza A, Sarnago S And Mayor F Jr

Activation of G-protein-coupled receptors (GPCRs) triggers receptor phosphorylation by G-protein-coupled receptor kinase (GRKs), which in turn promotes the binding of arrestins. GRKs and arrestins play key roles in the regulation of GPCRs being involved in desesitization/resensitization processes, receptor endocytosis and receptor signalling to mitogenic cascades. GRK2 expression is altered in several pathological conditions, but the molecular mechanisms that modulate GRK2 levels are unknown. We recently described that GRK2 is rapidly degraded by the proteasome pathway (Penela et al. (1998). J. Biol. Chem. 273, 35238-35244). Moreover, this process is enhanced by GPCR stimulation and is severely impaired in a GRK2 mutant that lacks kinase activity (GRK2-K220R). Interestingly, beta-arrestin function and c-Src-mediated phosphorylation of GRK2 are critically involved in GRK2 proteolysis. This pathway for the modulation of GRK2 stability puts forwad a feedback mechanism for regulating GRK2 levels anf GPCR signalling.

# Two step proteolytic activation of the *Aspergillus nidulans* zinc finger transcription factor PacC

M.A. Peñalva

The *A. nidulans* PacC zinc finger transcription factor mediating regulation of gene expression by ambient pH, in common with a number of other transcription factors, notably the transducer of the Hedgegog signal *Cubitus interruptus*, undergoes proteolytic processing activation. These with which *A. nidulans* can be manipulated genetically makes PacC ideally placed for understanding how a transcription factor prevents its activation in the absence of appropriate signal transduction.

Ambient pH signalling occurs only under alkaline conditions. In such circumstances, pH signal transduction activates the otherwise transcriptionally inactive 674 residue PacC translation product by a two step proteolysis mechanism which can be compared to regulated intramembrane proteolysis (Rip). In a first step, which is crucially regulated by ambient pH, the 72 kDa PacC translation product (PacC72) is converted to a 53 kDa intermediate (PacC53) lacking the 280 C-terminal residues. This step is catalyzed by a signalling protease, possibly PalB, one of the proteins of the ambient pH signalling pathway.

In a second, pH-independent step, this (committed) intermediate is converted to a 27 kDa processing product (PacC27) containing the  250 N-terminal by an as yet unidentified processing protease. This protease does not require the sequence at the cleavage site and appears to recognize at a distance sequence or structure determinant located upstream of this site. I shall discuss our latest efforts for the identification of both the signalling and the processing protease.

## Role of the HECT family of ubiquitin-protein ligases in nuclear transport

Rodríguez M.S., Haguenauer-Tsapis R. and Dargemont C.

Ubiquitin conjugation plays an important role in protein and RNA metabolism. The E3 or ubiquitin-protein ligases play a key role in substrate recognition. Previous studies using a yeast temperature-sensitive (ts) mutant of E1 indicated that the ubiquitin pathway is involved in poly(A)+RNA export from the nucleus and in the nuclear protein import. Moreover, Tom1p, a ubiquitin ligase from the HECT family has been shown to play a role in nuclear export of poly(A)+RNA. In order to identify nucleocytoplasmic transport pathways that may be regulated by ubiquitin conjugation, we decided to study the role of the HECT family of ubiquitin-protein ligases in such processes. Five HECT domain-containing proteins are encoded by *S. cerevisiae* genome: Rsp5p, Ufd4p, Tom1p, Hul4p and Hul5p. We systematically tested whether the ubiquitin ligases from this family are implicated in nuclear transport of proteins and RNAs in S. cerevisiae. We have used GFP-based reporters to analyse the role of HECT ligases in nuclear transport of proteins. Our results indicate that none of these ligases is involved in the importin a/b mediated-nuclear import of proteins nor in the Crm1-dependent nuclear export. However, fluorescence *in situ* hybridisation (FISH) analysis using Cy3 labelled oligo-dT, revealed that Rsp5p, a second member of this family of ubiquitin-protein ligases, is implicated in the nuclear export of poly(A)+ RNA. In order to identify the target protein of Rsp5p implicated in mRNA export, we first decided to determine the region potentially implicated in the recognition of such substrate. We have shown that repeats WW2 and WW3 of Rsp5 are crucial for poly(A)+ RNA export mediated by this HECT ubiquitin-protein ligase. This information is currently being used to identify protein substrate(s).

# The ubiquitin-conjugating enzyme UbcX is involved in Nedd4-2 dependent regulation of the epithelial Na channel EnaC

Christophe Debonneville and Olivier Staub

The epithelial Na+ channel ENaC plays a pivotal role in the renal regulation of $Na^+$ homeostasis and blood pressure (1). It is composed of three homologous subunits, each containing two transmembrane domains, a large extracellular loop and short cytosolic N- and C-termini. Each subunit contains a PY-motif (xPPxY) at its C-terminus, which have been shown to be deleted/mutated in Liddle's syndrome, an inherited form of human hypertension, and which serve as binding sites for the ubiquitin-protein ligase Nedd4-2 (2). Indeed, we have demonstrated that ENaC is a short lived protein, that becomes ubiquitinated on its alpha and gamma subunit, and that such ubiquitination regulates ENaC activity (3).

We have also shown that the aldosterone-induced Sgk1 kinase controls the interaction between ENaC and Nedd4-2 via phosphorylation of Nedd4-2, pointing at a physiologically relevant signalling pathway for this process (4). Several enzymes are involved in ubiquitination reactions, including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). We have now identified in a 2-hybrid screen, using the C-terminal region of Nedd4-2, an E2 enzyme, that we refer to as UbcX. We find that this enzyme is involved in ENaC regulation as well, and that it interacts with Nedd4-2, and is able to promote transfer of ubiquitin on Nedd4-2. Our data suggest that UbcX is the E2 enzyme acting in concert with Nedd4-2 in ENaC regulation.

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## CNOT4-mediated ubiquitination and the dynamics of RNA polymerase II transcription

H.Th.M. Timmers, G.S. Winkler, C.G.M. Zwartjes, Y.I.A. Legtenberg, F.M.A. van Schaik K.W. Mulder, C. Dominguez, and R. Boelens

Recent results underscore a direct role for ubiquitination in transcriptional activation pathways and in chromatin function.

The CCR4-NOT complex is a highly conserved global regulator of RNA polymerase II transcription. In yeast, several genes encoding subunits of this complex interact genetically and physically with the TFIID and Mediator complexes. In our laboratory, the human orthologues were identified and cloned to facilitate the biochemical analysis of the human CCR4-NOT complex, which contains about 8 subunits.

We solved the solution structure of the amino-terminal $C_4C_4$ RING domain of human CNOT4 and found that it possesses ubiquitin-protein ligase (E3) activity. This activity depends on the selective interaction of the RING domain with UbcH5B, a member of a large group of highly homologous ubiquitin-conjugating enzymes (E2s). Using a combination of NMR spectroscopy and mutational analysis, key residues for binding were identified delineating the interaction surfaces of CNOT4 and UbcH5B. This allowed us to succesfully design an altered specificity pair. Together, our analyses provide a framework to understand selectivity of E2-E3 protein interaction.

To investigate the mechanism by which the human CCR4-NOT complex regulates transcription, components of this complex were artificially recruited to promoter regions of a reporter construct. Using transient transfection assay we found strong and specific repression by the GAL4-CNOT2 subunit fusion protein indicating that promoter recruitment of the CCR4-NOT complex can result in transcriptional repression. We will discuss how CNOT4-mediated ubiquitination can contribute to the dynamics in pol II transcriptional regulation.

## The role of proteasomal degradation within the nucleus in the generation of systemic autoimmune responses

Anna von Mikecz, PhD, Min Chen, MD, Peter Hemmerich, PhD, Thomas Rockel, Gabriele Steinweger

Although proteasomes are abundant in the nucleoplasm little is known on proteasome-dependent proteolysis within the nucleus. Thus, we monitored the subcellular distribution of nuclear proteins in correlation to proteasomes which process endogenous proteins, regulate numerous cellular processes, and deliver immunocompetent peptides to the antigen presenting machinery.

Confocal laser scanning microscopy revealed that histones, splicing factor SC35, spliceosomal components, such as U1-70k or SmB/B', and PML partially colocalize with proteasomes in nucleoplasmic substructures, whereas centromeric and nucleolar proteins topoisomerase I, fibrillarin and UBF did not overlap with proteasomes. Specific inhibition of proteasomal processing with lactacystin induced accumulation of histone protein H2A, spliceosomal components, and PML, suggesting that these proteins are normally degraded by proteasomes. In contrast, concentrations of centromeric proteins CENP-A, -B, and nucleolar proteins remained constant.

Quantification of fluorescence intensities corroborated that nuclear proteins which colocalize with proteasomes are degraded by proteasome-dependent proteolysis within the nucleoplasm. Since the examined nuclear proteins possess similar half lifes, and represent prominent autoantigens of systemic autoimmune diseases such as *Lupus erytemathodes*, these data suggest that the proteasome proteolytic pathway is involved in processing of nuclear components, and thus may play an important role (1) in the regulation of nuclear structure and function, (2) in the generation of autoimmune responses against nuclear autoantigens.

## Timing of APC/C substrate degradation is determined by fzy/fzr specificity of destruction boxes

Amit Zur and Michael Brandeis

The APC/C, activated by fzy and fzr, degrades cell cycle proteins that carry RXXL or KEN destruction boxes (d-boxes). APC/C substrates regulate sequential events and must be degraded in the correct order during mitosis and G1. We studied how d-boxes determine APC/C-fzy/APC/C-fzr specificity and degradation timing. Cyclin B1 has an RXXL box and is degraded by both APC/C-fzy and APC/C-fzr, fzy has a KEN box and is degraded only by APC/C-fzr. We characterized the degradation of substrates with swapped d-boxes. Cyclin B1 with KEN instead of RXXL, was degraded only by APC/C-fzr. Fzy with RXXL instead of KEN, could be degraded by APC/C-fzy and by APC/C-fzr. Interestingly, APC/C-fzy, but not APC/C-fzr, specific degradation is highly dependent on the location of the RXXL. We studied degradation of tagged substrates in real time and observed that APC/C-fzr is activated in early G1. These observations demonstrate how d-box specificities of APC/C-fzy and APC/C-fzr, and the successive activation of APC/C by fzy and fzr establish the temporal degradation pattern. Our observations can further explain why some endogenous RXXL substrates are degraded by APC/C-fzy, while others are restricted to APC/C-fzr.

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