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Targets of programmed destruction: a primer to regulatory proteolysis in yeast

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Abstract. Proteasome-mediated proteolysis of defined target proteins is an important regulatory mechanism, which contributes to control of various essential pathways and programs of the eukaryotic cell. Here, I describe basic principles and mechanisms of regulatory proteolysis as observed in a prominent model cell, the yeast *Saccharomyces cerevisiae*. Selective proteolysis is important for balancing of a cell's proteins. It is used to directly limit or neutralize enzymes in response to defined signals. Proteasome-mediated degradation is also crucial for the regulation of gene transcription. Elimination of transcription factors as well as mobilization of transcriptional activators by limited proteolysis is involved in negative and positive control of transcription. Moreover, recent data indicate that the proteasomal degradation system may be spatially linked to the transcription machinery. Selective proteolytic destruction of regulators and structural proteins is an essential regulatory mechanism ideally designed for the regulation and correct execution of unidirectional processes as are the cell division cycle or the program of apoptotic cell death. Here the proteasomal degradation system acts on various levels.

Key words. Proteasome; ubiquitin; proteolysis; regulation; enzyme activity; transcription; cell cycle; apoptosis.

Introduction

Balancing of a protein's concentration is an important mechanism to regulate its activity. Changing of protein levels can be achieved by control of synthesis [in most cases at the level of gene transcription, in some cases at the level of messenger RNA (mRNA) translation] but also in an antagonistic way by stimulation or inhibition of proteolytic degradation. The major cellular tool for regulatory proteolysis in the cytoplasm and nucleus of the eukaryotic cells is the proteasome, a sophisticated nanomachine designed for the selective elimination of proteins (for reviews see [1–3] and the article by W. Heinemeyer et al. in this issue). Research over the last decade revealed that a large variety of different regulatory processes is essentially controlled by selective proteolysis via the proteasome. Here, as in research on proteasome structure, assembly and catalytic activities, work in the budding yeast *Saccharomyces cerevisiae* was a pacesetter for the whole field. Many processes of regulatory proteolysis have initially been studied in yeast; discovery of others greatly profited from research in this model. A comprehensive description of proteasome function in regulation of the yeast cell would exceed the scope of this article. Moreover, a variety of excellent reviews covering discrete topics of regulatory proteolysis in detail are found in the recent literature. Thus, focusing on selected examples, I will present an overview of the basic principles, mechanisms and objects of proteasome-mediated regulatory proteolysis in yeast.

Most proteins degraded by the proteasome are marked for destruction by tagging with multi-ubiquitin chains. Ubiquitination of proteasomal substrates is performed by a complex enzyme system that consists of E1 (ubiquitin activating-) enzymes, E2 (ubiquitin conjugating-) enzymes and, in most cases, E3 (ubiquitin ligating-) enzymes (for reviews see [4–6] and the article by S. Fang and A. M. Weissman in this issue).

Efficient degradation in many cases requires assistance by proteins that support the transfer of ubiquitin-marked

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substrate proteins to the proteasome complex. Such helpers may simply recruit ubiquitinated targets but may also, as in the case of the AAA-ATPase Cdc48, contribute to unfolding of substrates or their liberation from larger structures as protein complexes or membranes (for overview see the article by R. Hartmann-Petersen and C. Gordon in this issue and [7]).

Elimination of enzyme activity

Proteolysis is a straightforward and rapid process to directly down-regulate the activity of a protein. One of the best-studied examples of inactivation of an enzyme by proteasome-mediated destruction is degradation of the gluconeogenetic enzyme fructose-1,6-bisphosphatase (FBPase). When grown on non-fermentable carbon sources, yeast cells produce hexoses by the gluconeogenetic pathway. This anabolic pathway shares most of its enzymes with the antagonistic pathway, the glycolytic pathway. However, dephosphorylation of fructose-1,6-bisphosphate by FBPase is a step that is unique to gluconeogenesis. When cells are set back to fermentable carbon sources, FBPase activity has to be nullified to avoid a futile cycle of ATP consumption brought about by the combined action of FBPase and its glycolytic counterplayer phosphofructokinase. For this purpose FBPase in response to the glucose signal is partially inactivated (~50%) by phosphorylation and thereafter completely neutralized by proteasome-mediated degradation [8–10] (see also the article by D.H. Wolf in this issue)

Regulatory proteolysis in control of gene transcription

As in every type of cell, gene expression in yeast is controlled by a complex network of transcriptional regulation [11]. Cells modify their transcriptome during the course of defined cellular programs as, for instance, cell proliferation or differentiation but also in response to altered environmental conditions or after the exposure to stress. Transcriptional reprogramming requires modulation of the activity of transcriptional activators and repressors, including rapid adjustment of transcription factors at the protein level. Here, selective proteasome mediated degradation serves as an important regulatory mechanism.

The proteasome mediates down-regulation of gene expression

Transcriptional activators bind to specific promoter sequences with the objective to recruit the transcriptional machinery, the polymerase II holoenzyme complexes, to the respective target genes. Proteasome-mediated destruction is a rapid mechanism to limit or to abrogate the activity of transcriptional activator proteins with the aim of down-regulation of transcription of the corresponding target genes (fig. 1A). A well-studied example is Gcn4, a c-Jun-like transcriptional activator. Gcn4 is a master regulator of gene expression in *S. cerevisiae*. In response to deprivation of any of several amino acids Gcn4 induces the transcription of multiple genes of amino acid biosynthetic pathways; this mechanism is termed general amino acid control [12]. However, recent studies demonstrated that Gcn4 has a broader role and that Gcn4-dependent transcription is also stimulated by general starvation conditions and stress. Thus, exposure to DNA-damaging conditions [e.g. ultraviolet (UV) light or methylating agents (MMS)] can lead to induction of Gcn4-dependent transcription even when cells are kept on rich medium. In agreement with this data recent microarray studies uncovered that Gcn4 is implicated in control of gene expression of at least 1/10 of the whole yeast genome, including genes involved in DNA repair [13, 14].

The cellular concentration of Gcn4 is controlled by balancing its rate of synthesis and degradation. Under non-starvation conditions Gcn4 dependent expression is limited. For this purpose Gcn4 is set to a low level by two means: (i) Effective synthesis of Gcn4 is prevented by a unique mechanism of translational control [15], and (ii) rapid proteolytic destruction of Gcn4 protein via the ubiquitin-proteasome machinery, which can occur with a half-life of 5 min, is stimulated under non-starvation conditions [16]. Gcn4 ubiquitination requires the E2 enzyme Cdc34 and, as the ubiquitin ligase, SCF complexes activated by the F-box protein Cdc4 [17]. SCF complexes are sophisticated E3 enzymes, which are mainly responsible for tagging of cell cycle regulators during the G1, S and G2 phase (see later section on cell cycle regulation). SCF-mediated ubiquitination of cell cycle regulators takes place phase specifically. In contrast, Cdc4-SCF-mediated degradation of Gcn4 occurs constitutively throughout the whole cell cycle. These data indicate that SCF-mediated ubiquitination is primarily triggered on the level of the individual substrate protein and not by stimulation of the E3 enzyme. In agreement with this model Gcn4 degradation requires phosphorylation of specific residues in the Gcn4 activation domain, notably Thr105 and Thr165. This modification is executed by two cyclin-dependent protein kinases, Pho85 and Srb10 [17, 18]. Both kinases appear to additively contribute to signaling of Gcn4 degradation, as elimination of both kinase activities is required to stabilize Gcn4 to the same extent as caused by inactivation of Cdc4-SCF complexes.

In response to amino acid starvation or deprivation or inhibition of protein synthesis transcription of Gcn4-dependent target genes has to be induced. For this purpose the cellular level of Gcn4 is raised (i) by stimulation of

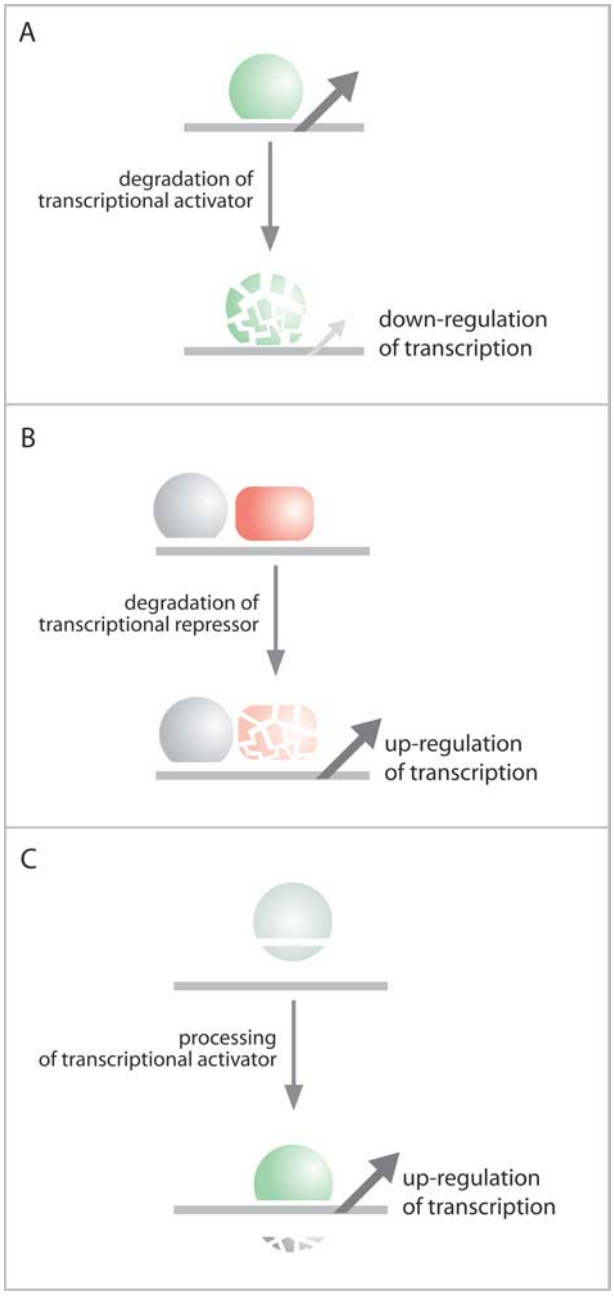

Figure 1. Proteasome-mediated regulatory proteolysis is involved in control of transcription. (*A*) Degradation of transcriptional regulators is used to abrogate transcription. (*B*) Inhibition of gene expression is nullified by proteolytic destruction of repressor proteins. (*C*) Precursor forms of transcriptional activators are matured to the active form by limited proteasome-mediated proteolysis, resulting in induction of transcription.

Gcn4 mRNA translation and (ii) reduction of its proteolytic destruction. For this purpose signaling of degradation by Pho85-dependent Gcn4 phosphorylation is inhibited. In contrast, Srb10-mediated degradation of Gcn4 occurs constitutively. Inactivation of Srb10 leads to an increase of the Gcn4 level by only twofold [18]. Derepression of Gcn4-dependent transcription by this means, however, is limited to only few Gcn4 target genes, suggesting that Srb10, which is an integral part of the RNA polymerase II holoenzyme complex, may in addition have a positive influence on Gcn4-dependent gene transcription [15].

The proteasome controls up-regulation of gene expression

It seems to be a paradox. Contrary to its native role of eliminating proteins, the ubiquitin-proteasome systems can cause up-regulation of the concentration of certain target proteins by inducing stimulation of their transcription. Here, two major mechanisms are so far well known: destruction of transcriptional repressor proteins and mobilization of transcriptional activators by limited proteolysis (fig. 1B/C).

That proteasome-mediated processes might contribute to stimulation of expression was even described in the case of Gcn4-dependent gene transcription. Impairment of proteasome activity, either by mutation of 19S cap subunits or mutations of the 20S core, did not affect Gcn4-mediated transcription when cells were starved for histidine but led to abolishment of UV- or MMS-stimulated Gcn4-dependent gene transcription on rich medium [19]. This effect, which is attributed to a defect in degradation of an unknown factor that inhibits Gcn4-dependent transcription, hints at the possible function of proteasomal pathways in stimulation of gene transcription.

A better-defined example of ubiquitin-proteasome system-mediated abolishment of transcriptional repression is degradation of the yeast MATα2 repressor protein. *S. cerevisiae* cells exist in three proliferative forms: two haploid types a and α and a diploid type a/α brought about by mating of haploids of opposite types. A unique genetic locus, the MAT locus encoding mating-type-specific regulators, governs cell identity [20]. Haploid cells are typified by expression of either MATa- or MATα-encoded sequences present at the MAT locus, whereas diploid cells are characterized by expression of both MAT cassettes in parallel. The MATα sequence encodes two transcriptional regulators α1 and α2, whereas the MATa sequence harbors a single gene encoding the a1 regulator. The MATα2 protein is a transcriptional repressor of specific genes in α cells. Homothallic haploid cells can switch their mating type by copying the MATa or MATα cassette from silent loci into the MAT locus. The phenotypic switch induced by this genetic alteration occurs within one cell cycle and requires fast adaptation of the concentration of the regulators involved. Thus, to neutralize MATα2 repression and to allow fast accumulation of MATa-specific proteins after a switch from MATα to MATa, the MATα2 repressor protein is rapidly elimi-

nated with a half life of ~4 min in a ubiquitin-proteasome-dependent manner. The primary signal mediating MATα2 degradation is a N-terminal 67 amino acid stretch, termed Deg1. The hydrophobic surface of an amphipathic helix present in the Deg1 sequence (amino acids 14–32) is thought to function as a key determinant for signaling of MATα2 repressor ubiquitination and degradation [21].

In diploid cells MATα2 is specified for repression of haploid-specific genes by forming a complex with the a1 protein. Like the MATα2 repressor, the a1 protein contains a Deg1-related sequence and is rapidly degraded in a ubiquitin-dependent manner in haploid a cells. a1/α2 heterodimeric complexes built in diploid cells are turned over with much lower rates than the free a1 and α2 proteins. The biological relevance of this behavior remains undefined. The proteolytic stabilization of a1/α2 heterodimers is thought to be the result of coverage of the amphipathic helix determinants of the Deg1 sequences of both subunits, which are involved in the a1/α2 interaction [21]. Masking of determinants that signal ubiquitination by integration of the substrate into protein complexes may be a general means to regulate the proteolytic stability of proteasomal substrates.

Transcription factor degradation can be locus dependent

For both the transcriptional activator Gcn4 and the Matα2 repressor evidence was obtained that transport to the nucleus is a prerequisite for their degradation. In non-starved cells Gcn4 is found in a small cytoplasmic fraction, which is proteolytically stable. The larger fraction of Gcn4 localizes to the nucleus. This fraction is rapidly turned over [22]. Nuclear transport of Gcn4 is triggered by two nuclear localization NLS sequences but does not require a functional general amino acid control system nor functional Pho85 and Srb10 kinases. As proved by stabilization of mutated Gcn4 that lacked the NLS sequences or of wild-type Gcn4 that was expressed in cells impaired in nuclear import, transport of Gcn4 to the nucleus is a prerequisite for its rapid degradation. Pho85 kinase, which stimulates Gcn4 degradation under non-starvation conditions, is localized within the nucleus independent of the presence or absence of amino acids. Taken together these data lead to the model that Pho85-dependent control of Gcn4 stability is restricted to the nucleus. Thus, regulation of Gcn4 synthesis, which occurs in the cytoplasm, and regulation of Gcn4 degradation are independent compartment-specific processes. A similar behavior has been found for the Matα2 repressor. As shown by Lenk et al., Ubc6/Ubc7-mediated ubiquitination and rapid turnover of a reporter protein containing the Deg1 signal requires its effective transport to the nucleus [23]. Endogenous Matα2 is exclusively localized in the nucleus, and its proteolytic destruction is impaired in nuclear import mutants. What is the purpose of such spatial restriction of degradation signaling? Arrival of transcriptional regulators at their locus of action, the nucleus, is a prerequisite for their proteasomal destruction. Thus, elimination of transcription factors that had no chance to execute their function is avoided.

Proteasome-mediated degradation processes may be tightly connected to the transcription machinery

Overexpression of potent transcriptional activators, such as Gcn4, can cause a general block in transcription. It is thought that this effect, termed squelching of transcriptional activation, is based on the sequestration of basal transcription factors by the overexpressed activator protein [24, 25]. Strict limitation of transcriptional activator levels may therefore be important for correct functioning of the transcription machinery. An ideal tool for this task is proteasome mediated proteolysis. Moreover, in addition to regulation of initiation of transcription precise control of gene expression may require exact and timely defined nullification of the transcription process. Initial work indicates that here the ubiquitin-proteasome system may have a crucial role. Experimental evidence was obtained that core components of the degradation system may be in direct spatial connection to the transcription machinery. The proteasome system may thus function beyond the scope of simply limiting the concentration of transcription factors that independent of the transcription machine float around in the nucleus and cytoplasm. Here an important observation was that Srb10 kinase, which signals Gcn4 degradation, is a component of the RNA polymerase II holoenzyme complex [18, 26]. This finding indicated that signaling of Gcn4 degradation can occur at the transcription machinery and may be an integral part of the transcription process. Srb10 seems to mediate degradation of Gcn4 only after binding of this transcription factor to promoter sequences. Such behavior may ensure that Srb10-stimulated Gcn4 degradation is limited to those Gcn4 molecules that have functioned as a transcriptional activator, a process by Tansey colorfully called the ‘black widow’ mechanism [26]. Coupling of steps of substrate degradation to transcription may not be restricted to the activation of degradation signals as is Srb10 mediated phosphorylation of Gcn4. In mammalian cells CNOT4 protein, a homologue of yeast Not4, was characterized as a subunit of the CCR4-NOT complex [27]. This complex acts as a repressor of RNA-polymerase II-mediated transcription. CNOT4 was revealed to be a RING finger E3 ubiquitin-ligase, which interacts with a subset of ubiquitin-conjugating enzymes

(hUbc5B, hUBC6 and hUbc9), evidencing that even ubiquitin-tagging processes may take place in spatial connection to the RNA polymerase complex. Moreover, evidence was obtained that the entire degradation system including the proteasome is physically connected to the transcription machinery. Recently, Morris et al. found components of the ubiquitin system as well as 19S cap and 20S core proteasome components associated with the *CDC20* promoter [28]. Periodic association of the proteasome to the *CDC20* promoter, which takes place antagonistic to the dissociation of cyclin-dependent kinase complexes (CDKs), is triggered by the CDK binding factor Cks1. Initial data obtained with proteolytically defective 20S proteasomal mutants may hint at a non-proteolytic role of the ubiquitin-proteasome system in *CDC20* transcription [28]. However, whether this is indeed the case or whether the proteasome is required for a local proteolytic process awaits further clarification (see also discussion by Lipford and Deshaies [29]). Besides limiting the rounds of RNA polymerase firing, ubiquitin-mediated degradation processes at the transcription machinery might serve other purposes. Degradation processes may for instance act as stimulatory switches, which allow the RNA polymerase to detach from pre-initiation complexes (discussed in [29]). Selective proteolysis may in addition play a role in promoter clearance. Moreover, some evidence for the implication of the ubiquitin-proteasome system in control of the transcription machinery beyond the scope of transcription factor elimination was obtained by the finding that the proteasome degrades the large subunit of RNA polymerase II upon damaging of DNA [30]. Taken together, the data suggest that only the tip of the iceberg is visible, and that clarification of proteasome-mediated processes at the transcription machinery awaits further research.

Activation of transcription factors by limited proteolysis

Besides elimination of repressor proteins, regulatory proteasomal proteolysis can contribute to induction of gene expression by causing mobilization of transcriptional activators (fig. 1C). For this purpose inactive precursors are processed to the active form by limited proteolysis. Such behavior was initially discovered in the mammalian system for the transcription factor NF-κB. To produce mature p50 NF-κB, the C-terminal domain of p105 precursor molecules is eliminated by destruction via the proteasome system. However, the N-terminal transcription factor domain of NF-κB precursors is excluded from proteolysis and left intact [31, 32]. Two models have been put forward to explain limited proteolysis of NF-κB. Model I suggested that p105 degradation starts from the C terminus and is terminated by a hypothetical stop-transfer signal in the center of p105 [33, 34] (for overview see [35]). The alternative model proposed that p105 is processed by cotranslational cleavage of a nascent NF-κB polypeptide chain associated with a second p105 precursor, thereby producing p50-p105 dimers [36, 37] (for overview see [35]). Because C-terminal ends of nascent polypeptide chains are hidden in the ribosomes, such processing of p105 precursors would require internal cleavage of the nascent polypeptide chain.

When expressed in yeast cells, p105 NF-κB precursors are proteasome dependently matured to p50 molecules of nearly identical size as in mammalian cells, suggesting that the proteasomal processing machinery is conserved from yeast to mammals. New clues to the aims and mechanisms of transcription factor mobilization by limited proteolysis came from studies on two distantly related homologues of NF-κB in yeast, Spt23 and Mga2 [38, 39]. These transcriptional activators share overlapping functions in expression of the *OLE1* gene encoding fatty acid desaturase. Spt23 and Mga2 proteins are synthesized as inactive p120 precursors. In contrast to NF-κB, which is a soluble protein, Spt23 and Mga2 are anchored to the endoplasmic reticulum (ER) by their C-terminal tail with the bulk N-terminal parts containing transcription factor domains facing the cytoplasm. Both factors are mobilized by proteasome-mediated processing. Their C-terminal membrane anchors are eliminated, leaving the transcription domains intact. Thus, processing results in release of soluble p90 proteins, which can reach the nucleus to start induction of target gene transcription. Processing of Spt23 occurs post-translationally directly at the ER membrane and is negatively feed-back regulated by unsaturated fatty acids, the products of Ole1, indicating that Spt23 may act as a sensor for ER membrane composition [38, 40, 41]. The membrane-embedded C-terminal tails of Spt23 and Mga2 are thought to be not accessible to degradation, leading to the conclusion that processing of Spt23/Mga2 is achieved by internal endoproteolytic cleavage. Based on this suggestion Rape and Jentsch put forward an interesting model for the mechanism of proteasome-mediated processing [35]. They proposed the formation of hairpin loop structures at the processing position, which reach into the proteolytic chamber of the 20S proteasome and are there attacked by the proteolytic sites of the proteasome. This model is in agreement with the architecture of the 20S proteasome. The gates at both distal ends of the 20S cylinder have a diameter of 1.3 nm, allowing the entrance of two side-by-side positioned peptide strands or hairpin loop structures. Recent elegant in vitro experiments on the mechanism of proteasomal substrate admission strongly support this idea. Originally, it had been thought that substrates are preferentially fed into the proteasome from their termini and subsequently degraded in a processive way [42, 43]. To challenge this model Liu et al. examined the degradation of tester pro-

teins consisting of naturally disordered protein chains bilaterally protected by fusions of non-degradable GFP (green fluorescent protein) moieties at both termini [44]. Secondly, they inspected circularized tester proteins that lacked open termini and were made up of a disordered protein stretch and a non-degradable GFP moiety. When incubated with activated (open) 26S proteasomes or latent (distal gates closed) 20S proteasomes in vitro, the disordered domains of theses tester proteins were degraded, whereas the tightly folded GFP domains remained unaffected. These results clearly proved that proteasomal degradation does not require substrates with accessible ends and can be initiated at an internal site. After endoproteolytic cutting the proteasome is thought to start processive degradation at both strands of the loop inserted into the proteolytic chamber; complete elimination of the membrane domain in case of Spt23 supports this model. These findings lead to the interesting question how particular parts of a target protein can be spared from destruction.

Several findings indicate that repulse of tightly packed domains that can resist the unfolding forces of the 19S-regulatory complexes (as e.g. the GFP moieties used by Liu [44]) may be a key to this puzzle [44–46]. Association with a protecting partner can be a means to avoid degradation or to exclude defined regions of a target protein from complete destruction. Interaction with the protecting partner may cause stabilization of the correct fold of a region that is left intact, making it inflexible and thus resistant to unfolding and degradation by the proteasome. In agreement with this model formation of homodimers is a major strategy in (cotranslational) processing of NF-κB and has also been proven to be implicated in posttranslational processing of yeast Spt23. Mutations in the IPT dimerization domain present in both transcription factors prevents their processing [41, 46]. Thus, the dimerization domain, which may tightly be packed in the dimers, is thought to act as a terminator of processive degradation of the N-terminal part of these transcription factors, whereas the C-terminal fragments produced by the endoproteolytic cut, including in case of Spt23 membrane-embedded domains, are completely degraded [35]. Interestingly, release of the processed subunit from its homologous, intact binding partner seems to require assistance of special chaperones. Indication exists that in the case of Spt23, this step and thus the launch of Spt23 from the ER membrane is supported by Cdc48 ATPase [41].

ATP-ase

Regulation of uni-directional processes: cell cycle control and induction of apoptosis

The cell division cycle is characterized by a defined sequence of highly coordinated morphological and mechanistic steps. When entering a new cycle, yeast cells are committed to complete the entire cycle. In S-phase DNA is synthesized and *S. cerevisiae* cells emerge the new daughter cell as a bud. After completion of DNA synthesis and transition through a second gap phase (G2), which in budding yeast is less significant, cells enter the M-phase. They attach the duplicated chromosomes to the mitotic spindle apparatus, segregate them into the mother and daughter cell and undergo cell division. The correct course of these processes is controlled by a complex regulatory program. Checkpoints are integrated into this program that survey the correct chronology of the steps and recognize failures induced by accident or harmful conditions.

Proteasome-mediated selective protein degradation is irreversible, and therefore a well-customized regulatory mechanism to control unidirectional processes as is the cell division cycle. Regulatory proteolysis is implicated in control of the cell cycle program on different levels. It is essentially required for the rapid and correct adaptation of the cells' protein equipment to phase-specific tasks. For instance, cyclin molecules appear and disappear in oscillating waves with a defined order [47]. The cyclins associate with certain kinase molecules, in budding yeast the Cdc28 protein, and modify them for specific tasks. Cyclin-dependent kinase complexes (CDKs) generated by these means act as the central governors of the cell cycle program. The ordered sequence of phase-specific CDKs may be viewed as a central biochemical watch controlling the correct course of the cell cycle processes. Proteolysis is required to (i) timely limit the function of the CDK species but (ii) also to allow the rebuilding of the ensuing CDKs, thereby representing both major tasks of regulatory proteolysis. Neutralization of proteins by degradation is an important mechanism to trigger certain cell cycle events. However, in certain cases disposal of proteins that may already have lost their activity by different means but whose presence would disturb perfect function of the machinery in the subsequent cycle can be the major aim of selective proteolysis.

Two large E3 enzyme complexes, the SCF and the APC (anaphase-promoting complex or cyclosome), which share some functional homology [48], are mainly responsible for tagging of substrates that function in execution and control of the cell cycle program (reviewed [49–53]. The SCF is active throughout the whole cell cycle, and substrates are marked for SCF-mediated ubiquitination by phosphorylation, whereas the APC is specifically activated for mitotic tasks. Here, I will describe some prominent examples of cell cycle regulation by the ubiquitin-proteasome system in yeast that are well understood and that had a strong impact on the general understanding of the cell cycle program in eukaryotes.

How to avoid re-replication: proteasomal degradation of Cdc6 in S-phase is required for accurate DNA reproduction

To maintain their genome, eukaryotic cells have to precisely coordinate duplication of the genetic material each time a cell passes through the division cycle. After entry into a new cycle, cells initiate replication of DNA at hundreds or even thousands of defined chromosomal sites, the origins of replication. Start of DNA duplication in the S-phase is preceded by the formation of pre-replicative complexes (pre-RC) in G1. Factors including ORC, Ccd6, Cdct1 and Mcm (minichromosome maintenance) proteins assemble in a series of ordered steps to build pre-RCs at the origins of replication. pre-RCs are prepared for activation by kinases at the transition to replication. After activation, they bind other components in an ordered way, as for instance unwinding factors and multiple eukaryotic DNA polymerases, which ultimately execute DNA replication.

Formation of pre-RCs is a key regulatory event for coordination of DNA replication. Cdc6 plays a critical role in this process and contributes to the strict prevention of DNA re-replication within a single division cycle. Origin recognition complexes (ORCs) composed of six subunits act as the initiator of replication. ORCs bind to specific DNA sites (in yeast A and B1 elements of the origins of replication). Then ORCs, most probably in an ATP-bound state, interact with the Cdc6 protein. Cdc6, which is an ATPase of the AAA family, is thought to act as a loader that uses ATP binding and hydrolysis to load a ring-shaped DNA processivity factor, assembled from Mcm2-7 proteins, the so-called clamp, onto DNA (reviewed in [54, 55]). Cdc6 is regulated through its interaction with ATP and by changes in its abundance or localization. It likely acts as a multimer in which ATP binding and hydrolysis induce conformational changes that result in the recruitment of Mcm proteins to DNA. On the other hand, the cellular level of Cdc6 is modulated during cell cycle progression. As cells pass into S-phase and origins start firing, Cdc6 molecules dissociate from pre-replication complexes, are marked for degradation by SCF^{Cdc4}-dependent ubiquitination and are subsequently eliminated via the proteasome [56–59]. As found for other substrates of SCF, Cdc6 ubiquitination is triggered by its phosphorylation, most probably via Clb-CDK activity. Due to the absence of Cdc6, the formation of pre-RC is inhibited until cells enter the ensuing G1-phase. It is therefore thought that proteolytic elimination of Cdc6 is a major mechanism to prevent re-replication. However, overexpression of Cdc6 in *S. cerevisiae* was not sufficient to induce reduplication, indicating the existence of other redundant mechanisms to avoid re-replication [54]. Interestingly, mammalian cells make use of a different mechanism for neutralization of Cdc6 after origins have started firing. Here, CDK phosphorylation induces the export of Cdc6 from the nucleus during S-phase [60, 61]. Cdc6 is then degraded during the subsequent mitosis via an APC^{Cdh1}-mediated mechanism [62, 63].

Controlling entry into mitosis

Proteasome-mediated destruction of defined regulators is used to trigger defined steps in cell cycle progression. The best-understood example is control of entry into anaphase. To maintain their genomic integrity during proliferation, cells have to correctly distribute the duplicated chromosomes to both daughter cells during mitosis. Failures in this process lead to aneuploidy, a situation that cannot be repaired and may have dire consequences. Genomic instability based on malfunctions of the machinery that guarantees segregation of chromosomes with high fidelity is a central motor of oncogenesis in human cells [64, 65]. The sisters of duplicated chromosomes produced during S-phase have to be untangled and correctly connected to microtubules of opposite polarity; each of the two kinetochores of a sister chromatid pair is linked to one of the two opposing spindle-organizing centers. Then in anaphase sister chromatids are pulled apart to opposing spindle poles. Correct achievement of the status of bi-oriented spindle connection, called amphitelic attachment, is supervised by the spindle damage checkpoint. A central attribute of this process is that the cohesion that holds sister chromatids together is resolved with correct timing, i.e. only after completion of correct spindle attachment (for a recent review see [42]). Cohesion complexes are built up of two coiled-coil proteins Smc1 and Smc3, which dimerize with their C-terminal tails, and a third player Scc1, which connects Smc1 / Smc3 dimers at their N-terminal heads [66, 67]. Therefore, cohesin complexes are thought to form ring-shaped structures, which encompass sister chromatids and hold them together in a topological way rather then by direct binding to chromosome structures [68] (reviewed in [42, 69]. The cohesion between sister chromatids has to be maintained during metaphase until all chromosomes are correctly connected to the mitotic spindle. Maintenance of sister cohesion is an essential prerequisite for the proofing of correct spindle attachment. When the cell enters anaphase, cohesion is resolved to allow separation of the chromatids. This step is triggered by a proteasome-mediated regulatory event, destruction of the Pds1 protein [70, 71] (reviewed in [50, 72]). Pds1, now also termed securin, functions as an inhibitor of a cysteine endoprotease, the separase Esp1. Ubiquitination of Pds1/securin is mediated by the anaphase-promoting complex (APC, so called because loss of its function results in inhibition of entry into anaphase). For this task the APC is activated by a specific regulator, the Cdc20 protein [73–75]. Proteasome-medi-

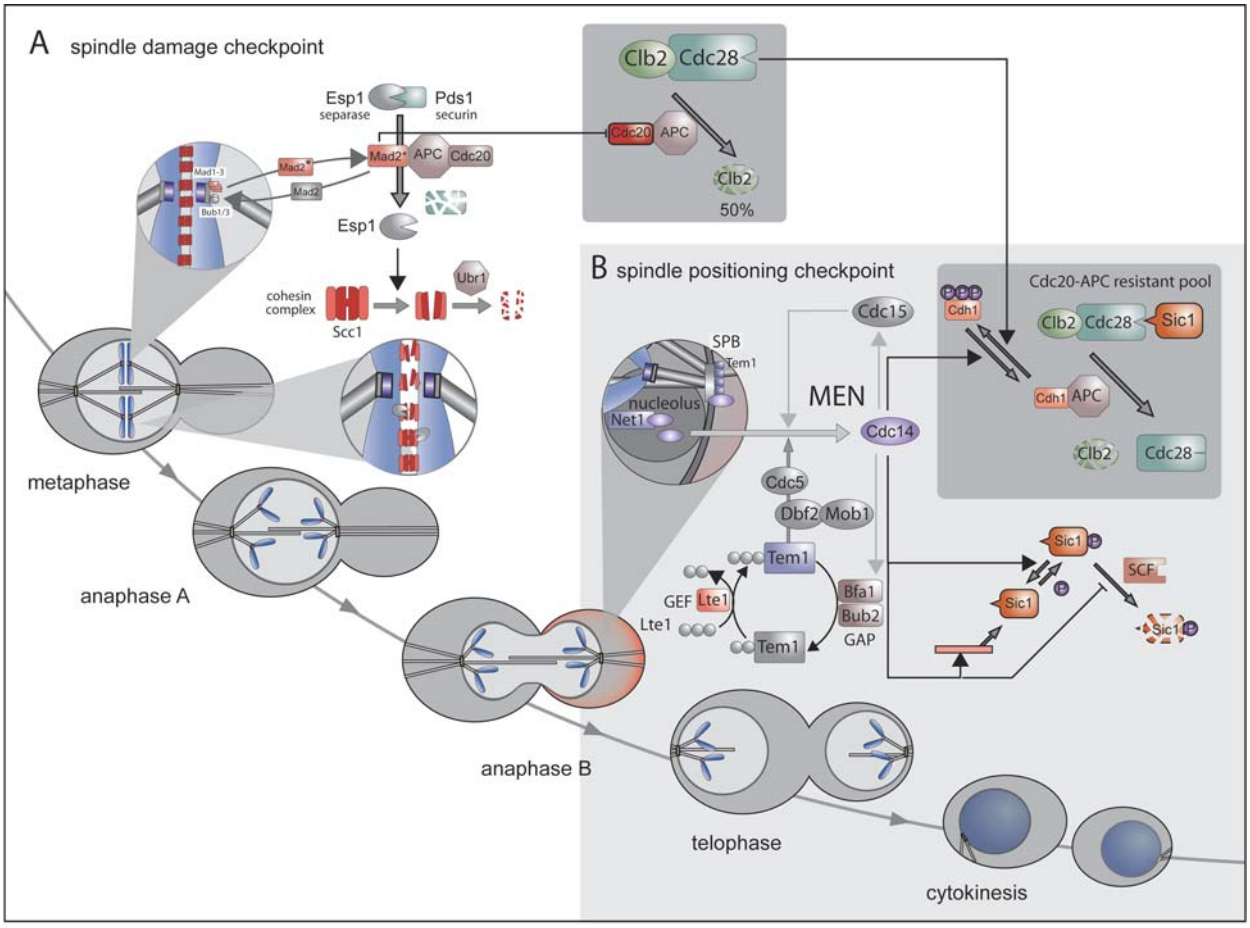

Figure 2. Proteasome-mediated steps regulate mitosis. (*A*) Pds1 degradation is a prerequisite for entry into anaphase. Cdc20-APC is inhibited in response to defective spindle assembly. Incomplete kinetochore-microtubule attachment or lack of tension at kinetochores, which is surveyed by the spindle damage checkpoint, produces a 'wait anaphase' signal presumably generated by an activated form of Mad2 protein. After completion of chromosome-microtubule attachment, the wait signal breaks down and Cdc20-APC triggers proteasome-mediated elimination of securin Pds1, which functions as an inhibitor of Esp1 separase. Esp1 is thus licensed to cleave the cohesin Scc1, leading to resolution of the cohesion between sister chromatids. This step is an essential prerequisite for segregation of chromosomes in anaphase. The C-terminal fragment of Scc1 is removed by a Ubr1-dependent proteasomal pathway. Disposal of remnant Scc1 fragments seems to be necessary for perfect functioning of the spindle in the ensuing cycle. (*B*) About 50% of Clb2 is proteolytically eliminated by a Cdc20-APC-mediated pathway in early mitosis. Upon arrival of the daughter nucleus in the bud cell, the mitotic exit network (MEN) is activated. Tem1, residing at the daughter spindle pole body, is stimulated by its GTP exchange factor Lte1, which is restricted to the bud cell. Activated Tem1, through Dbf2/Mob1, triggers release of Ccd14 phosphatase from its storage place, the nucleolus. Cdc14 initiates final inactivation of Clb-CDK complexes by different means. It stimulates the Cdh1-APC-dependent pathway of Clb2 degradation by dephosphorylation of inactive Cdh1 (also called Hct1). Cdc14 triggers accumulation of the CDK inhibitor Sic1 by (i) stimulating Swi5 dependent transcription and (ii) blocking SCF-mediated degradation of Sic1 protein. As inactivation of Clb-CDK is a prerequisite of mitotic exit, this pathway ensures that cytokinesis is induced only after arrival of the daughter nucleus in the bud cell (for further details see text).

ated elimination of Pds1 leads to release of Esp1 separase [71], which thereby is authorized to open the ring-shaped cohesion complexes by endoproteolytic cleavage of Scc1 at one or both of its cleavage sites [76, 77] (reviewed in [78, 79]). Thus, a distinguishing mark of this proteasome-mediated regulatory process is, that – in contrast to endoproteolytic cleavage of e.g. Spt23 – the proteasome delegates the job of processing to another, highly specific protease (fig. 2).

Interestingly the C-terminal fragment of the cohesin Scc1 produced by Esp1 clipping is degraded proteasome dependently via the Ubr1-mediated N-end rule pathway [80]. This step was found to be important to prevent chromosome instability. Here, the obvious task of proteolysis is disposal of a protein fragment to avoid that this remnant disturbs correct assembly or function of the mitotic apparatus in the ensuing cycle; for a similar purpose the spindle component Ase1 (in this case the full-length protein) is proteolytically eliminated in late mitosis [81].

Pds1 destruction and thereby the permission for sister chromatid separation is controlled by the spindle damage checkpoint. This checkpoint verifies correct assembly of

the mitotic spindle. As long as attachment of kinetochores to spindle microtubules is incomplete (presence of chromosomes that have not acquired a bi-oriented status or when both kinetochores of a sister chromatid pair are erroneously connected to microtubules originating from the same spindle pole) a 'stop' signal is generated that inhibits entry into anaphase until the failure is repaired (fig. 2).

Which deviations from the normal status are recognized by the spindle damage checkpoint is still a matter of debate. Studies in rat kangaroo PTK1 cells have shown that a single unattached kinetochore can induce inhibition of entry into anaphase. The stop signal, however, was neutralized after ablation of the unattached kinetochore by laser-assisted microsurgery and cells restarted progression through mitosis [82]. These results indicated that unattached kinetochores may be a central signaling element producing the 'wait anaphase' signal. On the other hand, skillful micromanipulation of chromosomes in spermatocytes of praying mantid undergoing meiosis pointed to another attribute. Here, presence of an X chromosome that by accident was not connected to its normal partner the Y chromosome and therefore could not acquire a bi-oriented status caused inhibition of entry into anaphase I. When such an unengaged X chromosome was hooked up with a microneedle and stretched, the stop entry into anaphase signal was neutralized and the cells started to continue the meiotic cycle [83]. These results indicated that the ability of correctly attached sister kinetochore pairs to be set under tension may be an important feature that is examined by the spindle damage checkpoint. Interestingly, data obtained from budding yeast support both ideas. Studies of kinetochore protein mutants revealed that these structures may play a central role in induction of the metaphase arrest signal in yeast [84]. Deletion of the kinetochore complex CBF3 subunit Ndc10 caused abrogation of kinetochore assembly and eliminated the mitotic delay induced by spindle-destabilizing drugs; though being unable to attach chromosomes to spindle microtubules the cells continued nuclear division. On the other hand, proof for the idea that tension may play a role in the spindle damage checkpoint of yeast cells came from video-imaging studies of spindle pole body and kinetochore movements during metaphase [85]. This study showed that (as found in other organisms) preceding to anaphase kinetochores were transiently pulled apart polewards. Moreover, the chromatids were elastically stretched during this process, suggesting that the ability of bi-oriented chromosomes to be set under tension may be a quality probed before cells start entry into anaphase.

A single mono-oriented sister chromatid pair, existing in parallel to a large number of correctly attached chromosomes, whose wait signal generators are silenced, can block entry into anaphase [82]. Therefore, the stop signal sent out from even one incorrectly oriented chromosome must inhibit the entire Pds1 degradation machinery. Due to these requirements it appears to be unreasonable that linkage between kinetochores and Cdc20-APC complexes is brought about by a direct physical interaction between both structures. Thus, the existence of diffusible compounds that transfer the stop signal is expected. Possible candidates for this task are found in a set of proteins, Mad1-3 and Bub1/3, whose loss of function resulted in the deficiency to induce metaphase arrest following drug-induced inhibition of microtubule assembly (reviewed in [86, 87]). All these factors bind and act on unattached kinetochores. The most promising aspirant is Mad2. As expected for a regulator that transfers the 'wait anaphase' signal, Mad2 was proven to show up at both platforms, unattached kinetochores and Cdc20-APC [88–91]. Mad2 is an abundant protein, which associated with Mad1 encounters unattached kinetochores with high dynamics. Studies in rat kangaroo cells revealed that release and rebinding of Mad2 at kinetochores occurs with a half-life time of ~25 s [88]. In vivo studies with mammalian cells [92] as well as yeast cells [93] proved that Mad2 is able to form a ternary complex with Cdc20-APC and block Pds1/securin ubiquitination.

Current models propose that during its contact with unattached kinetochores, Mad2 is transferred into an active form (Mad2*), which shuttles to Cdc20-APC and blocks Pds1 ubiquitination (for recent reviews on this topic and its limitations see [87]). It is thought that active Mad2 is relaxed at Cdc20-APC and thereafter travels back to unattached kinetochores to be reactivated (fig. 2). Such a scenario would allow a steady flow of stop information to the securin ubiquitination machinery until incorrectly attached chromosomes acquire their proper state. Then active Mad2 is expected to break down, and silencing of the ubiquitination machinery is nullified. Crucial elements of this model remain to be experimentally proven, and indication was obtained that the situation may be more knotty. For instance, the nature of activated Mad2 remained unresolved. All attempts to identify modified versions of Mad2 have at yet been unsuccessful. In vitro Mad2 is capable of tetramerizing. However, no experimental evidence has obtained that oligomerization of Mad2 may be part of the activation mechanism in vivo. On the other hand, studies in mammalian cells and yeast revealed that Mad2 can form associates with Mad3, Bub3 and Cdc20, suggesting that ternary protein complexes made up of these proteins may contribute to transduction of the wait anaphase signal [94, 95]. The situation is further complicated by the fact that Cdc20 itself cycles at kinetochores even with higher dynamics than Mad2 (half-life time ~2 s) [96], suggesting that in addition or alternatively to Mad2 the APC regulator Cdc20 may pick up information directly at unattached kinetochores. Taken together, the data indicate that the diffusible signal is

made up of a complex cooperation of different elements, which may provide overlapping functions. This interplay and the exact mechanism of transduction of the block Pds1 degradation signal await further inspection (for recent reviews see [86, 87].

Mitotic exit: degradation of B-type cyclins

Control of mitotic exit is a second crucial step of M-phase whose regulation essentially depends on the ubiquitin-proteasome system. Initiation of mitosis is promoted by high Clb2-CDK activity, whereas exit of mitosis requires a switch to a status of low Clb2-CDK activity delineating the G1 phase (see [97] and references therein). To guarantee down-regulation of Clb-CDKs at the end of mitosis, nature has evolved two independent redundant mechanisms: inhibition of Clb-CDK activity by a specific inhibitor protein, Sic1, and proteolytic elimination of mitotic cyclins.

As recently reported, Clb2 degradation in budding yeast mitosis occurs in two stages (reviewed in [98]). As found in all eukaryotes B-type cyclin degradation is initiated at the metaphase-anaphase transition. In early mitosis Cdc20-APC is activated to trigger Pds1 destruction and also becomes engaged in mediating destruction of a fraction of the Clb2-pool during anaphase [99, 100]. However, ~50% of Clb2 seems to be excluded from Cdc20-APC-mediated proteolytic destruction and persists until late mitosis [99] (fig. 2). It remains undefined how exclusion of this Clb2 portion from Cdc20-APC mediated proteolysis is achieved. The saved fraction of Clb2 is degraded later in M-phase when the cell is prepared to exit mitosis via a different, Cdh1-APC-dependent pathway [73, 101]. Cdc20-APC and Cdh1-APC activities are controlled by different means. The Cdc20 concentration oscillates in a cell cycle-dependent manner. Its level rises at entry to M-phase due to initiation of synthesis and is lowered in G1-phase by proteasome-mediated degradation [75, 102–104]. In contrast, the APC activator Cdh1 is present throughout the entire cycle with a constant concentration [103]. Cdh1 can be kept inactive by Clb-CDK-dependent phosphorylation and can be switched into the active status by dephosphorylation [101, 105, 106].

To ensure correct distribution of duplicated genomes to both daughter cells, chromosome segregation must always precede mitotic exit. Correct chronology of these processes is surveyed by the spindle-positioning checkpoint (reviewed in [107]). A central regulator of this checkpoint is Cdc14 phosphatase. Throughout almost the entire cell cycle (G1-, S-, G2- and early M-phase) Cdc14 is kept inactive by a specific inhibitor, the Net1 protein. Interaction with Net1 holds Cdc14 in a stand-by position at its depository, the nucleolus (fig. 2). Subsequent to arrival of the daughter nucleus in the bud cell Cdc14 is activated by its release from the nucleolus [108–110]. Cdc14 induces Clb-CDK inactivation by turning back the outcome of phosphorylation steps executed by mitotic CDKs. Though acting also on other proteins, dephosphorylation of three targets is crucial in Cdc14-dependent regulation of mitotic exit (fig. 2). Cdc14 up-regulates the level and thereby the activity of the Clb-CDK inhibitor Sic1 by two different means. Cdc14 dephosphorylates and thereby proteolytically stabilizes Sic1 [111, 112], which in the phosphorylated state is proteasome dependently degraded throughout the entire cell cycle via an SCF (Cdc4)-mediated pathway. Cdc14, on the other hand, promotes *SIC1* transcription by causing dephosphorylation and thereby nuclear up-take of the *SIC1* transcription factor Swi5 [111, 113, 114]. In addition to up-regulation of the Sic1 concentration Cdc14 phosphatase triggers proteolytic elimination of Clb2. For this purpose Cdc14 dephosphorylates Cdh1, thereby licensing this factor to bind to the APC and to recruit Clb2 to the ubiquitination machinery [73, 101, 105].

How is release and activation of Cdc14 linked to arrival of the daughter nucleus in the bud cell? Cdc14 functions as the ultimate effector of a sophisticated signal transduction pathway termed the mitotic exit network (MEN), which is a crucial regulatory pathway designed to control exit from mitosis (fig. 2). The MEN is activated by the RAS-like G-protein Tem1 (reviewed in [115]). After formation of the mitotic spindle Tem1 preferentially resides to the cytoplasmic face of the spindle pole body (SPB) that is destined to enter the newly formed daughter cell [116, 117]. Tem1 is kept inactive by an unusual GTPase-activating unit (GAP), a complex made up of two components Bub2 and Bfa1, which can prevent mitotic exit in response to defective spindle assembly. When duplicated nuclei are separated and the SPB bearing Tem1 enters the newly formed bud cell, the G-protein is brought into contact with its activator, the guanine-nucleotide exchange factor (GEF) Lte1, which only exists in the new daughter cell [116, 117]. Then through an unknown mechanism including Dbf2/Mob1 complexes active Tem1 triggers release of Cdc14 from the nucleolus, inducing final Clb2 degradation and Clb-CDK inactivation. Cdc14 in addition dephosphorylates Cdc15, which subsequently binds to the daughter SBP and triggers cytokinesis [118]. Activated Cdc15 is also thought to fire into a positive feedback loop that stimulates further Cdc14 release. The exact composition and interplay of MEN components, for instance the multiple role of polo kinase Cdc5, in control of MEN awaits further investigation (for further details see reviews by [115, 119, 120]). The story is complicated by the fact that two additional pathways have been discovered that contribute to control of Cdc14 release and regulation of MEN. The first pathway, called FEAR, is activated by the Esp1 separase and triggers temporary

Cdc14 release in early mitosis (anaphase) (for overviews see [119, 121, 122]). The second, so-called AMEN, which is centrally governed by the Amn1 protein, negatively controls MEN [123] (reviewed in [120]).

Apoptosis, a new field in ubiquitin-proteasome research in yeast: destructive processes regulate cell death

Apoptosis, a highly coordinated program of cellular suicide, plays an essential role in the development, homeostasis and maintenance of multicellular organisms. Apoptosis is implicated in elimination of superfluous cells and body shaping during development. It is essentially required for removal of harmful cells such as autoreactive immune cells, virus-infected cells or cells with unrepairable DNA damage bearing the risk of being transformed to malignant tumor cells. Because of its impact on human diseases such as acquired immunodeficiency syndrome (AIDS), cancer, autoimmune and neurodegenerative disorders, research on apoptosis has become a major issue in biomedical science [124, 125].

A central feature of apoptosis is its tight control and effectiveness. A variety of external and internal stimuli induce apoptosis, allowing selective and rapid removal of cells without damage of the surrounding tissue. External stimuli that can cause apoptosis are receptor ligands, various toxins [ethanol, reactive oxygen species (ROSs)] or toxic conditions (UV, γ-radiation). Apoptosis-stimulating internal signals can be brought about by damage such as mitochondrial leakage, mitotic catastrophe, replication failures or by specific processes, for instance developmental events. The cell processes complex information made up of external and internal pro- and anti-apoptotic stimuli to finally come to a decision of yes or no, i.e. whether to execute cellular suicide or not. During the course of the apoptotic program, cells undergo a set of typical cytological and morphological alterations. Phosphatidyl-serine, which in healthy cells exclusively localizes to the inner layer of the plasma membrane, flips to the outer leaflet in early apoptosis. The chromatin condenses and is concentrated close to the nuclear envelope (chromatin margination). The DNA is cut into short fragments, and the cell is broken down to smaller membrane-enclosed particles, the apoptotic bodies, which may harbor whole organelles (for instance mitochondria or fragmented nuclei). On the molecular level induction and execution of apoptosis is controlled by a sophisticated network composed of ligand receptor proteins, systems that recognize and signal internal damage, signal transducers, pro- and anti-apoptotic regulators and effector molecules that finally carry out the cytological processes of apoptosis (for review see [126]).

The ubiquitin-proteasome system in apoptosis of cells from metazoan organisms

One of the prominent tasks of the proteasome system is removal of abnormal and damaged proteins, suggesting that this system may have a fundamental function in elimination of waste and avoidance of intracellular disturbances that lead to induction of apoptosis. On the other hand, the irreversibility of proteolysis suggested ubiquitin-proteasome-mediated degradation of defined targets to be an ideal regulatory tool for control of the unidirectional program of apoptosis. The ubiquitin-proteasome system was thus expected to play an important role in the cell death machinery. Recent research in metazoan cells has indeed confirmed this idea (see recent reviews in [127–129]). Proteasome-mediated regulatory proteolysis can contribute to regulation of apoptosis on different levels. It may directly negatively control regulators that stimulate induction of apoptosis but also affect inhibitor proteins that prevent induction of the cell death program, and it may influence the level of apoptotic regulators by contributing to control of their transcription (see previous section). Moreover, cellular pathways or programs, for instance cell cycle or DNA damage response, that are tightly linked to the cell death program depend on proteasome-mediated regulation. Based on these suggestions the ubiquitin-proteasome system was expected to fulfill multiple functions in control of apoptosis and to regulate both pro- and anti-apoptotic processes. Initial studies with proteasome inhibitors supported this view. Dependent on the cell line application of proteasome inhibitors resulted in either stimulation [130–133] or prevention of apoptosis [134, 135]. Depending on conditions, for instance cell line, genetic background, nature and concentration of the proteasome inhibitor, proteasome inhibition by chemicals seems to influence anti- and pro-apoptotic processes to a different extent, in one case resulting in stimulation, in the other case avoidance of apoptosis. Further evidence for functions of the ubiquitin-proteasome system in apoptosis was obtained by the finding that factors involved in apoptosis were identified to constitute components of the ubiquitin-dependent degradation machinery. It was found for instance that loss of the von Hippel-Lindau tumor suppressor gene (VHL), which codes for a component of the SCF-related VHL/Elongin-B ubiquitin ligase complex (VCB complex), leads to inhibition of apoptosis in renal carcinoma cells [136–138] and that RING finger-containing members of the IAP (inhibitor of apoptosis) family are E3/ubiquitin ligases (see below).

Moreover, studies in metazoan cells demonstrated the involvement of ubiquitin-proteasome-mediated degradation in regulation of a variety of components of the cell death machinery. The current view is that cell proliferation and apoptosis are controlled in a strict antagonistic

way. In response to DNA damage the cell stops cell cycle progression to allow repair. If this is impossible, the cell undergoes apoptotic suicide. A key regulator of this decision and therefore a crucial factor for maintenance of genomic integrity and prevention of tumor development is the tumor suppressor protein p53 [139–141]. This regulator mainly acts as a transcription factor that controls expression of many target genes, including induction of pro-apoptotic genes and repression of anti-apoptotic genes. In addition p53 may act through transcription-independent mechanisms. Its activity is mainly regulated at the post-translational level by changing its proteolytic stability [142, 143]. In unstimulated cells p53 is kept low by targeting its proteasome-mediated destruction via the RING finger-containing ubiquitin ligase Mdm2 [142–144]. In addition to destruction, p53 ubiquitination triggers its export from the nucleus, thereby keeping p53 away from its transcriptional tasks [145, 146]. In response to the absence of growth factors, oncogene activation, DNA damaging agents or hypoxia, the p53 level increases to either induce growth arrest or initiate apoptosis. For this purpose p53 degradation is down-regulated. This can be achieved (for instance in response to irradiation or application of chemotherapeutic drugs) by p53 phosphorylation, which prevents its interaction with Mdm2. Alternatively, for instance after oncogene activation, p53 degradation can be neutralized by Mdm2 inactivation through expression of a specific inhibitor, ARF [147, 148].

Proteins of the Bcl2 family are important regulators of apoptosis, including both pro-apoptotic factors (Bax and Bak subfamily and BH3-only proteins) and anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w). These factors function as key regulators of the integrity of mitochondria and are thought to organize cytochrome C release from the mitochondrial intermembrane space to the cytoplasm. Liberated cytochrome C stimulates caspase 9 activation, thereby triggering an important internal signaling event in induction of apoptosis (for overview see [126, 149]). Pro-apoptotic proteins of the Bcl-2 family are involved in many types of apoptotic cell death. Moreover, the presence of anti-apoptotic members of the Bcl-2 family appeared to be an important prerequisite for survival of each type of cell. Balancing the ratio between pro- and anti-apoptotic Bcl-2 family members is a crucial factor in regulation of many types of apoptosis. Here, the proteasome fulfills an important role. For instance, it was found that degradation of Bcl-2 is a key regulatory event in tumor necrosis factor α (TNF-α)-induced apoptosis of human endothelial cells [150]. In this pathway TNF-α triggers dephosphorylation of Bcl-2 and thereby its subsequent ubiquitination. On the other hand, elimination of the pro-apoptotic factors Bax and Bik via the ubiquitin-proteasome system appeared to be a mechanism that prevents induction of cell death and may be used as a survival mechanism of some tumor cells [151, 152]. Another important class of proteins that clearly demonstrates the involvement of the ubiquitin-proteasome system in regulation of apoptosis is the IAP family. Members of these bona fide inhibitors of apoptosis are not only controlled by proteasome-mediated degradation but appeared to execute functions in ubiquitination. One member, the 528 kDa murine protein BRUCE, harbors a ubiquitin-conjugating domain (UBC) and was characterized to function as an E2-ubiquitin-conjugating enzyme [153]. Other IAP members, e.g. XIAP, cIAP1 and cIAP2, possess RING finger domains typical for E3-ubiquitin ligases. In response to apoptotic stimuli these IAPs perform autoubiquitination and are degraded [154, 155]. In addition to triggering their own elimination, IAPs can induce ubiquitination of other molecules, including proteins of the caspase family. Caspases are cysteine proteases that play a central role in initiation of almost any type of apoptotic cell death. They are activated from inactive precursors by (auto)-proteolytic processing. Different type of caspases can cooperate as signaling molecules in a cascade causing amplification of the pro-apoptotic signal but are also crucial executioners of the cell death program by causing proteolytic cleavage or degradation of various target proteins (for overview see [126]). xIAP, cIAP1 and cIAP block apoptotic death through binding and inhibition of caspases and can in addition promote ubiquitination and proteolytic destruction of caspase molecules through their E3 activity [154, 156] (for recent reviews see [127–129]).

Apoptosis in budding yeast

Apoptosis in metazoan cells is well established and a field of intensive research. Apoptotic cell suicide seemed to be useless for monocellular microorganisms because here it would result in death of the entire individual. However, on second thought one could imagine that programmed elimination of individual cells from a colony or culture can be an evolutionary advantage even for monocellular species. The initial view of absence of an apoptotic program in yeast was supported by the finding that no obvious orthologues of players of apoptosis, such as e.g. Bcl-2 family members or caspases, were found in the *S. cerevisiae* genome.

In 1997, however, Madeo et al. obtained evidence for the existence of apoptotic cell death in budding yeast. They showed that *S. cerevisiae* cells that harbored a special point mutant allele of the AAA-ATPase gene *CDC48* (*cdc48*^{S565G}) died with the typical attributes of apoptosis. When cultivated on glucose medium for several days, a large fraction of *cdc48* cells underwent cell death, accompanied by resolution of plasma membrane asymmetry, chromatin condensation and margination, cutting of DNA into small pieces and even cell fragmentation [157].

Two years later the involvement of Cdc48 in apoptosis was confirmed in the mammalian system by the finding that a human orthologue, the VCP protein, is required for prevention of apoptosis in mammalian cells [158]. Even these initial data on yeast apoptosis link cell death to the ubiquitin-proteasome system. Phenotypic analysis in yeast revealed Cdc48 ATPase to play a role in a series of unrelated processes such as homotypic membrane fusions, activation of transcription factors (see previous section) and, as shown recently, regulation of mitotic exit and spindle disassembly [41, 159–162]. On the molecular level Cdc48 was characterized to use ATPase activity to unfold proteins or dissociate proteins from large structures [163], thereby playing a crucial role in making substrates accessible to the proteasome [160, 164–166] (reviewed in [7]). Interestingly, in mammalian cells another factor that is connected to apoptosis is engaged in a similar task. The Bcl2 binding anthanogene-1 (BAG1), which harbors a UBL (ubiquitin-like) domain, is involved in transfer of substrates – for instance delivery of aggregation-prone proteins – to the proteasome system (reviewed in [7, 127]). Existence of an apoptotic program in yeast was further proven by the finding that yeast cells showed an ability to respond to apoptotic regulators from the mammalian cell. When pro-apoptotic factors of the Bcl-2 family, for instance Bax, were expressed in yeast, the cells did not tolerate the presence of these factors and died [167]. A closer inspection of cells overexpressing Bax demonstrated that this effect is based on induction of a cell death that is accompanied by typical apoptotic phenotypes such as phosphatidyl-serine exposition, membrane blebbing, chromatin condensation and DNA fragmentation [168]. These effects and cell death, however, were prevented by the coexpression of the anti-apoptotic factor Bcl-xL. As in mammalian cells, Bax is targeted to the mitochondrial membranes of the yeast cell [169] and causes release of cytochrome C [170]. This release, however, seemed not to be the central deadly effect, because Bax could induce lethality even in yeast cells that expressed a GFP-tagged cytochrome C that was not released from mitochondria [171]. In addition to Bcl-2 members, expression of caspases [172] and Apaf1 [173], which is a factor involved in cytochrome C-triggered activation of caspase 9, were also lethal for the yeast cell. Taken together, these data showed that the yeast cell may possess an ancient apoptotic program that is activated by these mammalian regulators. Apoptosis in budding yeast was further established by the discovery of a caspase-related protease in this species [174]. Based on low sequence similarities with mammalian caspases, a family of metacaspases had been defined with members in protists, fungi and plants [175]. The *S. cerevisiae* member, now called Yca1/Mca1 protein, is a protease with specificity for caspase substrates. In response to apoptotic stimuli Yca1 is activated in a caspase-typical manner by proteolytic processing and clearly contributes to apoptosis in yeast. Conditions or signals that lead to induction of apoptosis in yeast are exposure to ROSs, DNA damage, nutrient limitation and cell aging.

For identification of potential proteasomal substrates Ligr et al. undertook a screen for genes whose overexpression was toxic for cells that due to 20S proteasomal mutations were defective in proteasome-mediated degradation [176]. Further inspection of 62 candidate genes showing the expected phenotype of high expression lethality (HEL genes) yielded six genes [Nsr1, Ppa1, Sar1, Stm1 and the two unknown ORFs (open reading frames) Hel10 and Hel13], whose overexpression in proteasome-deficient strains resulted in induction of cell death with all the hallmarks of apoptosis. In each of these cases cell death was associated with phosphatidyl-serine exposure, DNA fragmentation, chromatin-condensation and -margination, whereas no apoptotic phenotypes were detected for the other 56 HEL candidates. Thus, these six genes constitute a first set of endogenous yeast genes with a pro-apoptotic role. Induction of apoptosis by these HEL genes may be more or less direct. Accumulation of such proteins may on the one hand damage the cell in a way that apoptosis is initiated as a final response. Apoptosis-inducing Hel proteins may, on the other hand, include integral regulators of apoptotic pathways whose activation leads directly to execution of the cell death program.

Due to following findings the Stm1 protein is thought to constitute a direct activator of apoptosis: Stm1 shows direct connection to DNA damage and repair. It is a DNA-binding protein, which preferentially associates with G-rich quadruplex DNA in vitro and appeared to be associated with telomeres in vivo [177]. Cells lacking the *STM1* gene show enhanced sensitivity against certain DNA-damaging conditions, for instance UV irradiation or induction of double strand breaks by the radiomimetic drug bleomycin [176]. Stm1 interacts with Mec3 [W. Hilt unpublished data], which is a kinase that plays a crucial role in DNA-damage signaling. As revealed in null mutants, Stm1 is required to prevent certain types of DNA damage. On the other hand, Stm1 has a pro-active role in ROS-induced apoptotic cell death. Cells lacking Stm1 cells show reduced sensitivity with a clear decrease in appearance of apoptotic cells when treated with subtoxic concentrations of hydrogen peroxide [176]. Thus, though showing no obvious sequence similarity, Stm1 shares several overlapping features with the tumor suppressor p53. Stm1 is involved in the decision between execution of DNA repair and induction of apoptosis. Moreover, Stm1, like p53, is post-translationally controlled by proteasome-mediated proteolysis. Recent work links Stm1 to the yeast caspase Yca1 [W. Hilt, unpublished data].

Another interesting protein causing the HEL phenotype is Sar1. Sar1 is a small GTPase involved in control of

vesicle transport from the ER to the Golgi compartment (reviewed in [178]). Several lines of evidence were obtained that blocking of the secretory pathway may lead to induction of apoptosis [179]. Answering the questions how Sar1 is controlled by the ubiquitin-proteasome system and why enhancing its level leads to induction of apoptosis awaits further research.

Interestingly, rapid destruction of the replication factor Cdc6 can serve as a pathway to apoptosis [180]. Here, Cdc6 was either destroyed through a caspase-dependent extrinsically induced pathway or by a ubiquitin-proteasome-mediated process that was stimulated by treatment with the DNA-damaging drug adozelesin. The latter pathway, which in mammalian cells is independent of p53, is conserved in *S. cerevisiae.* In proliferative cells that have committed to undergo apoptosis, DNA replication is often uncoupled from mitosis through the premature activation of mitotic CDKs. DNA damage-induced Cdc6 degradation via the proteasome may contribute to such uncoupling and constitute a ancient pathway of cell death response.

Future prospects

Sequencing of the *S. cerevisiae* genome has uncovered ~6000 potential ORFs. Comparitive sequencing reduced these ORFs to a number of 5727 genuine genes coding for proteins with a size larger than 50 amino acids [181]. 50% of the genes detected are annotated as unknown ORFs; a large number of the rest are less characterized concerning their function. Research on these functionally less or uncharacterized genes will lead to the discovery of new proteasomal substrates. A recent proteome-wide analysis detected more than 1000 yeast proteins that exist in ubiquitinated forms [182]. Further work will clarify how tagging of new proteasomal substrates for degradation is signaled, reveal the players involved and resolve the detailed mechanism of their proteolytic destruction. Such work will lead to the discovery of new pathways that are regulated by the ubiquitin-proteasome system. Integration of such steps of regulatory proteolysis into larger cellular networks – presumably including use of computational modeling and systems biology – will greatly enhance our understanding of cell function and regulation.

In recent years proteasomes have become interesting targets for pharmacological intervention. Proteasome inhibitors are now in clinical trials as pharmaceuticals for the chemotherapy of certain types of cancer [183, 184]. The better understanding of defined pathways of regulatory proteolysis will open new opportunities for development of strategies for the treatment of severe diseases. Future research in yeast will provide further basic insight into this field.

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