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Minireview

Regulation of the G1 to S transition by the ubiquitin pathway

Lauren M. DeSalle, Michele Pagano*

Department of Pathology and Kaplan Comprehensive Cancer Center, MSB 548, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA

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Abstract This year the most prestigious prize in medical sciences, the Lasker Award, has been presented to the three scientists who discovered the ubiquitin pathway: Aaron Ciechanover, Avram Hershko, and Alexander Varshavsky [Nature Med. 6 (2000) 1073–1081]. During a time when the scientific community was focused on understanding how proteins were synthesized, they intently pursued the novel idea that cells were programmed to selectively destroy proteins. Their work led to the identification of an elaborate system of protein degradation targeting a myriad of cellular substrates. A small protein called ubiquitin is at the center of this process. Although the ubiquitin pathway was first described in the early 1980s, it has only more recently advanced to the forefront of basic research as a significant regulatory network within the cell. The field continues to grow as new ubiquitination enzymes and novel functions of this system are identified. Scientists are focused on elucidating the mechanisms by which cells deploy the ubiquitin pathway to control levels of selected proteins, such as cell cycle regulatory proteins, transcription factors and signaling molecules. Accelerated or decelerated rates of degradation of particular substrates participate in the genesis of many human diseases. Thus, understanding the mechanisms that confer specificity to the ubiquitin system will allow the development of novel therapeutic approaches to target aberrations in this pathway underlying tumorigenesis and other human pathologies.

Key words: Ubiquitin; SCF complex; Cell cycle; Cancer; Skp2

1. Introduction

Targeted protein degradation by the ubiquitin-proteasome pathway plays a vital role in monitoring the abundance of many short-lived regulatory proteins (reviewed in [2]). A polyubiquitin chain is built onto one or multiple lysine residues of a substrate to target it for capture and degradation by the 26S proteasome. Then the proteolytic core of the proteasome processes the ubiquitinated substrate into small peptides. The conjugation of ubiquitin, a small (76 amino acid) heat-stable polypeptide present in all eukaryotes, to a protein substrate is executed by a series of well-defined enzymatic reactions. The three enzymes of this cascade were purified by affinity chromatography of a crude reticulocyte extract over a ubiquitin-Sepharose column. The enzymatic activity of the three eluates (E1, E2, and E3) was characterized [3]. The ubiquitin-activating enzyme (E1) forms an ATP-dependent thioester bond with ubiquitin and transfers it to one of many ubiquitin-conjugating enzymes (E2s or Ubcs). E2s form a thioester bond with activated ubiquitin and transfer it to a substrate with the help of one of many ubiquitin ligases (E3s or Ubls). Some ubiquitin ligases such as the Hect proteins (homologous to E6-AP C-terminus) can form a thioester bond with ubiquitin as an intermediate in the transfer of ubiquitin from the E2 to the substrate (reviewed in [4]). This tight ubiquitin regulatory cascade is evolutionarily conserved from yeast to mammals. Combinatorial interactions among different E2s and different E3s generate a large number of specific substrate targeting complexes that regulate the stability of key proteins (Fig. 1). The exact temporal and spatial destruction of substrates by ubiquitin-mediated degradation is consistent with its task in controlling cell division, transcription, cell signaling and development.

Progression through the eukaryotic cell cycle is defined by a timed succession of distinct events (reviewed in [5]). A cell's regulatory machinery must guarantee completion of DNA replication in the S (synthesis) phase before the chromosomes are segregated into daughter cells at the end of the M (mitosis) phase. This periodic movement through the cell cycle is orchestrated by programmed oscillations in the activity of a family of serine/threonine protein kinases called cyclin-dependent kinases (cdks). The activation of a cdk is dependent on its association with a cyclin regulatory subunit; inactivation is regulated by its association with a cdk inhibitor (cki). Furthermore, a fine control by upstream cdk regulatory kinases and phosphatases as well as by a transcriptional network mediates the fluctuation in cdk activity. Cdks and their regulators are internal sensors of mitogenic and antimitogenic signals. Their rapid response to stimuli such as growth factors, DNA damage, or cell–cell contact inhibition is critical for controlled proliferation. Of particular interest for this review is the rapid and temporal destruction of cellular regulatory proteins by ubiquitin-dependent degradation to advance unidirectional transitions through the cell cycle.

Two distinct ubiquitin conjugation pathways mediate cell division by affecting the following events: (1) transition from G1 to S phase, (2) sister chromatid separation during anaphase, and (3) exit from mitosis. The first event in G1/S, initially characterized in *Saccharomyces cerevisiae*, requires the E2 enzyme, Cdc34 (or Ubc3), and an E3 ligase complex

*Corresponding author. Fax: (1)-212-263 8211.
E-mail: paganm02@med.nyu.edu

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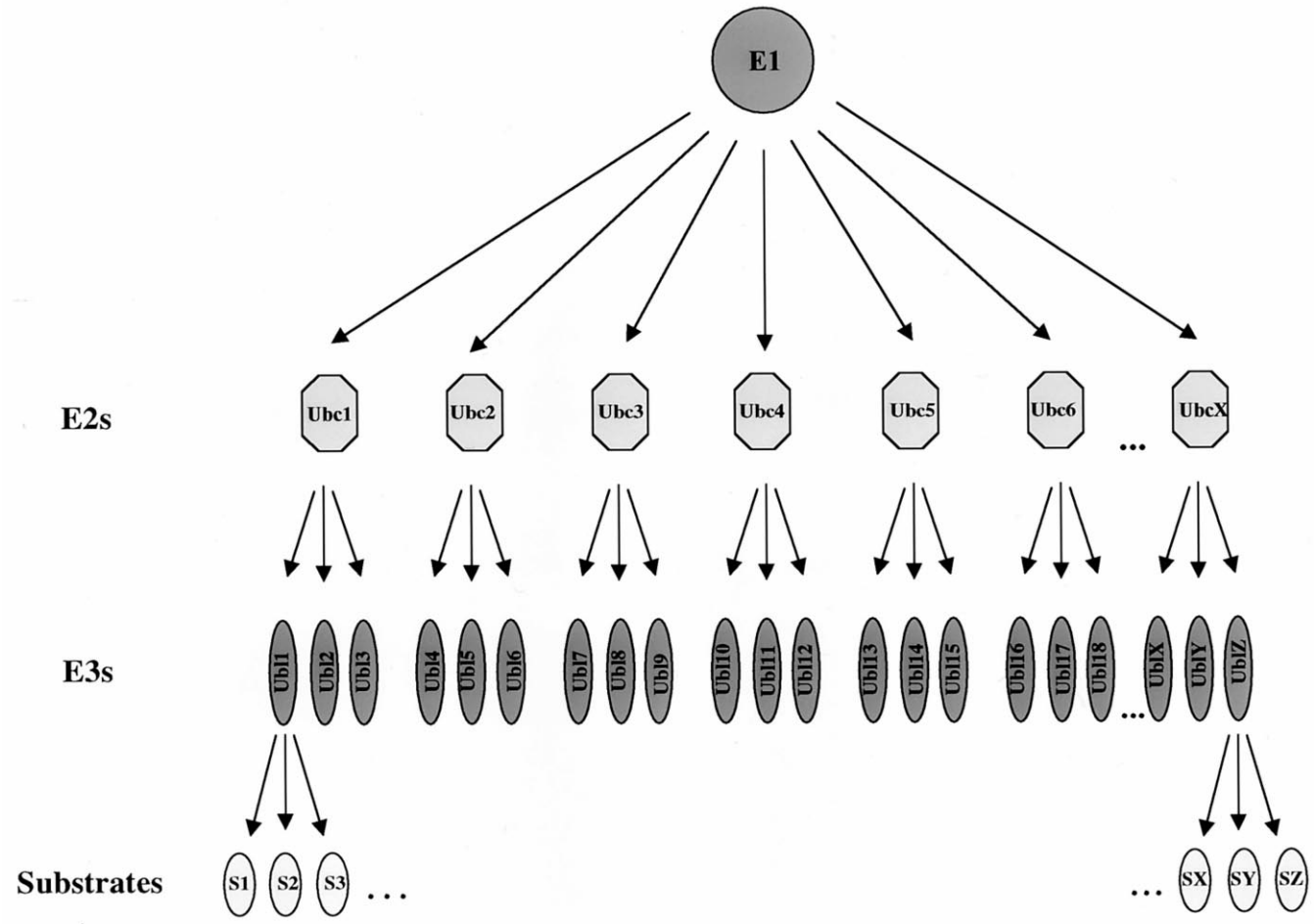

Fig. 1. Schematic representation of the hierarchy of the ubiquitin pathway. The ubiquitin-activating enzyme (E1) transfers activated ubiquitin to one of many ubiquitin-conjugating enzymes (E2s or Ubcs). With the help of one of many ubiquitin ligases (E3s or Ubls), an E2 transfers ubiquitin to the substrate(s) to target it for degradation. The dashed arrows suggest the variety of combinatorial interactions between different E2s and different E3s that are possible. The complexity and specificity of this pathway increases with each enzymatic reaction in the cascade.

termed SCF^{Cdc4} (Skp-cullin-F-box protein) to activate DNA replication. The two mitotic events require a large multi-protein E3 complex called the APC/C (anaphase promoting complex/cyclosome) in combination with one of two distinct E2s (Ubc10 or Ubc4). It regulates mitosis by affecting chromosome and spindle dynamics and by regulating the activity of mitotic cdks (reviewed in [5,6]). The focus of this review is the role of the mammalian SCF ubiquitin ligases in the control of the G1/S transition. Following a brief description of the convergence of the cell cycle with the ubiquitin pathway is a discussion of our current understanding of mammalian SCF complexes.

2. Historical perspective

A consensus on how the cell cycle is thought to function emerged in the late 1980s. Genetic experiments in *Schizosaccharomyces pombe* (fission yeast) and *S. cerevisiae* (budding yeast) indicated that the eukaryotic cell cycle was defined by transitions from one state (G1/S) to another (G2/M). Cell division cycle (Cdc) mutants were identified in yeast that could prevent cells from replicating their DNA, while other mutants prevented mitosis. Biochemical experiments using extracts from *Xenopus laevis* that accurately reproduce the cell cycle in vitro provided an experimental system that advanced our understanding of M phase regulation. (These data were originally reviewed in [7,8]; refer to [9] for a current review of the cell cycle.)

Initially, a complex called maturation promoting factor or M phase promoting factor (MPF) was identified and had the ability to release *Xenopus* oocytes from meiotic arrest. MPF was purified as two components, a kinase (found to be homologous to *S. pombe* Cdc2) and a cyclin subunit. This latter was shown to be the regulatory subunit required for the catalytic activation of Cdc2. At the time that MPF was purified, cyclins were divided into two classes, A and B, based on their size, kinetics of appearance in the cell cycle, and sequence homology. The destruction of these cyclins during M phase was responsible for the loss of kinase activity that allowed for exit from mitosis. This was the first suggestion that selective proteolysis played an important role in the cell cycle.

A more direct indication of the relationship between regulation of the cell cycle and ubiquitin-dependent degradation emerged from insight into the role of Cdc34 in budding yeast. Cdc34 was known as an essential regulator of the G1/S transition. In 1988, Goebl and colleagues discovered that Cdc34 also had ubiquitin-conjugating activity. It could catalyze the covalent attachment of ubiquitin to histones H2A and H2B in

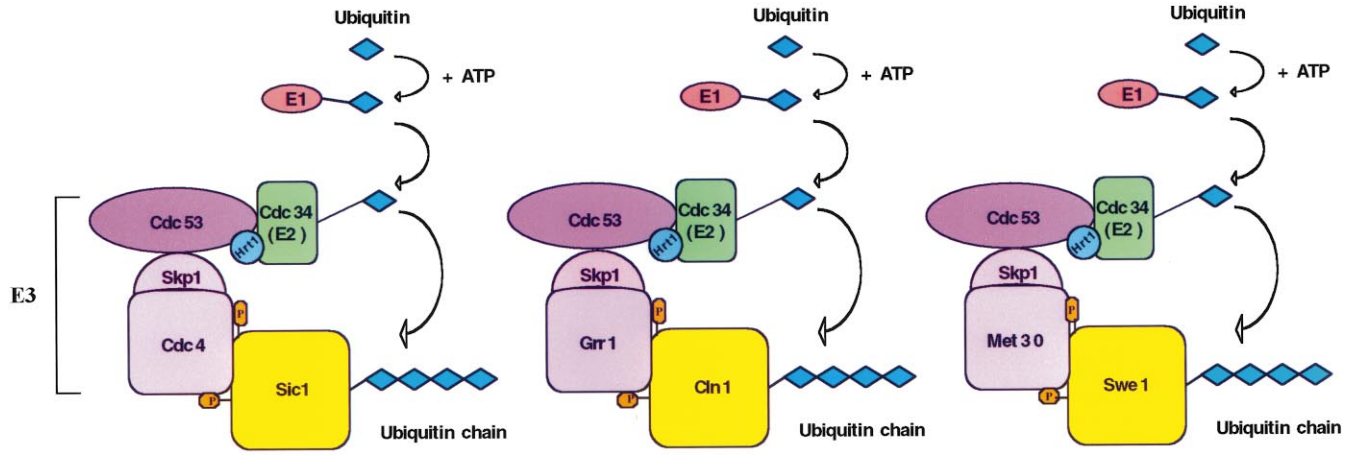

Fig. 2. Schematic representation of budding yeast SCF^{Cdc4}, SCF^{Grr1}, and SCF^{Met30}. These ubiquitin ligases are called SCFs because each is formed by three major subunits: Skp1, Cdc53 (also called CulA), and one of many F-box proteins. SCF ligases also contain a small RING finger protein subunit called Hrt1 through which they bind to the ubiquitin-conjugating enzyme Cdc34. SCF^{Cdc4} binds phosphorylated Sic1 through the F-box protein Cdc4 (which also targets Cdc6, Far1, and Gcn4 for degradation). SCF^{Grr1} binds phosphorylated Cln1 through the F-box protein Grr1 (which also targets Cln2 and Gic1/2 for degradation). SCF^{Met30} binds phosphorylated Swe1 through the F-box protein Met-30.

vitro [10]. Curiously, the significance of the in vitro ubiquitin-conjugating activity of Cdc34 on histones remains unclear to this day since H2A and H2B are long-lived cellular proteins. Importantly, Cdc34 has proven fundamental to our knowledge of the SCF system of ubiquitin-dependent degradation.

3. SCF^{cdc4} in *S. cerevisiae*

The classic example of SCF-dependent degradation emerged from genetic studies in *S. cerevisiae*. In this yeast, entry into S phase requires the activity of the S phase promoting complex Clb5–Cdc28 (where Clb5 is an S phase kinase and Cdc28 is an *S. cerevisiae* cdk). This complex is assembled during G1 phase, but is inactive due to high levels of Sic1, a Clb5–Cdc28 inhibitor. The loss of Cdc34, as well as Cdc4 or Cdc53 (also cloned in screens for cell cycle mutants), each results in a failure to initiate DNA replication despite high levels of Cln–Cdc28 kinase activity (Clns are the G1 cyclins in this yeast). Data from several groups explained this arrest in G1 as the inability to eliminate Sic1. Sic1 was blocking the activity of the downstream Clb5–Cdc28 kinase, whose activation is necessary for DNA replication [11,12]. These findings suggested that Sic1 must be degraded in late G1 by a proteolytic pathway requiring the ubiquitin-conjugating activity of Cdc34, as well as functional Cdc4 and Cdc53 [13,14]. Further experiments demonstrated that the Cdc34-mediated degradation of Sic1 is triggered by the phosphorylation of Sic1 by Cln–Cdc28 [15–18].

The idea that a multiprotein complex directed Sic1 degradation became apparent after the discovery that Cdc34 physically associated with Cdc53 and Cdc4 [19,20]. Additional genetic experiments led to the identification of Skp1, which was isolated as a suppressor of a Cdc4 mutant [21]. Human Skp1 had been identified as an interactor of the cyclin A/Cdk2 complex (S phase kinase interacting protein 1) but its function was not understood [22]. Then in a screen for suppressors of Cdc4 mutants, Harper's and Elledge's groups found that different Skp1 mutants could arrest yeast in either G1 or G2. An alignment of Skp1-interacting proteins, including Cdc4, Met30, Grr1, and β-Trcp, resulted in the identification of a Skp1-interaction motif called the F-box, because it was also found in cyclin F [21]. In addition to its function in Sic1 proteolysis, Skp1 was shown to have a role in Cln2 stability. The degradation pathways of Sic1 and Cln2 both require the same proteins, Cdc34, Skp1, and Cdc53. However, the F-box protein required for Cln2 degradation is Grr1, not Cdc4 [19,23,24]. From these observations came the F-box hypothesis: multiple SCF-dependent proteolytic pathways exist where Skp1 binds to different F-box proteins, each targeting particular substrates for degradation (Fig. 2) (reviewed in [5,6,25]).

Finally, in 1997 two independent groups reconstituted Sic1 ubiquitination in vitro using purified components of the SCF^{Cdc4} ligase (Skp1, Cdc53, and Cdc4) in the presence of ubiquitin, E1, Cdc34, and Cln/Cdc28 (this latter complex is required to phosphorylate Sic1) [17,18]. This was the first characterization of the specific SCF ligase subunits that control the ubiquitination of a distinct substrate.

4. SCF subunits

4.1. F-box proteins

Analysis of several SCFs has revealed common structural and functional themes. The F-box protein confers substrate specificity by recruiting a particular target to the core ubiquitination machinery. The hallmark of an F-box protein is a conserved domain of approximately 40 amino acids that mediates binding to Skp1 and defines the F-box. A family of 49 mammalian F-box proteins was identified using Skp1 as the bait in a yeast two-hybrid screen and by searching DNA databases with the conserved motif [26,27]. They were divided into three classes based on additional protein–protein interaction domains C-terminal to the F-box that are thought to be involved in the binding of the substrate. Fbws contain WD-40 domains, Fbls contain leucine-rich repeats, and Fbxs contain no or different motifs (Fig. 3). The gene nomenclature follows a four-letter system of reference: *FBXW*, *FBXL*, and *FBXO*. This recent identification of a large family of mammalian F-box proteins promises a diverse pool of SCF-regulated substrates involved in a range of cellular processes.

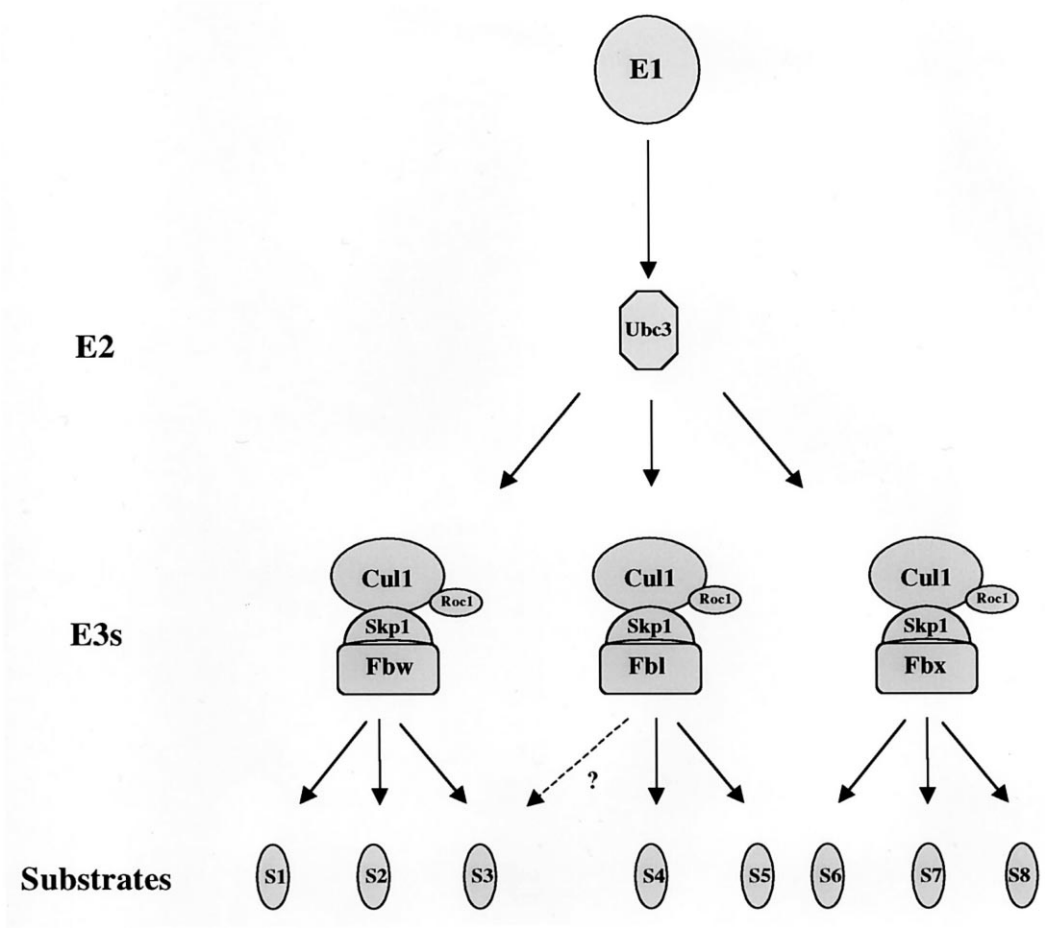

Fig. 3. Schematic representation of different mammalian SCF complexes. Mammalian F-box proteins are divided into three classes based on conserved motifs C-terminal to the F-box: Fbws (containing WD-40 repeats), Fbls (containing leucine-rich repeats), and Fbxs (containing different motifs or no motif). The question mark indicates the possibility that a specific substrate can be targeted by more than one SCF. See text for details.

Several genetic models of loss of function have been generated that implicate individual F-box proteins in the regulation of embryonic development and cell proliferation. *Fbxw4*, which encodes the protein Dactylin, is disrupted in the mouse dactyplasia mutant. Normally, Dactylin is thought to target for destruction a suppressor of limb formation to allow for the appropriate level of cell proliferation. However, in Dactylin-deficient mice this suppressor is not degraded and cell proliferation is greatly diminished, resulting in the absence of hands and feet or the development of malformed single digits [28]. This Dac phenotype resembles the human autosomal dominant split hand/foot malformation diseases. This is the first in vivo model demonstrating the importance of a mammalian F-box protein in vertebrate embryogenesis. Knockouts of Slimb [29,30], the *Drosophila* homolog of β-Trcp (Fbw1a), and of murine Skp2 (Fbl1) [31] have contributed significantly to current insight into the physiological function of distinct SCF complexes (see Sections 5.1 and 5.2).

4.2. Skp1

Skp1 is a critical scaffold protein that binds one Fbp, on one hand, and one cullin (i.e. Cdc53), on the other [18,32,33]. Some evidence suggests that Fbps and cullins can bind one another in a Skp1-independent manner, however the presence of Skp1 stabilizes the entire complex. While there are several Skp1 family members, the mode of binding of these homologs with the different F-box proteins and their biological significance are currently unknown (reviewed in [34]). The crystal structure of Skp1 bound to the human F-box protein Skp2 was recently solved [35] and provides insight into the mechanism of this interaction. Skp1 interacts with Skp2 via a dual interface. The core interface is a superhelical arrangement of the C-terminal region of Skp1 with residues 109–151 comprising the F-box of Skp2. Each binding partner contains amino acids that map to this core region and are highly conserved in their respective family. Mutation of these residues results in the disruption of binding. This suggests that the structure of the core interface is maintained in most, if not all, combinations of Skp1 and F-box protein family members. The variable interface is a large hydrophobic groove formed by two helices of the F-box, helix 4 of Skp2, and the C-terminal tail of Skp2. Only two residues in this second interface are conserved in their respective family member. However, reasonable evidence suggests that an analogous hydrophobic interface will be maintained among interactions between other F-box proteins and Skp1 homologs. The Skp1/Skp2 structure is rigid so as to properly position the substrate and the interacting E2 and ensure transfer of ubiquitin from the E2 to the substrate.

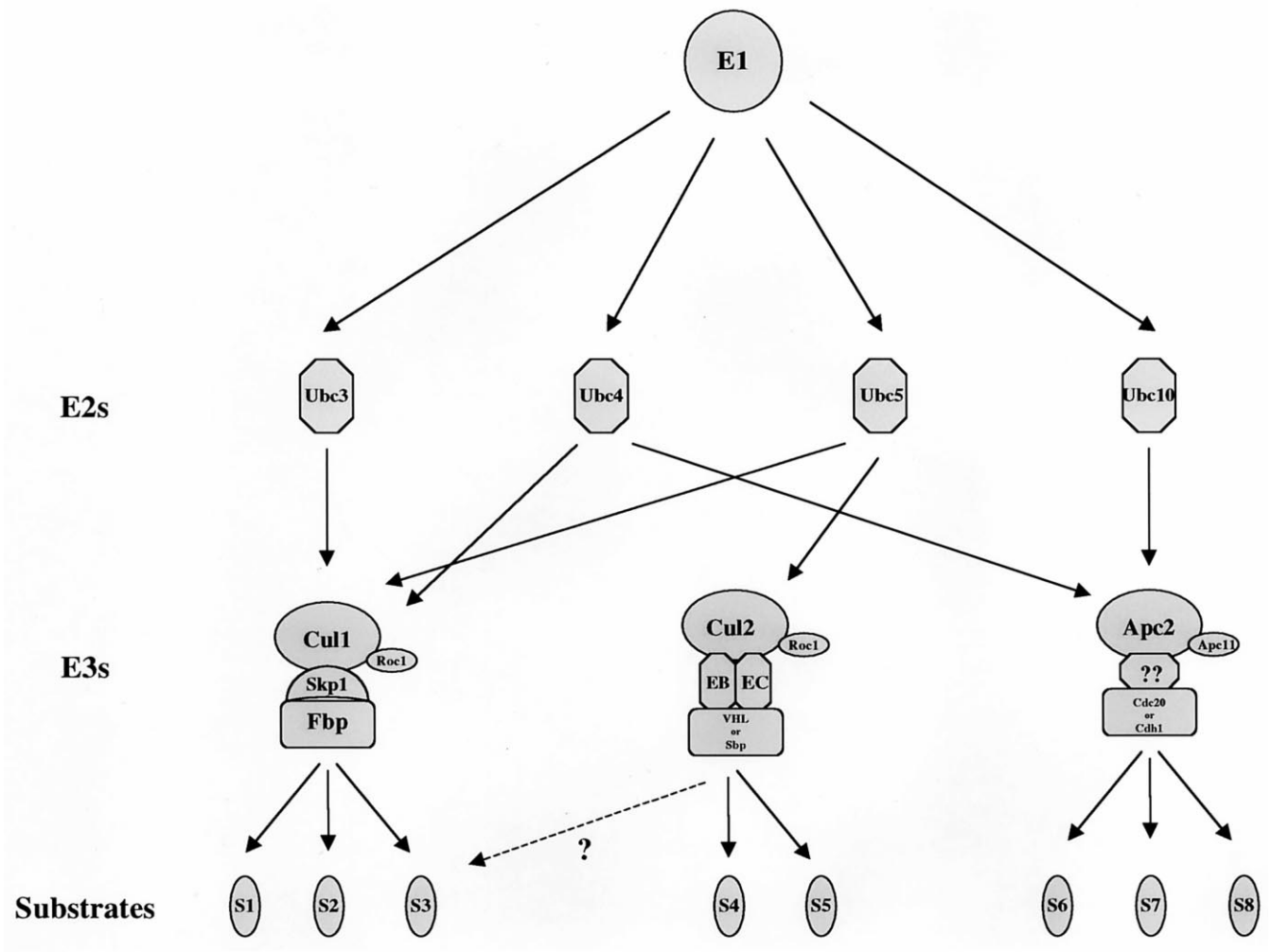

Fig. 4. Schematic representation of different mammalian cullin-based E3 ligases. Ubiquitin ligases can be subdivided based on the cullin subunit. Cul1 forms an E3 ligase together with Roc1, Skp1 and one of many F-box proteins. Cul2 forms an E3 ligase together with Roc1, elongin B, elongin C, and VHL or one of many Socs-box-containing subunits. APC2 (a cullin family member) and APC11 (homolog to Roc1) as well as other APC subunits (not indicated here) form an E3 ligase with Cdc20 or Cdh1. The double question mark is because the subunit directly bound by Cdc20 and Cdh1 is not known. The different complexes can assemble with different E2s as indicated. The question mark indicates the possibility that a specific substrate can be targeted by more than one cullin-based E3 ligase. See text for details.

4.3. Cullins

Cul1 was originally identified in nematodes in a screen for genes inhibiting hyperplasia [36] and is homologous to Cdc53 (also called CulA) in yeast. A family of four additional *Caenorhabditis elegans* and six human genes were identified in databases by searching with the Cul1 sequence. Cul1 is the best characterized member of the family and is the only one that interacts with Skp1 [37]. The name cullin comes from the verb 'to cul' in the sense of 'to sort'. In fact, at the time that Cul1 was characterized in nematodes, the complexity of the SCF system was only beginning to reveal itself. Because cullins were similar to Cdc53, and Cdc53 was a subunit of the SCF, Kipreos and Hedgecock [36] predicted that each cullin was part of a distinct ligase that culled or sorted numerous substrates for destruction.

Cul1 has three domains that mediate its association with other components of the SCF (reviewed in [34]). The least conserved domain among cullin members is the N-terminal region which in Cul1 mediates its binding to Skp1. The second and most highly conserved domain is present in the extreme C-terminus of all cullins and mediates the attachment of a small ubiquitin-like protein, Nedd8 (Rub1 in yeast). Covalent modification of cullins by the Nedd8 pathway appears to enhance the ubiquitin-ligating activity of some SCF ligases (see Section 4.5) (reviewed in [38]). A third domain downstream to this Skp1-binding region is called the cullin homology (CH) region or Cdc34/RING-H2 subunit-recruiting domain. This domain is conserved among all cullins and binds to Roc1/Rbx1 (Hrt1 in yeast) (see Section 4.4) ([39] and reviewed in [4,5]). Other cullins also bind to Roc1 and form different ligases with different specificity (Fig. 4). Cul-2 associates with a complex of elongin C (a protein similar to Skp1), and elongin B (an ubiquitin-related protein), and either the VHL (Von Hippel Lindau) tumor suppressor protein or a Socs (suppressor of cytokine signaling)-box-containing protein. Together, this complex is referred to as the VBC-like ligase, in which VHL and probably Socs proteins carry out the role of the substrate-targeting subunit performed by Fbps in SCF complexes (reviewed in [4,5]). It is likely that other cullins form similar complexes and that the SCF pathway is a

prototype system for a larger class of multi-component cullin-based ubiquitin ligases. Interestingly, APC/C contains a cullin subunit (Apc2) and RING finger protein (Apc11) that targets a specific set of substrates.

Targeted deletion of the *Cul1* gene in *C. elegans* and in mice has provided useful genetic tools for the analysis of the function of this gene. In *C. elegans*, the loss of Cul1 results in increased proliferation and hyperplasia in all larval tissues examined [36]. This suggests that Cul1 is an essential negative regulator of cell cycle. Additionally, Cul1 is required for developmentally regulated cell cycle exit (G1 to G0 transition), however the loss of function of Cul1 in quiescent cells does not have an effect on cell cycle re-entry (G0 to G1 transition). Mice carrying a deletion in *Cul1* die around embryonic day 6.5 and cyclin E protein levels, but not mRNA are increased in these cells (as determined by immunohistochemistry and in situ hybridization, respectively) [40,41]. The mechanism of developmental arrest in $Cul1^{-/-}$ embryos is unknown. The accumulation of cyclin E is not likely to be the cause, since cells have a tolerance for high levels of this cyclin [42,43]. It is likely that the emerging role of Cul1 in SCFs is as a core component that supports the targeting of multiple substrates, which probably accumulate in these $Cul1^{-/-}$ cells. Very recently, it has been reported that *Cul1* is a direct transcriptional target of Myc and that enforced expression of Cul1 or antisense p27 oligonucleotides is capable of overcoming the slow growth phenotype of Myc-deficient mouse embryonic fibroblasts [44]. This indicates that p27 is a major obstacle to cell cycle progression in the absence of Cul1.

4.4. Roc1/Rbx1/Hrt1

Roc1 directly associates with Cul1 on one hand and with Ubc3, Ubc4, or Ubc5 on the other [45,46]. However, Roc1 and the related Roc2 can bind all cullins. They both contain a conserved C-terminal RING finger (RING-H2), a cysteine-rich fold surrounding structurally critical zinc ions. Mutation of conserved cysteine and histidine residues in this domain abrogates the ubiquitin ligase activity of Roc1-containing complexes in vitro and disrupts its binding to Ubcs, but not to Cul1 [45,46]. It has been suggested that one function of the RING finger is to stabilize the interaction between Cul1 and Ubcs to facilitate ubiquitin transfer from the E2 to the substrate [47] (reviewed in [34]). This RING finger architecture is a common structural feature of non-Hect ubiquitin ligases. Most E3s contain a RING finger that appears fundamental to their catalytic activities. In fact, mutation in the RING finger domain of the ubiquitin ligases Cbl and Mdm2 abrogates their ability to promote degradation (reviewed in [4]). Interestingly, it has recently been demonstrated that the RING domain of c-Cbl and the HECT domain of E6AP (the ligase for the tumor suppressor p53) both recognize the same structural elements of UbcH7 [48].

4.5. Nedd8/Rub1

Nedd8 (neural precursor cell-expressed developmentally down-regulated) was first reported as a novel mRNA abundant in fetal mouse brain [49]. Nedd8 is an 81 amino acid protein that is 60% identical and 80% homologous to ubiquitin and it is one member of an expanding class of ubiquitin-like molecules. Both Nedd8 monomers and Nedd8 conjugated proteins could be detected by Western blot with an antibody specific to Nedd8 [50]. Mutational analysis revealed that, like ubiquitin, Nedd8 is also processed at its C-terminus to expose a glycine residue essential for conjugation. The mechanism of activation and conjugation of Nedd8 to its substrate parallels the ubiquitin cascade. The Nedd8-activating enzyme is a heterodimer consisting of Uba3 that is homologous to the C-terminal portion of E1 and APP-BP1 which is 56% similar to the N-terminal portion of E1. It contains the critical cysteine residues for thioester linkage to Nedd8 [51,52]. Despite its name, human Ubc12 does not conjugate ubiquitin but is the Nedd8-conjugating enzyme [51–53]. All known targets of Nedd8 conjugation in eukaryotic cells are cullin family members. In fact, all cullins are covalently modified by the Nedd8 pathway [54] and this appears to increase the activity of some cullin-based ligases (reviewed in [38,55]).

Loss of function of Rub1, the Nedd8 homolog in *S. cerevisiae*, produces viable yeast that exhibit slow growth. Progression through the cell cycle is impaired but, clearly, Rub1 is not essential for growth in budding yeast [56]. However, when the activity of the SCF is compromised by mutation of Cdc34, Cdc4, Cdc53, or Skp1 in combination with deletion of *Rub1*, the yeast display a lethal phenotype. This suggests the cells are unable to modify Cdc53, as a rubylation-defective Cdc53 displays a similar deficient phenotype when expressed with mutant Cdc34.

In contrast to the slight phenotype in budding yeast, it has recently been reported that the Nedd8 pathway is essential for cell viability in fission yeast [57]. Forced expression of a neddylation-deficient mutant form of Pcu1 (the Cul1 homolog) and depletion of Nedd8 both result in defective cell growth and the stabilization of Rum1, a cki in this yeast. Disruption of the genes encoding the *S. pombe* E1 and E2 enzymes for Nedd8 conjugation also has a lethal phenotype. These data support the hypothesis that neddylation is a shared and critical modification for cullin-based ubiquitin ligases.

5. Mammalian SCF complexes

Thus far, two human SCF complexes have been characterized in great detail, $SCF^{\beta-Trcp}$ and SCF^{Skp2} (Fig. 5).

5.1. $SCF^{\beta-Trcp}$

$SCF^{\beta-Trcp}$ is the first mammalian ubiquitin ligase with an assigned biological function. β-Trcp (β-transducin repeat-containing protein/Fbw1a) was identified as a binding protein of the HIV-1 viral protein, Vpu, and through this association it participates in a novel endoplasmic reticulum-associated degradation pathway that regulates CD4 proteolysis [58]. β-Trcp was also identified along with at least six other novel human F-box proteins in a yeast two-hybrid screen for Skp1 interactors [26]. Human β-Trcp is homologous to *Xenopus* β-TrCp [59], *Drosophila* Slimb [29], and *C. elegans* Lin-23 [60]. $SCF^{\beta-Trcp}$ targets β-catenin [61–64], a proto-oncogene, and IκBα [63,65–69] and IκBβ [70], both components of the NF-κB (nuclear factor-κB) signaling pathway, for phosphorylation-dependent ubiquitination and destruction. $SCF^{\beta-Trcp}$ also mediates the processing of p105, another player in the NF-κB network [71,72]. These known substrates of the $SCF^{\beta-Trcp}$ ligase are players in distinct cellular pathways controlling proliferation or inflammation. A β-Trcp homolog exists, called β-Trcp2, that is 78% identical and 85% homolo-

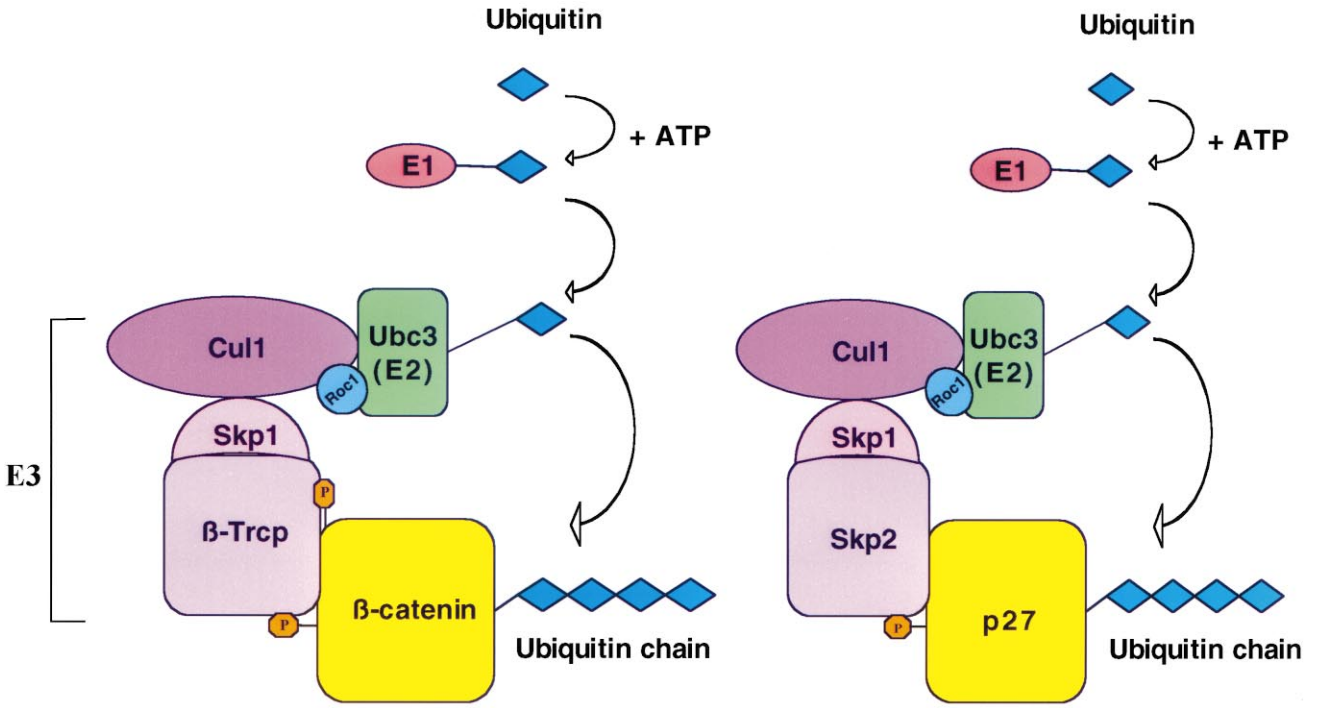

Fig. 5. Schematic representation of mammalian $SCF^{\beta-Trcp}$ and SCF^{Skp2}. $SCF^{\beta-Trcp}$ and SCF^{Skp2} bind their specific phosphorylated substrates, β-catenin and p27, respectively. $SCF^{\beta-Trcp}$ binds β-catenin through the F-box protein β-Trcp. SCF^{Skp2} binds p27 through the F-box protein Skp2. $SCF^{\beta-Trcp}$ also targets IκBα and IκBβ for ubiquitin-dependent degradation. SCF^{Skp2} also targets free, unbound cyclin E. See text for details.

gous to β-Trcp. It appears to target some substrates (IκB and β-catenin) for destruction, at least in overexpression experiments [73,74].

5.1.1. $SCF^{\beta-Trcp}$ targets the NF-κB pathway. The NF-κB network is a complex regulatory system that plays a central role in the regulation of genes that function in inflammation, cell proliferation and apoptosis (reviewed in [75]). NF-κB is a heterodimeric protein composed of a p65/RelA subunit and a p50 subunit. It is sequestered in the cytoplasm in an inactive form by a member of a family of inhibitory proteins, termed IκBs, which mask its nuclear localization signal. In response to various stimuli (i.e. viral infection or inflammatory cytokines) a signal transduction cascade is triggered, and IκBα and IκBβ [70] are phosphorylated by IKK (IκB kinase). Specifically, IκBα is phosphorylated on Ser-32 and Ser-36 and IκBβ is phosphorylated on Ser-19 and Ser-23 [76–81]. These serine residues are located in a N-terminal consensus phosphorylation region, DSGXXS, that is found in both proteins and is conserved in other β-Trcp substrates. The interaction between IκBα and β-Trcp is also dependent on an aspartic acid residue at position 31 in the DSGXXS motif [82]. The phosphorylated forms of IκBα and IκBβ are recognized by β-Trcp, allowing their ubiquitination and degradation. The degradation of NF-κB inhibitory subunits liberates cytoplasmic NF-κB to enter the nucleus and activate the expression of target genes. Additionally, the $SCF^{\beta-Trcp}$-mediated processing of p105 to the active subunit p50 requires the phosphorylation of the C-terminus of p105 (residues 918–934). This region shares similar features with, but is not identical to, the DSGXXS motif [72].

A highly conserved signaling pathway exists in *Drosophila* that is analogous to that of NF-κB. Dorsoventral patterning in early embryos is regulated by Dorsal, the NF-κB homolog. In response to activation the IκB-like protein, Cactus, is degraded and Dorsal translocates to the nucleus where it activates downstream genes. Slimb-deficient embryos express markedly reduced levels of these response genes [68]. Therefore, it is likely that Slimb/β-Trcp is required for Cactus/IκB degradation in vivo in flies.

5.1.2. $SCF^{\beta-Trcp}$ targets β-catenin. $SCF^{\beta-Trcp}$ regulates the stability of β-catenin, a downstream signaling factor in the Wnt pathway of development and proliferation (reviewed in [83]). In the absence of Wnt signaling, glycogen synthase kinase 3β (GSK) induces the degradation of cytoplasmic β-catenin via the ubiquitin-proteasome pathway. The adenomatous polyposis coli (APC) tumor suppressor promotes the binding of β-catenin to axin/conductin. Axin/conductin dramatically accelerates β-catenin turnover by enhancing the phosphorylation activity of GSK [84]. GSK phosphorylates Ser-33 and Ser-37 in the N-terminus of β-catenin. A complex of APC, axin/conductin, and GSK is essential for β-catenin phosphorylation. Phosphorylated β-catenin is recognized by the F-box protein β-Trcp and through the F-box of β-Trcp, the other subunits of the SCF complex, as well as the E2, are recruited to β-catenin for its ubiquitination. In response to the activation of Wnt signaling, degradation of free β-catenin decreases because of inactivation of GSK. Unphosphorylated β-catenin accumulates in the cytoplasm and is then translocated into the nucleus where it generates an active transcriptional complex with a member of the Lef-1/Tcf-1 family of transcription factors. Downstream targets of these transcription factors include proto-oncogenes such as c-*myc* and *cyclin D1* [85–87].

Aberrations in this pathway can lead to deregulated growth and oncogenesis (see below).

Genetic experiments in *Drosophila* provide some of the first in vivo evidence suggesting the physiological function of β-Trcp/Slimb. *Slimb* (supernumerary limbs) was initially identified in a screen for recessive mutations that alter normal adult patterning in flies [29,88]. The characterization of several mutants implicated Slimb as a negative regulator of the Hedgehog (Hh) and Wingless (Wg) signaling pathway, the *Drosophila* version of the Wnt pathway. Loss of function of Slimb results in the accumulation of Ci (Cubitus interruptus), a transcription factor that activates the expression of Hh-responsive genes, and Armadillo (Arm), the fly homolog of β-catenin [29]. Given the fact that Slimb is an F-box protein, it was suggested that Slimb was part of an SCF targeting Arm and Ci for ubiquitination and degradation.

It has recently been suggested that Slimb restricts centrosome duplication during the cell cycle [30]. A mutant *Drosophila* line named *centrosome replication-defective* (*crd*) displays overduplication of centrosomes and polyploidy. This is typical of defects in mitotic progression. The *crd* gene is inserted into the 5′-untranslated region of the previously identified *slimb* locus. It is not clear whether Slimb is localized on centrosomes, however, it is known that Cul1 and Skp1 are there [89,90]. Interestingly, the Skp2 knockout in mice also exhibits multiple centrosomes and polyploidy (see Section 5.2) [31].

Genetic mutations or altered protein expression of β-catenin or APC have been implicated in human cancers. They result in increased levels of β-catenin, which in turn lead to increased Lef-1/Tcf-1 transcriptional activity and deregulated proliferation [86]. An accumulation of β-catenin has been found in several malignancies and is often the result of β-catenin mutations that abolish the serine phosphorylation sites required for its degradation [91]. Additionally, mutation of Asp-32 of β-catenin (corresponding to Asp-31 in IκBα which is essential for its association with β-Trcp) has been detected in human cancers including synovial sarcomas (D32Y) [92], hepatocellular carcinomas (D32N, D32Y and D32V) [93], hepatoblastomas (D32T) [94], pilomatricomas (D32Y and D32G) [95], and prostate cancer (D32Y) [96]. Transgenic mouse models show that β-catenin stabilization leads to hair tumors when expressed in epidermal cells [97], and colon adenomas when expressed in the intestines [98]. Furthermore, mice carrying an inactivating mutation in the murine APC gene develop multiple intestinal adenomas and are prone to develop mammary tumors [99]. Thus, both the wild type and mutated forms of β-catenin are associated with malignant transformation when they accumulate because of a defect in degradation. Fluorescence in situ hybridization (FISH) was used to map *β-Trcp* to chromosome position 10q24 [100]. This locus is frequently altered in tumors, however no deletions, amplifications, or translocations of *β-Trcp* were identified in a screen of 42 human tumor cell lines and 16 breast tumors. This lack of gross genomic alteration does not exclude the possibility that β-Trcp is a target of genetic alteration in malignancy.

5.2. SCF^{skp2}

Skp2 (S-phase kinase-interacting protein), also called Fbl1, is a human F-box protein that was originally discovered as an interactor of the cyclin A/Cdk2 complex [22]. Biochemical and somatic cell genetic experiments have implicated Skp2 in the ubiquitin-dependent degradation of the cki p27 both in vitro [101,102] and in vivo [101,103]. In response to mitogenic stimuli, p27 is phosphorylated on threonine 187 by Cdk2 [104–106] and consequently is recognized by Skp2 [101,103]. Through the F-box of Skp2, the other subunits of the SCF (Skp1, Cul1, and Roc1) are recruited and p27 is ubiquitinated to be targeted for destruction. In contrast to wild type p27, the T187A phosphorylation mutant cannot be ubiquitinated. Moreover, immunodepletion of Skp2 from extracts abrogates ubiquitin-conjugating activity and addition of recombinant purified Skp2 rescues p27 ubiquitination activity. Many data underscore the importance of Skp2 in regulating cell proliferation through its regulation of p27 degradation. Expression of both Skp2 message [22] and protein [101,107] peaks in S phase and then decreases as cells progress through G2 phase. Skp2 overexpression in fibroblasts promotes progression from quiescence to DNA synthesis in the presence of low serum [102]. A decrease in p27 protein levels is also observed in these cells. However, quiescent cells expressing a mutant p27 (T187A) that cannot be phosphorylated and degraded suppress entry into S phase. Additionally, the expression of a Skp2 F-box mutant that cannot form a stable ligase complex is defective in inducing S phase and cannot eliminate ectopically produced wild type p27 [101,102]. Finally, experiments that block the function of Skp2 in vivo through microinjection of anti-Skp2 antibodies and antisense oligonucleotides targeting Skp2 mRNA result in an inhibition of S phase entry [22] and a stabilization of endogenous p27 [101]. These data strongly suggest that Skp2 plays a critical role in G1 to S phase progression through its regulation of p27 proteolysis. Recently, Skp2 has also been shown to be required for the ubiquitination of cyclin E in its free, non-Cdk2-bound form [31]. It has been suggested that Skp2 is the F-box receptor for other cellular regulators (e.g. cyclin D1, p21, Myb, and E2F-1), however limited data are currently available to support the hypotheses that these proteins are Skp2 substrates [31,107,108].

Skp2 is the first F-box protein whose physiological function has been analyzed in a mouse knockout model [31]. $Skp2^{-/-}$ mice are smaller than their littermates, and $Skp2^{-/-}$ cells exhibit hyper-accumulation of p27 and free cyclin E. Other cell cycle proteins including those suggested to be Skp2 substrates (E2F-1 and cyclin D1) are not elevated in Skp2-deficient cells [31]. Mice lacking Skp2 exhibit enlarged nuclei with polyploidy and multiple centrosomes. Thus, Skp2 may control chromosome replication and centrosome duplication.

Skp2 overexpression has been observed in transformed cell lines [22], although Skp2 overexpression, per se, is not sufficient to transform cells. Skp2 and activated Ras cooperate in in vitro transformation assays. Additionally, studies demonstrate that the injection of cells overexpressing Skp2 and activated Ras into nude mice induces tumors [109]. Furthermore, it has recently been demonstrated that Skp2 cooperates with Ras in an in vivo model of lymphogenesis [110]. The mechanisms of cooperation between Skp2 and Ras to induce transformation are quite complex. It is likely, however, that the accelerated degradation of multiple Skp2 targets contributes to the oncogenic process.

A reduction in p27 levels is necessary, but apparently not sufficient in the formation of carcinomas and lymphomas. Mice lacking p27 are larger than control animals and develop some spontaneous tumors, specifically in the pituitary [111–113]. Furthermore, p27-deficient mice are susceptible to tumor

formation in multiple tissues when challenged with carcinogens [114]. Numerous studies have shown that decreased levels of p27 are of independent prognostic significance in some cancers (reviewed in [115]). Additionally, the most aggressive of these cancers have enhanced p27 degradation activity. It is possible that the destabilization of p27 observed in many types of aggressive human cancers [116–119] is due to a corresponding increase in the levels of Skp2 [109,110,120,121]. Thus, the inverse correlation between low expression of p27 and high levels of Skp2 may be an important prognostic indicator.

6. Concluding remarks

Given the intricate temporal and spatial controls of the ubiquitin-proteasome pathway, it is expected that aberrations in this regulatory system are implicated in the pathogenesis of several human diseases [1,122,123]. Mutations that affect either the enzymatic machinery of the ubiquitin pathway or the ability of a specific substrate to be targeted for destruction can result in the dysregulation of cellular proliferation and other biological processes. Scientists are interested in designing inhibitors that block the ubiquitination. Because of the wide-ranging role of the ubiquitin pathway in many basic cellular processes, it may be difficult to limit the potentially negative effects of inhibitors that target the general ubiquitination machinery. For example, inhibitors of the proteasome are likely to affect several cellular regulatory systems non-specifically. In contrast, inhibitors of ubiquitin ligases will more specifically block distinct selected cellular processes. Ongoing efforts to understand the mechanisms of specificity that govern this pathway as well as the identification of new ubiquitin-proteasome substrates promise continued discoveries that basic researchers should look forward to translating to the clinic.

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