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Review

A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin

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Abstract

The SCF (Skp1–Cullin–F-box) E3 ubiquitin ligase family was discovered through genetic requirements for cell cycle progression in budding yeast. In these multisubunit enzymes, an invariant core complex, composed of the Skp1 linker protein, the Cdc53/Cul1 scaffold protein and the Rbx1/Roc1/Hrt1 RING domain protein, engages one of a suite of substrate adaptors called F-box proteins that in turn recruit substrates for ubiquitination by an associated E2 enzyme. The cullin–RING domain–adaptor architecture has diversified through evolution, such that in total many hundreds of distinct SCF and SCF-like complexes enable degradation of myriad substrates. Substrate recognition by adaptors often depends on posttranslational modification of the substrate, which thus places substrate stability under dynamic regulation by intracellular signaling events. SCF complexes control cell proliferation through degradation of critical regulators such as cyclins, CDK inhibitors and transcription factors. A plethora of other processes in development and disease are controlled by other SCF-like complexes, including those based on Cul2–SOCS-box adaptor protein and Cul3–BTB domain adaptor protein combinations. Recent structural insights into SCF-like complexes have begun to illuminate aspects of substrate recognition and catalytic reaction mechanisms.

Keywords: Cullin; F-box; Skp1; SOCS-box; BTB domain; APC/C; Ubiquitin; Proteasome; Cell cycle

1. Introduction

The ubiquitin–proteasome system is a crucial determinant of virtually all biological processes in eukaryotes [1]. This modification and degradation system controls the stability of numerous regulators including cell cycle proteins (e.g., cyclins, CDK inhibitors and replication factors), transcription factors (e.g., IκB and β-catenin), tumor suppressor proteins (e.g., Rb and p53), oncoproteins (e.g., Myc and Jun), membrane proteins (e.g., growth factor receptors) and many more. Degradation of a protein by the ubiquitin–proteasome pathway entails two successive events: (i) the covalent attachment of a chain of ubiquitin moieties to the substrate protein and (ii) the ATP-dependent proteolysis of the substrate by the 26S proteasome [2]. Ubiquitin transfer requires the activity of E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligase) enzymes. Ubiquitin is first bound in a high-energy thioester bond to a cysteine residue of the E1 enzyme in an ATP-coupled reaction. Then ubiquitin is transferred from the E1 enzyme to an active site cysteine residue of the E2 enzyme, again as a thiolester linkage. In the final critical step mediated by an E3 enzyme, ubiquitin is linked to the substrate via an isopeptide bond between the C-terminal glycine of ubiquitin and a selected lysine residue of the substrate. Repeated transfer of additional ubiquitin molecules to successive lysines on each previously conjugated ubiquitin, typically on Lys48, generates a polyubiquitin chain. The polyubiquitin tag, minimally a chain of four ubiquitin monomers, is recognized by the 26S proteasome, which unfolds substrates in an ATP-dependent manner and channels the unfolded polypeptide chain into its catalytic lumen, where a host of protease

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sites rapidly degrade the protein into short peptides [3]. The specificity of ubiquitin-dependent proteolysis derives from the many hundreds of E3 ubiquitin ligases that recognise particular substrates through dedicated interaction domains. Targeting motifs on substrates are typically short primary sequence elements often referred to as degrons [4].

The burgeoning cohort of E3 enzymes can be subdivided into two general classes, based on one of two characteristic protein motifs, the HECT domain and the RING domain [2]. Ubiquitin ligases of the HECT (*h*omologous to *E*6-AP *C*-*t*erminus) domain family, such as the archetypal E6-AP enzyme or the yeast Rsp5 enzyme, themselves function as ubiquitin carriers. That is, the HECT catalytic domain forms a thioester intermediate with ubiquitin prior to transfer to the bound substrate [5]. In contrast, the RING domain class of E3 does not have inherent catalytic activity, but rather uses the Zn-binding RING structural motif to recruit and direct an E2 enzyme towards specific substrates, which are often recognized by associated substrate recruitment subunits [6,7]. Another Zn-binding domain called the PHD finger, recently identified in several E3 enzymes [8], is a likely structural variant of the RING finger [9]. A domain called the U-box that appears to act as an E2 recruitment factor is also is structurally related to RING domains [10,11]. SCF-like complexes are the archetype for the RING ubiquitin ligase family.

In this review we describe the key subunits of SCF complexes and how homologous complex architectures and different substrate specificity factors have diversified the functions of this remarkable enzyme family. Concepts and variations in SCF-dependent regulation are illustrated with selected examples of substrate recognition by SCF complexes, with a focus on cell cycle and signal transduction pathways. Other reviews are recommended for further details [12–14].

2. A brief history of SCF

The initial insight into SCF-dependent proteolysis came from analysis of the budding yeast *Saccharomyces cerevisiae* cell division cycle (*cdc*) mutants *cdc4*, *cdc34*, and *cdc53* [12]. These mutants arrest with unreplicated DNA and multiple elongated buds at the non-permissive temperature because they fail to degrade the B-type (Clb)-Cdc28 cyclin-dependent kinase (CDK) inhibitor Sic1, which prevents entry into S phase [15]. Sic1 is degraded upon its phosphorylation by G1 cyclin (Cln1/2)–Cdc28 complexes in late G1 phase [15,16]. In addition, the G1 cyclins themselves are degraded in a Cdc34 and phosphorylation-dependent manner [17–20]. Further genetic and biochemical studies in yeast revealed that Cdc53, Cdc4 and two other proteins, Skp1 and Rbx1 (a.k.a. Hrt1 or Roc1), form an E3 ubiquitin ligase complex that acts in concert with the E2 enzyme Cdc34 to regulate the G1/S phase transition [12,13,21,22]. Skp1 was first identified in a complex with human cyclin A–Cdk2 in conjunction with another associated protein, Skp2 [23] and was then independently isolated through its genetic and physical interactions with yeast Cdc4, yeast Ctf13 and human cyclin F [24,25]. Sequence alignment of cyclin F, Skp2 and Cdc4 unveiled a shared motif of approximately 40 residues, termed the F-box (for cyclin F), which is a conserved binding site for Skp1 [24]. Significantly, the degradation of Sic1 and Cln2 relies on both common (i.e., Cdc34, Cdc53 and Skp1) and divergent (Cdc4 versus Grr1) components [15,24,26]. This observation was generalized as the "F-box

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Ybr158w=Cst13/Amn1 166  V-FEIP-EIVENIIKMIVSLE(56)LFSCMMVNRLWLN-VTRP----FLFKSL
Ybr203c            187  I-NDLPVEIIAKILSEFELGRDQKTLVRCLYVSKKFYK--ATK----IVLYRL
Ybr280c             17  D-AGLSPDIVQATLPFLSSDD----IKNLSQTNKYYNT-LLDFDHSKILWHEL
Ydr131c              2  F-DKLPYEIFKQIAWRIPQED----KISLTYVCKRSYE-SIIP----FIYQNL
Ydr219c             17  L-TNLPLNLLFRILSHLDMND----LQNIGKTCTLLRM-LANE---NIVYRNA
Ydr306c            112  K-MVLPWEIQHRIIHYLDIPE(18)NMNYLLVCRNWYAMCLPK---LYYAPAL
Yfl009w=Cdc4       275  I-TSLPFEISLKIFNYLQFED----IINSLGVSQNWNK-IIRKS--TSLWKKL
Yil046w=Met30      184  I-SILPQELSLKILSYLDCQS----LCNATRVCRKWQK-LADD---DRVWYHM
Yjl149w             49  L-TKLPDELMQEVFSHLPQPD----RLQLCLVNKRLNK-IATK----LLYRRI
Yjl204c=Rcy1         4  L-LKVP-EIVTNIASYLSTVD----YLSFQQVNKRVYA-IING---KNDSKYW
Yjr090c=Grr1       317  L-NMLPSEILHLILDKLNQKYD---IVKFLTVSKLWAE-IIVK---ILYYRPH
Ylr097c            100  L-EILPDDILLRIIKKVILMSGES-WVNLSMTCSTFSKLCFHD---SVPFKTF
Ylr224w              8  L-MDLPLEIHLSLLEYVPN-E-------LRAVNKYFY--VLHN----HSYKEK
Ylr352w             14  LGAAIPPEIVYQILTYQFRDL(19)VKSNLTVNKTFSH-ICQV----LIYRYC
Ylr368w             16  I-DHLPPEIWLCISKLVGTSD----LHNLCLINRRLYL-TITS---DEIWKRR
Yml088w=Ufo1         8  L-QDLPPEILINIFSHLDEKD----LFTLQELSTHFRN-LIHD---EELWKNL
Ymr094w=Ctf13        9  F-LELPIDIRKEVYFHLDGNF(71)VLDCFKVNHLYDGTLIDA----LEWTYL
Ymr258c              4  Q-DQDIFIVFSHASLFLNQND----LLSLSLTSKKMHD-MIAI---PRLYSNI
Ynl230c=Ela1        22  V-SNVPYHLLKRILQKVKIPQ----LLKLEKSN--VLL-IFDD---DELWLEF
Ynl311c             57  L-MCLPTKVLLLILRTLDFNT----LVTLCQVNSRFYN-LITN---EFLFQNV
Yor080w=Fcl1/Dia2  221  V-GNLPIEILPIIFQRFTTKE----LVTLSLVCNKWRDKILYH---LDCFQEF

Consensus               L sxLPxEILxkILsyLDxxD    LLxLxxVCKRWyx LIdd   dxLWKxL
                        I t v  dLi rVf hve  e    iV V   Srkfr  Iaee   e VFq a
                        a   f   Vv nli kip  s    vi I   N  ln  Vlss   s iYr v
                        v   m     q   na   t       a       k   vt    t fln f
                        f             rf                              y   m
                        w
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Fig. 1. Alignment of 21 identifiable F-box sequences from the budding yeast *S. cerevisiae*. Starting residues of the F-box in each protein are indicated. Consensus sequence indicates the most favored residues in capital letters and moderately well tolerated residues in lower case. Conserved hydrophobic residues are shaded. See also Ref. [39].

hypothesis", which postulated that Skp1 links F-box-containing proteins to a core ubiquitination complex and that in turn F-box proteins recruit substrates for ubiquitination via their specific protein–protein interaction domains, such as the WD-40 repeats in Cdc4 or the leucine-rich repeats (LRR) in Grr1 [24]. The first direct link between Cdc53 and protein degradation was the finding that Cdc53 interacts with a fraction of the G1 cyclin Cln2, whose instability and ubiquitination in vivo depend on Cdc53 [19]. Physical and genetic interactions between Cdc4, Cdc34 and Cdc53 suggested that these components might form an E2–E3 complex [19,27]. At the same time, genetic evidence in the nematode worm *C. elegans* implicated the Cdc53 ortholog CUL-1 as negative regulator of cell division, suggesting that the Cdc53/cullin family played a conserved role in protein degradation and cell cycle control [28]. Subsequent studies demonstrated direct interactions between Skp1, Cdc53, Cdc34 and various F-box proteins and reconstitution of specific ubiquitin ligase activity against phospho-Sic1 [29–31]. Specific complexes were thus designated SCF, for *S*kp1–*C*dc53/cullin–*F*-box protein, with the F-box protein indicated by a superscript, as in SCF^{Cdc4}, SCF^{Grr1} and so on. F-box motifs are found in many otherwise unrelated proteins that bear a variety of different protein interaction domains [12]. In yeast, at least 21 proteins contain a discernable F-box motif (Fig. 1), many of which also contain recognizable protein interaction domains such as LRRs (Fig. 2). Many other protein interaction domains are represented in F-box proteins from other species (Fig. 2).

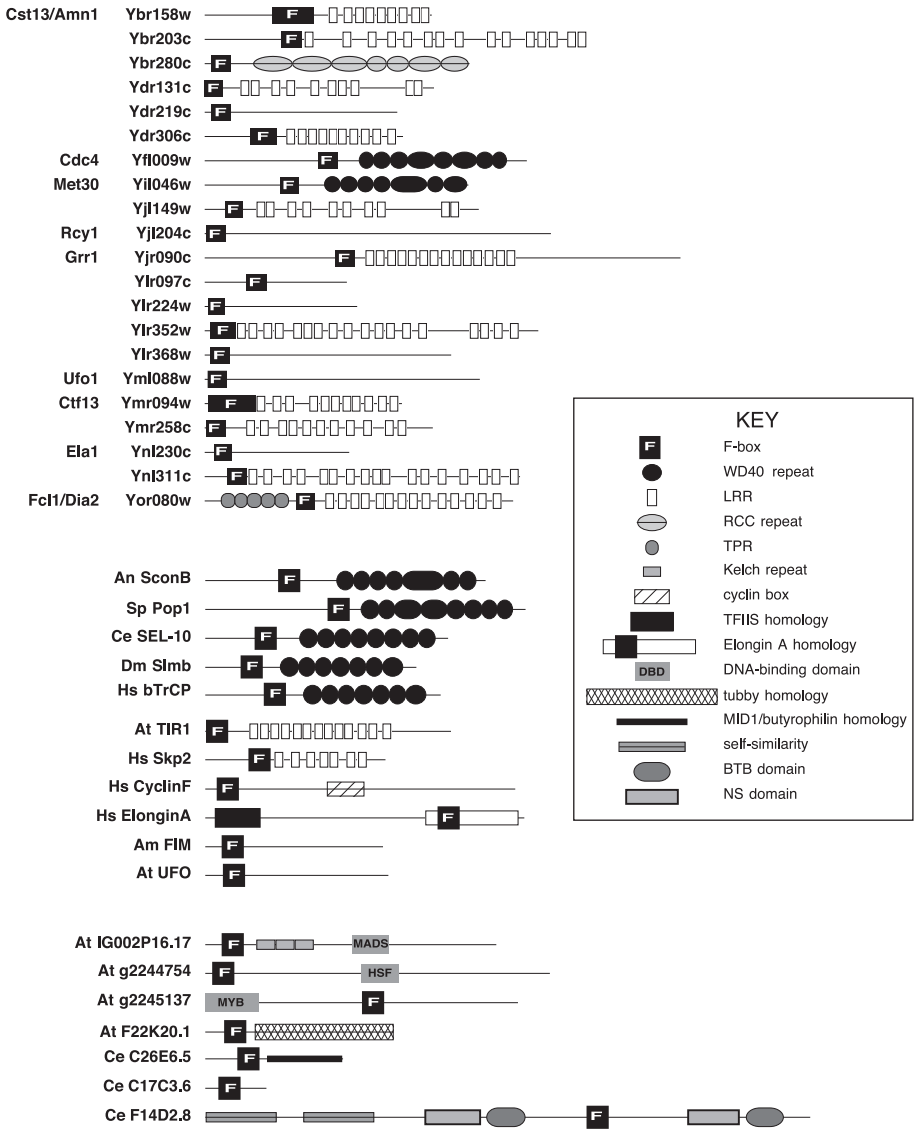

Fig. 2. Schematic representation of all 21 identified F-box proteins from the budding yeast *S. cerevisiae* and selected F-box proteins from other species. Legend to protein interaction domains is shown in the box. Six previously unrecognized LRR domain-containing F-box proteins in budding yeast were identified in this analysis: Ybr203c, Ydr131c, Yjl149w, Ymr094w (Ctf13), Ymr258c, Ynl311c. See also Refs. [39,446].

The widespread prevalence of F-box proteins suggested that this system might have sweeping regulatory powers [24], particularly as most SCF substrates are recognized in a phosphorylation-dependent manner, and hence directly linked to intracellular signaling pathways. These predictions have been more than amply fulfilled.

In a convergence of results from many laboratories, the RING domain protein Rbx1, also called Roc1 or Hrt1, was the last of the SCF core components to be identified [32–36]. Rbx1 was purified in association with the VHL tumour suppressor protein, which is a subunit of a related Cul2-based ubiquitin ligase, and subsequently found in SCF complexes [32,33]. In parallel, Rbx1 was isolated as a component of human SCF complexes, termed ROC1 [35,36], and as a Cdc53 associated protein, termed Hrt1 [34]. In yeast, conditional *rbx1* mutants arrest with a similar pre-replicative, multibudded phenotype as other SCF mutants, and are defective for degradation of Sic1, Cln2 and the polarity protein Gic2 in vivo [33,34,37]. Purified Rbx1 potently stimulates Cln1/2 ubiquitination by recombinant SCF^{Grr1}, as well as Sic1 ubiquitination by recombinant SCF^{Cdc4} [32–34]. The RING domain is defined by a series of eight conserved cysteine and histidine residues that bind zinc ions in a cross-brace arrangement and occurs in hundreds of different eukaryotic proteins (Fig. 3) [6]. Two subtypes of RING motifs can be distinguished, one carrying seven cysteines and one histidine at position 4 (RING-HC), and one carrying six cysteines and two histidines at positions 4 and 5 (RING-H2). In addition to Rbx1, ubiquitin ligase activity is associated with many other RING domain

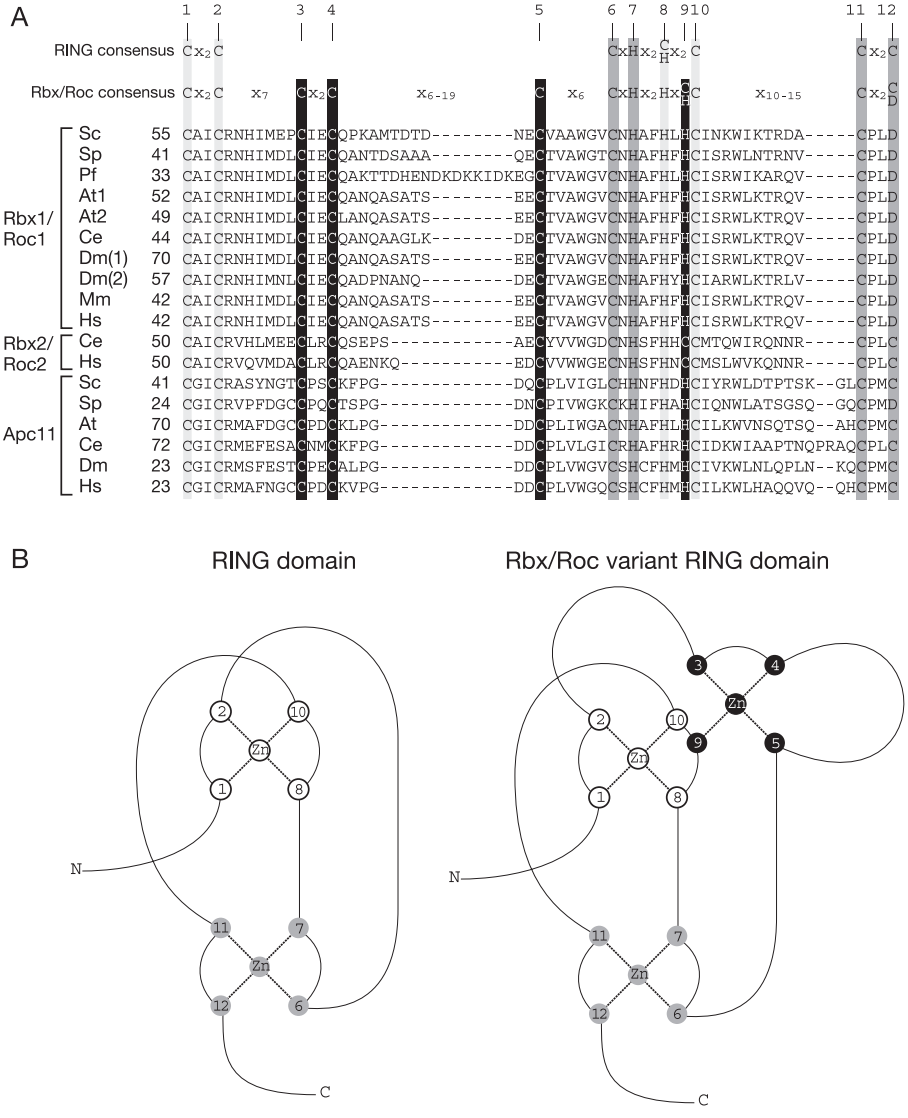

Fig. 3. The Rbx/Roc/Apc11 subfamily of RING domains. (A) Alignment of representative RING domain sequences. The generic RING domain consensus is shown at the top, while the Rbx/Roc RING domain consensus is shown below. Numbers indicate residues that chelate Zn ions, while shading corresponds to Zn atoms in schematic shown in part B. (B) Schematic representation of the RING domain cross brace structure (left) and the three Zn atoms coordinated by the Rbx/Roc RING domain subfamily (right). Solid lines represent the protein backbone, while dotted lines represent Zn chelation. Numbered circles correspond to residues in part A.

proteins including c-Cbl, Mdm2, Hrd1/Der3, Ubr1, Brca1 and Bard1, suggesting that it is a diagnostic feature of many E3 ubiquitin ligases [7,38]. However, not all RING domain proteins are E3 enzymes [6].

SCF complexes are built in a modular format that is conserved from yeast to humans. Skp1 binds both the F-box motif in the substrate recognition subunit and the N-terminus of Cdc53, thereby bridging the substrate to the core catalytic complex, whereas the C-terminus of Cdc53 binds to Rbx1, which serves as a docking site for the E2 enzyme, Cdc34 (Fig. 4). While adhering to this format, the repertoire of SCF-like components has also diversified considerably through evolution (Table 1) [12,24,39–43]. As most F-box proteins appear to recognize multiple substrates, the potential regulatory reach of SCF-like systems is patently vast. Whereas there is only a single Skp1 in budding and fission yeast, this gene family has also expanded substantially through evolution [44,45]. Multiple cullins are also present in all species, ranging from four in budding yeast to at least nine in mammals [28,46–48]. Given that Rbx family proteins bind to all known cullin family members, with the possible exception of Cul4B, it is likely that each will form bona fide ubiquitin ligases [35,36,48,49]. Despite the plethora of RING domain proteins, Rbx1 has only two obvious homologs (Fig. 3), which in some circumstances appears to contribute to substrate selectivity [35,50]. As detailed below, SCF core subunit variants serve to elaborate SCF-like function by combinatorial interactions with different classes of adapter proteins.

3. Neddylation, the CSN complex and Cand1

Analysis of SCF-associated factors has identified other protein components that modulate SCF activity. The conserved ubiquitin-related protein called Rub1 in yeast and plants and Nedd8 in other species is covalently linked to a conserved lysine residue in the conserved C-terminal region of all cullins, except Cul4B and Apc2 [51]. Rub1 facilitates SCF activity, as first shown by the synthetic lethality of *rub1* mutations with conditional alleles of SCF components in budding yeast [52,53]. Disruption of the Nedd8 pathway results in pleotropic defects, including loss of viability in fission yeast, arrested or abnormal development in nematodes, resistance to the hormone auxin in plants and division arrest and embryonic lethality in mammals [52–59]. Puzzlingly, however, other than synthetic phenotypes, the pathway is completely dispensable in budding yeast [52]. Neddylation is catalyzed by the bipartite activating enzyme called APPBP1-hUba3 in

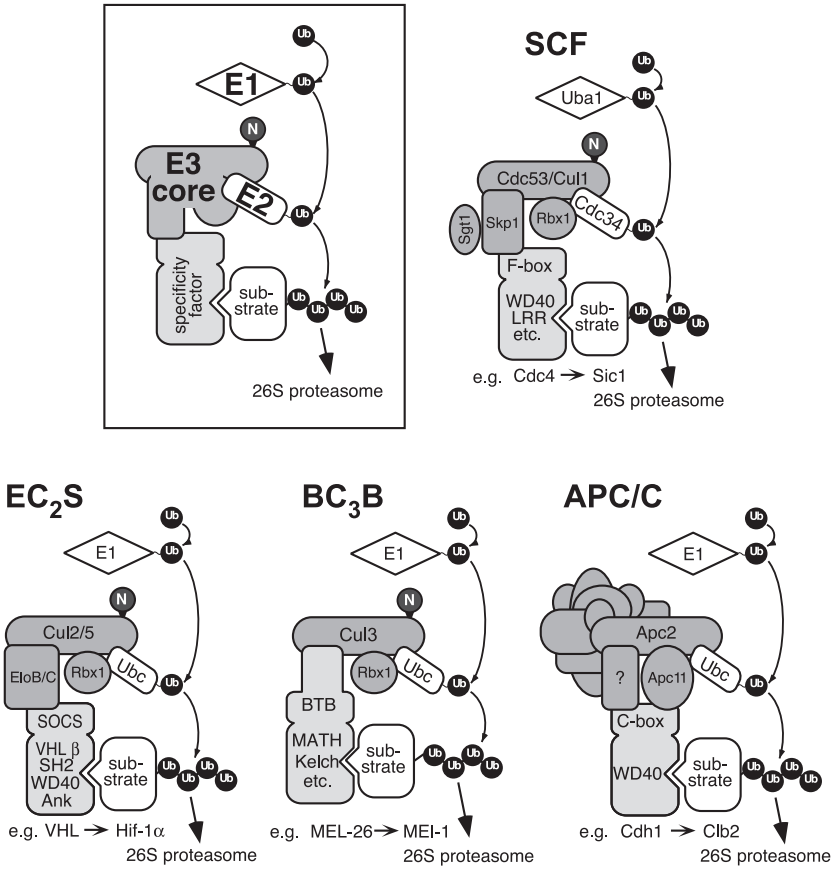

Fig. 4. The cullin–RING domain ubiquitin ligase superfamily. The generic cullin–RING-H2 architecture and that of each specific SCF-like complex, along with a representative adaptor subunit and substrate, are shown.

Table 1
SCF and SCF-like subunits in selected species

Organism	Skp1/BTB-like linkers			Cullin subfamily proteins						F-box-like adaptor proteins		RING
	Skp1-like	EloC-like	BTB proteins	CUL1	CUL2	CUL3	CUL4	CUL5	other[k]	references	F-box proteins[o]	SOCS/BC-box proteins[u]	Rbx/Apc11[@]
S. cerevisiae	1[a]	1	3[g]	1	0	1[j]	0[j]	0	2	a	21[p]	2[v,w]	2
S. pombe	1[a]	1	3[h]	1	0	1	1	0	1	l	16[q]	2[v]	2
C. elegans	21[a,b]	2[e]	105[h]	1	1	1	1	1	1	m	326[r] (203[s])	6[x]	3
D. melanogaster	7[c]	1	141[h]	1	1	1	1	1	2	a	31[s]	11[y]	2
A. thaliana	21[d]	1[d]	50[h]	1[i]	1	2	1	0	6	d	703[d]	4[z]	3
H. sapiens	1[a]	3[f]	208[h]	1	1	1	2	1	4	m,n	36[r] (109[s])	41[t]	3

BTB domain proteins are listed under Skp1/BTB-like linkers but also act as F-box-like adaptor proteins. Some tallies have been updated by BLAST searches at GenBank.
[a][44]; [b][45]; [c][115]; [d][449]; [e]protein IDs (PIDs): 13435329, 17570235; [f]PIDs: 5032161, 3688092, and 10334449 (with 27484210 as a fourth candidate); [g][117]; [h][118,119]; [i]a number of the "other" cullins appear to be partial CUL1 orthologs; [j]Cul3 may be a descendant of a CUL3/CUL4 precursor and thus may belong to both cullin subfamilies; see Ref. [44]; [k]partial, frame-shifted or highly divergent homologs; includes Apc2 homologs, [46,47]; [l][430]; [m][28]; [n]reviewed in Ref. [51]; [o]F-box protein tallies may include the BC-box protein ElonginA which also contains a divergent F-box (see text); [p][39]; this study; [q]T. Toda, personal communication; [r][334]; [s]SMART database (http://smart.embl-heidelberg.de/); [u]Tallies include canonical SOCS-box proteins, Rad7/Muf1-like proteins and/or ElonginA homologs; [v]Rad7 and ElonginA [435]; [w][450]; [x][108,434], also PIDs 17551502, 17555920, 7511669 and 17568839; [y][108], also PID 17864658; [z]PIDs 25370823, 4388727, 13374849 and 23297087; [108]; [@][32,34,35].

humans and Ula1-Uba3 in budding yeast and a dedicated E2 enzyme called Ubc12, but as yet a cognate E3 has not been found [53,60,61]. The reaction may in part be stimulated by weak recruitment of the charged Ubc12 to cullin complexes by Rbx1 [52,62–65]. The structure of human APPBP1-Uba3 has provided valuable insight into the mechanism of activation and transfer of ubiquitin-like proteins [66]. Rub1/Nedd8 modification is not essential for SCF assembly per se, but rather appears to facilitate E2 recruitment, the efficiency of substrate ubiquitination and as described below, the regulation of complex assembly [52,54,65,67–71].

Rather than a passive assembly or maturation step, Nedd8/Rub1 conjugation is dynamic. An entity called the COP9/signalosome or the CSN, first discovered in plants as a repressor of photomorphogenesis [72], catalyzes cleavage of Nedd8 from cullins [73]. The catalytic activity of the Csn5/Jab1 subunit, a metalloprotease with a JAMM motif, is required for this step [74]. The CSN is a multiprotein complex that resembles both the proteasome lid and the translation initiation factor eIF3, and is implicated in a number of disparate functions including photomorphogenesis, cell cycle progression, proteolysis, phosphorylation, signal transduction, transcriptional activation, and nucleocytoplasmic transport [72]. Many CSN functions are likely accounted for by effects on cullin activity. For instance, in *Arabidopsis* the CSN is required for SCF^{Tir1} to mediate responses to the hormone auxin and for SCF-dependent steps in flower development [75,76]. Although CSN mutants accumulate hyperneddylated cullins, this effect is accompanied by attenuated rather than enhanced SCF function, implying that SCF activity in vivo depends on active cycles of Nedd8/Rub1 modification [72,73].

Insight into one molecular role of Nedd8/Rub1 modification has emerged from analysis of a recently discovered SCF-associated protein, originally named Tip120A for its association with TATA binding protein (TBP) in transcription complexes, and renamed Cand1 [77–80]. Cand1 binds to the unmodified cullin–Rbx1 complex and occludes Skp1, thereby preventing SCF assembly. Conversely, Nedd8 modification blocks the Cand1 interaction and thereby stabilizes the active Skp1–Cul1–Rbx1 complex. Because Cand1 can dissociate Skp1 and Nedd8 can dissociate Cand1, the inactive and active complexes exist in equilibrium [77]. Consistent with this model, knockdown of Cand1 by RNAi in vivo increases the cellular content of Skp1–Cul1 complexes [77,78]. However, genetic evidence in *Arabidopsis* suggests that Cand1 is also a positive regulator of SCF function [81,82]. The negative and positive roles of Cand1 in regulating SCF might be reconciled if Cand1 serves in part to limit ectopic autoubiquitination of F-box proteins, which if allowed to run amok would deplete F-box protein pools. Finally, multiple proteasome subunits appear to associate with Tip120A [83], perhaps reflecting a role for Cand1 in recycling of inactive SCF complexes.

4. Other SCF interactions

Several other interaction partners for SCF complexes have been described. An auxiliary SCF subunit called Sgt1 was first discovered genetically in yeast as a suppressor of *skp1*^{ts} defects [84]. Like Skp1, Sgt1 physically interacts with both SCF subunits and with the CBF3 kinetochore complex, and in turn is required for CBF3 function and for full activity of the SCF^{Cdc4} complexes [84]. In addition, Sgt1 homologs have been linked to the induction of plant pathogen resistance and auxin regulation by SCF pathways [85–87]. However, Sgt1 may not be a core SCF subunit as it does not exhibit the full spectrum of *scf* mutant phenotypes. Indeed, recent evidence suggests that Sgt1 may be a co-chaperone for Hsp90 [88]. Consistently, formation of the Skp1–Ctf13 complex requires Hsp90 activity [89].

In a functional downstream connection in ubiquitin-dependent proteolysis SCF subunits, and indeed other unrelated E3 components, associate with the 19S regulatory cap of the proteasome [90,91]. Moreover, a specific connection to SCF complexes is mediated by the proteasome binding protein Cic1 [92]. Both Cdc4 and Grr1, which are normally unstable due to SCF-dependent autoubiquitination [93,94], become stabilized in a *cic1Δ* mutant strain [92]. Substrates may thus be channeled directly from the E3 to the proteasome. In addition to enhancing substrate flux, these connections are probably critical for E3 regeneration, given the ability of the proteasome to selectively strip ubiquitinated subunits from multiprotein complexes [95,96].

5. The cullin–RING ubiquitin ligase superfamily

The cullin–RING-adaptor theme is recapitulated in at least five other related multisubunit ubiquitin ligases (Fig. 4, Table 1). The complex permutations and combinations of the SCF-like ubiquitin ligases belie a coherent nomenclature. Although some standardizations have been proposed, such as SCF1 for Cul1 complexes, SCF2 for Cul2 complexes and so on [97], none are as yet uniformly accepted. For clarity of discussion here, with the exception of the APC/C, we designate SCF-like complexes according to the original SCF nomenclature, based in order on the Skp1-like linker, the cullin and the adaptor class. Where necessary, homologs of the different core subunits are designated by their gene name and number, with the original gene set as the default. For example, an *E*loC–EloB–*C*ul2–Rbx1–*S*OCS box protein complex is termed EC_2S, followed by the name of the SOCS-box protein in superscript.

5.1. APC/C

Many insights into the ubiquitin system have come from investigation of the Anaphase Promoting Complex/Cyclosome (APC/C), the E3 complex that controls the M/G1 transition though ubiquitination of cyclins and other mitotic effectors. As the APC/C has been extensively reviewed elsewhere [14,98], we limit our discussion to overall similarities between the SCF and APC/C. That the cullin–RING architecture might be a recurrent motif in E3 construction came with the discovery that the APC/C contains a cullin-like subunit called Apc2 and a RING-H2 subunit Apc11 [46,47]. This notion was consolidated upon subsequent discovery of Rbx1 as a core subunit of the SCF complex [32–36]. As for SCF complexes, the core catalytic activity of the APC/C resides in the Apc2-Apc11 duo, which exhibits nonspecific ubiquitin ligase activity [99,100]. Moreover, like SCF complexes, the APC/C targets different classes of substrates via association with one of at least three different variable subunits, namely Cdc20/Fzy, Cdh1/Hct1/Fzr and the meiosis-specific protein Ama1 [14,98,101]. All three APC/C adaptors contain WD-40 repeats, although substrate recognition depends on other regions as well [102]. Cdc20 and Cdh1/Hct1 bind substrates directly through short motifs called the D-box and the KEN-box, respectively [103–106]. Despite overall similarity to SCF complexes, the APC/C differs in other respects as it contains at least 11 subunits, none of which are recognizable as an Skp1/EloC homolog [46,47]. Furthermore, APC/C activity is primarily controlled at the level of complex assembly, rather than substrate level modification [14,98,102]. Lastly, unlike other cullins, Apc2 is not modified by Nedd8. The distant relationship between the SCF and APC/C nevertheless presaged the existence of other cullin–RING ubiquitin ligase complexes.

5.2. EC_2S

A closely related class of SCF-like enzymes is built on the ElonginC (EloC)–Cul2 core complex, analogous to the Skp1–Cdc53/Cul1 duo. EloC was originally isolated as a part of a transcriptional elongation complex that also contains the ubiquitin-like protein Elongin B (EloB), which binds tightly to EloC, and a SOCS-box containing protein Elongin A (EloA) [107]. In a manner analogous to the Skp1–F-box protein interaction, EloC binds to a family of substrate specific adaptors that contain a motif called the Suppressor of *C*ytokine Signaling (SOCS)-box, which forms a binding site for EloC, and variety of substrate specific protein interaction domains [108]. The prototype EC_2S complex is based on the Von Hippel–Lindau (VHL) tumour suppressor protein, which targets the hypoxia-responsive transcription factor HIF-1α for degradation under normoxic conditions (see below) [109]. The F-box and the SOCS-box exhibit strict specificity for their cognate cullins [110,111]. Unlike the usual N-terminal position of the F-box, the SOCS-box typically resides at the C-terminus. Despite these differences, crystal structures of the VHL–EloB/C complex and the Skp1-Skp2 complex reveal that the EloC-SOCS-box and Skp1-F-box interfaces are topologically related (see below). Other SOCS-box proteins also have associated ubiquitin ligase activity [112–114]. Further details of the EC_2S

ubiquitin ligase family have been extensively reviewed [108].

5.3. BC_3B

A recent variation on the SCF theme has emerged from several simultaneous findings that Cul3 and Rbx1 form ubiquitin ligase complexes with BTB domain containing proteins. Significantly, a BTB domain fold is embedded within the EloC and Skp1 structures [115,116]. In an interesting variation on SCF complex architecture, BTB proteins combine a Skp1-like adaptor domain and a substrate recognition domain in a single polypeptide [117]. These complexes are termed BC_3B ubiquitin ligases, for BTB–Cul3–BTB, reflecting the dual contribution of the BTB protein. Two hybrid screens uncovered 11 BTB protein partners for CUL-3 from nematodes [97], while analogous screens uncovered 13 BTB protein partners for human Cul3 [118]. In parallel, purification of Cul3/Pcu3 complexes from the fission yeast *S. pombe* captured all three predicted BTB domain proteins, called Btb1, Btb2 and Btb3 [119]. BTB proteins contain a host of protein interaction domains, including MATH, Kelch and ANK domains [117]. A number of small BTB proteins do not have an obvious interaction domain, and may serve different functions, perhaps including interference with the assembly of functional BC_3B complexes [97]. As for Skp1–Cul1 and EloC–Cul2 subcomplexes, the specificity for the BTB domain is dictated by the N-terminal residues of Cul3 [97,120]. The best characterized BC_3B complex is based on the *C. elegans* BTB protein MEL-26, which targets MEI-1/katanin for degradation at the end of meiosis (see below). The BC_3B system has recently been elaborated to human cells with the discovery that the BTB protein Keap1 targets the pro-survival transcription factor Nrf2 for degradation under non-stress conditions [121]. The possible functions of BTB domains in mitosis, cytoskeletal control and transcription have been reviewed recently [117].

5.4. Other cullin complexes

Three other cullin-based E3 complexes have been partially characterized. It has been discovered that several SOCS-box proteins that bind Cul2, including VHL, can also bind Cul5 [122]. At least one SOCS-box protein, an LRR-containing protein called Muf1, binds exclusively to Cul5 to form an active EC_5S^{Muf1} complex [122]. Cul4A also appears to participate in multiple ubiquitin ligase complexes. The human homolog of de-etoilated-1, a negative regulator of photomorphogenesis, associates with Cul4A, Ddb1, Roc1 and a protein called constitutively photomorphogenic-1 to target the proto-oncogenic transcription factor Jun for ubiquitin-dependent proteolysis [123]. Cul4A regulates nucleotide excision repair by targeting a protein that binds damaged DNA, called Ddb2, and the Cockayne Syndrome protein CSA, both of which interact with Ddb1 [124–126]. Human Cul4A is also implicated in DNA replication control through degradation of the initiation factor Cdt1 [127]. In fission yeast, Cul4A/Pcu4 regulates DNA replication through degradation of Spd1, a nuclear retention factor for ribonucleotide reductase [128]. Finally, a more recently identified cullin, Cul7, forms an SCF-like ubiquitin ligase with Roc1, Skp1 and the F-box protein Fbw6/Fbx29 [48]. Although the targets of SC_7F^{Fbx6} are not known, $Cul7^{-/-}$ mice die at birth of severe vascular defects [129]. Most, if not all, cullins thus function as ubiquitin ligases that target substrates via dedicated adaptor subunits.

5.5. Non-cullin complexes

A number of variant complexes appear to have abstracted some but not all subunits from cullin-based ubiquitin ligases. In addition to its primary role in SCF complexes, Skp1 also has cullin-independent roles in yeast through its association with Ctf13 in the CBF3-kinetochore complex [25,130], its interactions with non-Fox-box proteins Rav1 and Rav2 in the vacuole-associated RAVE complex [131], and its association with the F-box protein Rcy1 in regulation of vesicle trafficking [132,133]. In metazoans, the F-box protein Emi1, which is an inhibitor of the APC/C (see below), interacts with Skp1 but apparently not with other SCF core subunits [134]. Skp1 is thus a general adaptor for protein interactions. Diversification of subunit function has also occurred for other SCF-like complexes. For example, aside from its substrate recruitment role in the EC_2S^{VHL} complex, VHL appears to modulate deposition of the fibronectin matrix [135] and also directly stabilize microtubules [136]. A distinct mutational hotspot that compromises this latter function predisposes to haemangioblastoma and pheochromocytoma, as opposed to renal carcinomas caused by mutations that disrupt the VHL–HIF-1α interaction [136]. It is also likely that many BTB domain proteins have non-proteolytic functions. In particular, a number of bona fide transcriptional regulators contain BTB domains, usually in conjunction with the common class of C_2H_2 zinc finger domains that bind DNA, but it is not known whether Cul3 interacts with or affects the activity of these factors [137].

A class of E3 enzymes based on Skp1, F-box proteins, RING domain proteins and Sgt1-like proteins has also begun to emerge. An Sgt1-like protein called SIP has been discovered in complexes with the human homolog of the RING-domain protein seven in absentia (Sina), called Siah, Skp1 and a putative WD40 repeat-containing F-box protein called Ebi [138,139]. In human cells, it appears that Ebi recognizes and ubiquitinates β-catenin in parallel to the phosphorylation-dependent $SCF^{\beta\text{-}TrCP}$ pathway (see below). Because Siah expression is induced by genotoxic stress in a p53-dependent manner, it may help restrain proliferation under such conditions [138,139]. The Siah-based complex is subtly re-wired in *Drosophila* where Sina and Ebi catalyze ubiquitination of Ttk, a transcriptional repressor that

functions downstream of Ras/EGFR in eye development [140]. Rather than Ebi, another adaptor protein, Phyllopod, appears to recruit Ttk to Sina for ubiqutination; because Phyllopod expression is induced by Ras, the pathway responds at the appropriate time in eye development to eliminate Ttk [141]. Ebi also impedes entry into S-phase in neuronal precursor cells [142], and serves in the EGFR pathway, where it eliminates another transcriptional repressor, Suppressor of Hairless (SuH) [143]. A degron for the Sina/Siah family of E3s has recently been determined [144]. While the precise architecture of Sina/Siah complexes remains to be worked out, the fact that the cullin–Rbx1 subunits can apparently be replaced by another RING domain protein again attests to the malleable nature of multisubunit E3 enzymes.

6. Budding yeast SCF complexes and substrates

The budding yeast system has been in the vanguard for deciphering the principles of SCF-dependent regulation, beginning with the original concept that selectivity is determined by association of different F-box proteins with the SCF core complex [24]. Budding yeast contains at least 21 known or predicted F-box proteins, most of which are of unknown function (Figs. 1 and 2). Here, we briefly describe the best understood examples of yeast SCF complexes, their corresponding substrates and mode of regulation.

6.1. SCF^{Cdc4}

Budding yeast SCF^{Cdc4} targets a number of important regulatory proteins, including the CDK inhibitors Sic1 and Far1, the replication protein Cdc6 and the transcription factor Gcn4 [12]. From the inception of the SCF field, Sic1 has served as a model substrate, such that the mechanics of its recognition by Cdc4 are understood in considerable detail. As described, Sic1 is a B-type cyclin-Cdc28 inhibitor that is crucial for the establishment of the CDK-free window in G1 phase, which in turn is necessary for assembly of pre-replicative origins of DNA replication [15,145–148]. In late G1 phase, Sic1 is targeted to Cdc4 upon its phosphorylation, primarily by Cln1/2–Cdc28 kinases, with possible contributions from other CDK enzymes [15,16,149–153]. At the end of mitosis, Sic1 is reexpressed and maintained in an unphosphorylated stable state by the anti-CDK phosphatase Cdc14, which is activated by the mitotic exit network [154,155]. Defects in Sic1 degradation preclude DNA replication, as occurs in *scf* mutants, whereas cells that lack Sic1 exhibit a high degree of genome instability because of incomplete loading of origins of DNA replication in G1 phase [15,156]. Sic1 is thus a linchpin in the control of replication origins by CDK activity (Fig. 5A) [148].

Sic1 contains nine consensus CDK phosphorylation sites, seven of which are located in a necessary and sufficient N-terminal targeting domain of ~90 residues [157]. Sic1 appears to entirely lack secondary structure regardless of its phosphorylation status [158]. The C-terminal inhibitory domain of Sic1 presumably acquires secondary structure when it binds to Clb–Cdc28 complexes, as is the case for the mammalian CDK inhibitor p27 [159]. In contrast to well-studied phosphorylation-dependent interactions in which phosphorylation on a single site generates dedicated binding epitope [160], at least six CDK sites on Sic1 must be phosphorylated in order for Cdc4 binding to occur [158]. The dramatic transition in binding between five and six phosphorylation events is independent of which sixth site is phosphorylated, and operates both in vitro and in vivo. Exploration of the sequence space for high affinity binding of short peptides to Cdc4, beginning with a high affinity site derived from human cyclin E, yielded a consensus binding site termed the *C*dc4 *P*hospho-*D*egron (CPD), I/L-I/L/P-pT-P-⟨K/R⟩, where ⟨⟩ indicates disfavored residues [158]. Surprisingly, basic residues in the +2 to +5 position of the CPD, which perfectly conform to the consensus for an optimal CDK phosphorylation site [161], are highly non-optimal for interactions with Cdc4 (Fig. 6A). In addition, endogenous Sic1 sites contain mismatches to preferred bulky hydrophobic residues at the −1 and −2 positions. The consensus CPD sites thus binds Cdc4 with ~1 μM affinity, whereas naturally occurring Sic1 sites bind weakly if at all [158]. The structural basis for these unusual interaction properties is elaborated below.

The deliberate selection against Sic1 sites explains why multiple phosphorylation events are required for the Sic1–Cdc4 interaction. Critically, if individual phosphorylation events are distributive rather than processive, as appears the case, the interaction must exhibit a sixth order dependence on kinase concentration, provided that a competing phosphatase back reaction occurs [158,162]. In turn, this dependence generates a sigmoidal dose–response curve, sometimes referred to as ultrasensitivity [162]. That is, gradual increase in kinase activity can lead to a sudden increase in Sic1 degradation (Fig 6B). Ultrasensitivity is a crucial nonlinear property of many signaling pathways, as for example in the MAPK-based pathway that dictates *Xenopus* oocyte maturation [162,163]. Ultrasensitivity in Sic1 degradation has two main ramifications. First, because a high level of kinase activity is mandated, elimination of Sic1 is rendered resistant to random fluctuations in kinase activity, i.e., biochemical noise, that might otherwise trigger premature replication, or worse, local re-replication. Second, the sigmoidal response curve ensures Sic1 is eliminated over a narrow range of kinase concentration. These ideas were tested by re-engineering Sic1 such that all of the multiple endogenous non-optimal CPD sites within Sic1 were replaced with a single optimal CPD site [158]. A single CPD motif indeed allows efficient Sic1 degradation, but because the single site is no longer resistant to fluctuations in kinase activity, many cells eliminate Sic1 prematurely, with resultant genome instability [158]. As numerous other SCF substrates require multiple phosphor-

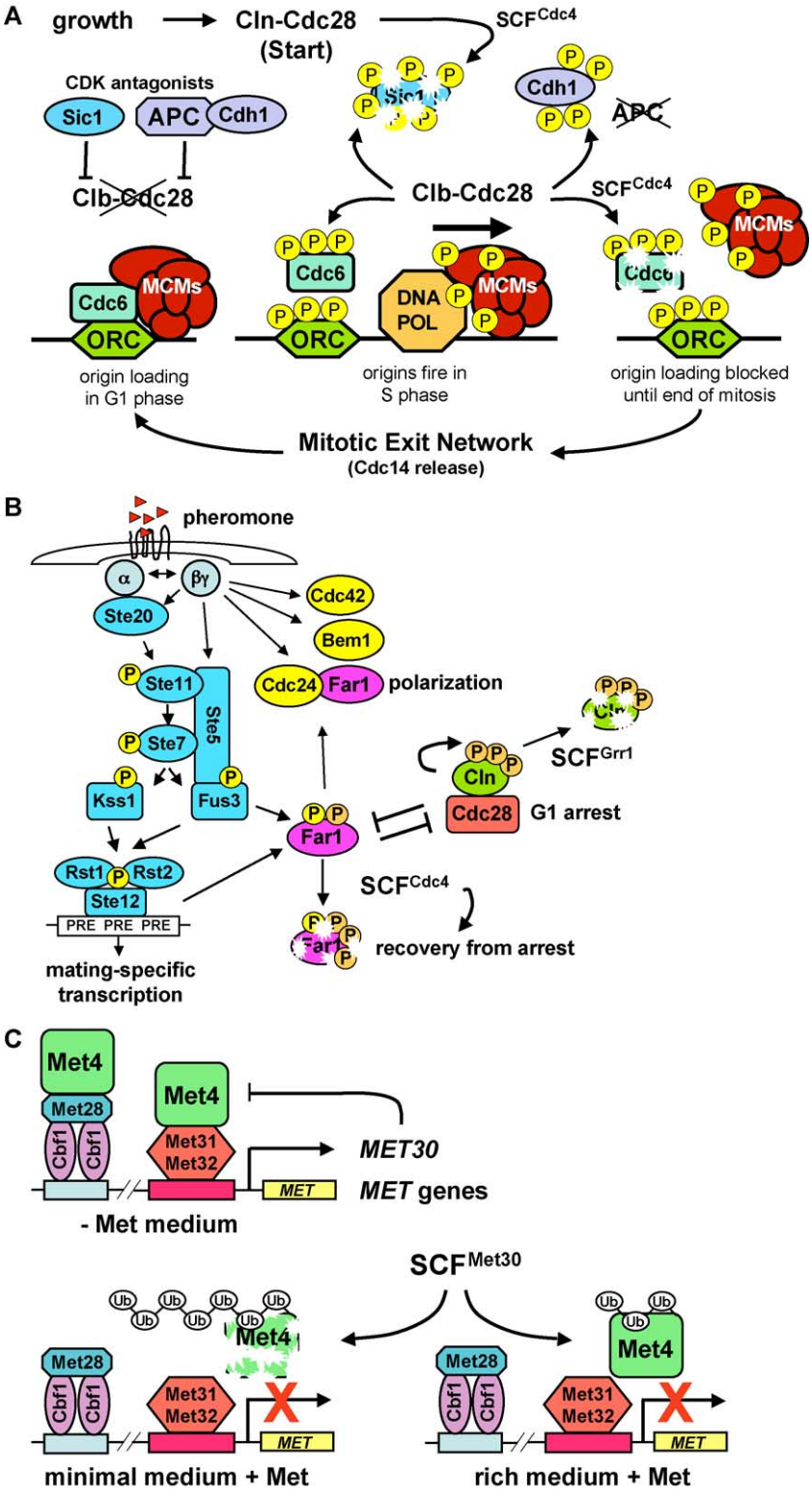

Fig. 5. Examples of SCF pathways in yeast. (A) Sic1 and control of DNA replication by CDK activity. See text and [22,148] for details. (B) Far1 mediates G1 arrest and polarization in the mating response. See text and Ref. [447] for details. Phosphorylation by Cln–Cdc28 and/or Fus3 targets Far1 to SCF^{Cdc4}, while autophosphorylation targets Cln1/2 to SCF^{Grr1}. (C) Met4-dependent gene regulation. Under methionine limitation, Met4 is recruited to *MET* gene promoters by the Cbf1–Met28 and Met31–Met32 complexes, where it activates transcription. Under conditions of methionine excess, Met4 is targeted to SCF^{Met30}, largely through Met4-dependent control of *MET30* expression. In minimal medium, Met4 appears to be polyubiquitinated and degraded, whereas under rich nutrient conditions, Met4 is dissociated from transcriptional complexes by SCF^{Met30}-dependent oligoubiquitination. Under the latter conditions, Met4 is still able to activate some genes, most notably *SAM1* which is essential for AdoMet production in rich medium.

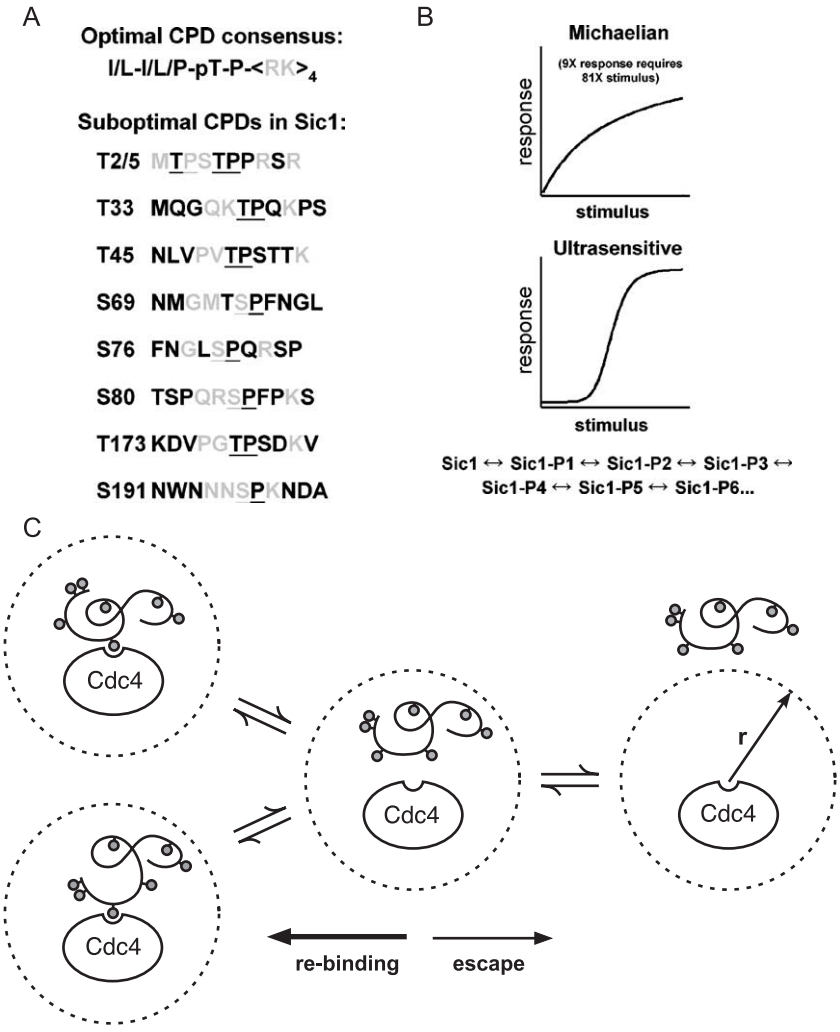

Fig. 6. Multisite phosphorylation of Sic1 drives its recognition by Cdc4. (A) Optimal Cdc4 phosphodegron (CPD) sequence, where ⟨⟩ indicates disfavored residues, and suboptimal natural CPD sites in Sic1. Mismatches to the CPD are shown in grey; phospho-Thr is favored over phospho-Ser in model peptide studies [158]. (B) Distributive multisite phosphorylation engenders a cooperative or ultrasensitive response, as opposed to the predicted Michelian hyperbolic response for single site interaction. Ultrasensitive responses are resistant to noise and confer switch-like behavior. See Ref. [162] for details. (C) Model for high affinity interaction of multisite phosphorylated Sic1 with Cdc4. Rapid rebinding of phospho-Sic1 at redundant low affinity sites is highly favored over diffusion away from Cdc4. See Ref. [437] for details.

ylation events for recognition, this model may be broadly applicable.

A second budding yeast CDK inhibitor, Far1, is also targeted to SCF^{Cdc4} by Cln–Cdc28-dependent phosphorylation [164]. Upon pheromone stimulation, the MAPK Fus3 phosphorylates and activates Far1 to inhibit Cln–Cdc28 kinases and thereby impose a G1 phase arrest [165,166]. Because Far1 specifically inhibits the Cln–Cdc28 kinases, an inherent antagonism engenders an all or none response to mating pheromone—either Cln–Cdc28 kinases are inhibited by Far1, leading to G1 arrest, or Far1 is targeted for degradation by Cln2–Cdc28 kinase-dependent phosphorylation, leading to recovery from G1 arrest (Fig. 5B). In addition to its role as a CDK inhibitor, Far1 serves as a scaffold protein for several membrane associated factors that drive polarized morphogenesis towards the mating partner [167]. In spite of its predominant localization in the cytoplasm during mating, Far1 actively shuttles between compartments [168], such that its degradation occurs exclusively in the nucleus by virtue of the nuclear localization of Cdc4 [169]. Although mutation of a single phosphorylation site in Far1, Ser87, results in considerable stabilization and increased sensitivity to pheromone [170,171], multiple other suboptimal CPD sites also appear to contribute to Far1 recognition (G. Chen and M.T., unpublished data). Because Fus3 is a proline-directed kinase and thus able to phosphorylate CPD-like sequences, activation and degradation of Far1 may be coupled events, perhaps dependent on the integration of signals from Cln–Cdc28 and Fus3.

Another Cdc4 substrate, Cdc6, establishes pre-replicative complexes at chromosomal origins of DNA replication in G1 phase cells [148]. Cdc6 is highly unstable in vivo, with different dependencies at different cell cycle positions

[172,173]. Both SCF^{Cdc4} and multiple CDK phosphorylation sites on Cdc6 are required for its degradation throughout most of the cell cycle [172–175]. Cdc6 instability in late G1 and S phase is dictated by Cln–Cdc28-mediated phosphorylation on N-terminal CDK sites, while later in G2/M phase degradation requires Clb–Cdc28-dependent phosphorylation on C-terminal CDK sites [173,175]. These alternate modes suggest that recognition of Cdc6 by Cdc4 may be affected by changes in conformation or steric accessibility resulting from cell cycle-regulated Clb–Cdc6 interactions [175]. Prior to Start, Cdc6 degradation proceeds by a mysterious route that is independent of CDK, SCF and APC/C activity [173]. Cdc6 degradation is one of several redundant mechanisms that prevent DNA re-replication, including phosphorylation of origin recognition complex (ORC) subunits, nuclear exclusion of Mcm replication proteins and binding of Clb5 to Orc6 [176,177].

A final well-characterized Cdc4 substrate, Gcn4, is a transcriptional activator of amino acid biosynthetic genes. In non-starvation conditions, Gcn4 is rapidly degraded by SCF^{Cdc4} [178]. Two different cyclin-dependent kinases, Pcl5–Pho85 and Srb10–Srb11, phosphorylate Gcn4 and thereby dictate its instability [179–181]. Gcn4 contains a high affinity CPD site at Thr165 [158], mutation of which partially stabilizes the protein [179], and four weak CPD sites, all of which must be eliminated for full stabilization [180]. The Cdc4 recognition mechanism is thus highly tunable, such that the number and nature of CPD sites can be varied, as can the targeting proline-directed kinases.

6.2. SCF^{Grr1}

Two of the primary substrates for SCF^{Grr1} are the G1 cyclins Cln1 and Cln2, both of which are recognized upon Cdc28-dependent autophosphorylation on multiple sites [18,19]. Consistent with this autocatalytic targeting mechanism, Cln2 is unstable throughout the cell cycle, including in pre-Start G1 cells [182]. Grr1 was identified in a genetic screen for mutants defective in Cln1 degradation [26], and later shown to form an SCF complex with specific activity towards phospho-Cln1 and -Cln2 [29,31,33,183]. Basic surface residues in the LRR-domain of Grr1 are crucial for recognition of phospho-Cln2, although the precise Grr1 phosphodegron and how it might differ from the Cdc4 phosphodegron has yet to be defined [184,185]. The presumptive F-box protein for Cln3, which appears to be targeted in a Cdc34-dependent manner [17], has not been identified. At the G1/S transition, SCF^{Grr1} also catalyzes ubiquitination of Gic2 which, together with its homolog Gic1, is a downstream effector of the Rho-related GTP-binding protein Cdc42 that initiates polarization of the actin cytoskeleton during bud emergence [186]. Gic2 instability, and probably that of Gic1, depends on its phosphorylation by an unknown kinase, an event which occurs only when Gic2 is activated by Cdc42 [186]. The coupling of Gic2 activation and proteolysis probably serves to restrict Gic2 activity to a narrow window in G1 phase [186].

In addition to its cell cycle functions, Grr1 regulates transcriptional responses required for nutrient adaptation, including induction of glucose transporters encoded by the *HXT* genes [187]. The complex pathway whereby Grr1 controls *HXT* gene expressed has recently been resolved. Upon glucose stimulation, Grr1 catalyzes the elimination of a protein called Mth1, which normally prevents the phosphorylation of a repressor of *HXT* genes called Rgt1 [188]. Once phosphorylated, Rgt1 no longer binds to promoter DNA, thereby allowing transcriptional activation [188,189]. In the first example of regulation at the level of SCF assembly, the Skp1–Grr1 interaction is abrogated by glucose [183]. Finally, Grr1 is also implicated in the induction of amino acid permease genes [190].

6.3. SCF^{Met30}

Met30 is an essential WD40 repeat containing F-box protein that was originally identified as a repressor of methionine biosynthesis genes [191]. *MET* gene expression is controlled by recruitment of a transcriptional activator, Met4, which is tethered to DNA via its interactions with various DNA binding subunits (Fig. 5C) [192]. Under conditions of methionine excess, SCF^{Met30} inactivates the transcriptional activator Met4 [31,193]. The effects of ubiquitination on Met4 are complex. Under minimal medium conditions, methionine causes the rapid polyubiquitination and elimination of Met4 via the conventional proteasome-dependent route [193]. In contrast, in rich medium in the presence of methionine, Met4 is oligoubiquitinated and inactivated without protein degradation [194]. The mechanism of this effect appears to involve disruption of protein interactions on promoter DNA, as oligoubiquitinated Met4 does not cross-link to *MET* gene sequences even though all Met4 remains in the nucleus in repressive conditions [195]. This complex dual mode of regulation has apparently evolved to enable expression of the S-adenosylmethionine (AdoMet) synthetase encoded by *SAM1*, and perhaps other genes, in rich medium [195]. In contrast to cells grown on minimal medium, in rich medium, production of AdoMet is limiting for growth. How the fate of Met4 is switched between degradation and oligoubiquitination depending on growth conditions remains an unsolved puzzle. Unlike the conditional phosphodegrons recognized by Cdc4 or Grr1, Met30 appears to recognize Met4 constitutively via a primary sequence element [196]. Instead, changes in *MET30* expression, which is under Met4 control, help govern the level of Met4 activity in a negative feedback loop [193,197]. Also, unlike other SCF substrates such as Sic1, Met4 appears to ubiquitinated on a single lysine residue [198]. In an intriguing cell cycle connection, Met4 down-regulation appears to be the essential function of Met30 for the G1/S transition,

presumably because unbridled Met4 activity leads to expression of one or more genes that antagonize Start [196]. Met30 has also been linked to phosphatidylserine transport to mitochondrial membranes, although the mechanism underlying this effect remains to be elucidated [199].

6.4. Other fungal SCF complexes

Most of the other 21 identifiable budding yeast F-box proteins interact with Skp1, Cdc53 and Rbx1 [39,200]. Although many of these F-box proteins initially appeared not to bear protein interaction domains [12], close sequence inspection reveals that at least six additional F-box proteins contain cryptic LRR repeats (for a total of 11 LRR containing F-box proteins), suggesting that their structure indeed conforms to the F-box hypothesis (Fig. 2). However, with few exceptions, deletion of most F-box protein genes in yeast causes little or no phenotype. The F-box protein Dia2/Fcl1 may have minor role in turnover of ectopically expressed cyclin E [201], and is required for maintenance of genome integrity (D. Blake and M. T., unpublished). An F-box protein called Ufo1 targets the Ho endonuclease, which effects the double stranded break needed to initiate mating type switching, for elimination in a manner that depends on Mec1 and other components of the DNA damage response [202]. The F-box protein Mdm30 appears to prevent mitochondrial aggregation by regulating the levels of Fzo1, a GTPase that catalyzes mitochondrial fusion [203]. Finally, a protein that contains a cryptic split F-box motif, called Cst13/Amn1, inhibits the mitotic exit network [204]. Although it is unclear whether Amn1 actually forms an SCF complex, the protein does contain LRRs that may target potential substrates (Fig. 2). In addition to directing substrate ubiquitination, most F-box proteins appear to undergo auto-ubiquitination reactions, possibly as a means to ensure sufficient amounts of the SCF core complex are available for productive substrate interactions [93,94,200].

Several fission yeast F-box proteins have been characterized. The equivalent of SCF^{Cdc4}, called the $SCF^{Pop1/2}$ complex, catalyzes phosphorylation-dependent ubiquitination of the Cdc6 homolog, Cdc18, and of the Sic1 analog, Rum1 [205–208]. In contrast to redundant replication control in budding yeast, stabilization of Cdc18 by mutation of CDK consensus phosphorylation sites is sufficient to induce re-replication [209]. $SCF^{Pop1/2}$ activity in vivo requires heterodimerization of Pop1 and Pop2 [208]. As homomeric complexes exhibit ubiquitin ligase activity in vitro, heterodimerization with Pop2 may function in part to localize Pop1 to cytoplasmic targets [210]. Pop1/2 also mediate degradation of the S-phase cyclin Cig2 in a phosphorylation-dependent manner [210,211]. The only other fission yeast F-box protein to be characterized to date is Pof3, a homolog of Dia2/Fcl1 that is also implicated in genome stability [212].

Analysis of sulfur metabolism pathways in *Neurospora crassa* has revealed the equivalents of Skp1 and Met30, called Scon-3 and Scon-2, respectively [213,214]. Similarly, in *Aspergillus nidulans* the equivalent factors are SconC and SconB, respectively [215,216]. The F-box motif was in fact first noted in comparisons of Scon-2 homologs, but its significance at the time was not appreciated [217]. The only other characterized F-box protein in these species is a Cdc4 homolog in *N. crassa*, called FWD1, which is required for degradation of the circadian clock protein FRQ [218].

7. A survey of metazoan cullin complexes and substrates

Several mammalian SCF-like pathways, each of which has its own subtleties, are now understood in detail. As in yeast, substrate recognition generally depends on substrate level modification, but in addition to phosphorylation, substrate hydroxylation and glycosylation, and the presence of additional protein cofactors, have been found to dictate recognition. Here we briefly review the best-characterized metazoan SCF and SCF-like complexes, with emphasis on mammalian systems.

7.1. $SCF^{\beta\text{-}TrCP}$

The first SCF pathway dissected in detail in mammalian cells is based on the WD40 repeat containing F-box protein β-TrCP [219]. Initial studies revealed that β-TrCP catalyzes the phosphorylation-dependent ubiquitination of both the NFκB inhibitor IκBα, as targeted by the IKK kinases, and the proto-oncogenic transcription factor β-catenin, as targeted by the GSK3/adenomatous polyposis coli/axin complex [220–222]. All known substrates of β-TrCP contain a close match to the canonical DSGXXS motif, originally identified in IκBα, within which both serine residues must be phosphorylated for efficient recognition [220]. Homodimeric SCF complexes containing either of the two known isoforms, β-TrCP1 or β-TrCP2, mediate ubiquitination of IκBα, the degradation of which frees the associated NFκB to translocate into the nucleus and activate transcription of pro-inflammatory and survival genes [219,223]. Subsequently, it was discovered that ubiquitination of the p105 NFκB isoform by $SCF^{\beta\text{-}TrCP}$ enables limited proteolytic processing of its C-terminus by the 26S proteasome to liberate the transcriptionally competent p65 isoform [224,225]. In the first example of viral subversion of a host SCF pathway, the HIV-encoded protein Vpu was found to couple the membrane protein CD4 to β-TrCP, again in a phosphorylation-dependent manner [226]. In further transcriptional connections, β-TrCP mediates the phosphorylation-dependent elimination of ATF4, a cAMP-dependent transactivator of the bZIP class [227], and

the TGF-β activated transcription factors, Smad 3 and Smad4 [228,229]. Finally, the repertoire of β-TrCP substrates has recently been expanded to include two critical cell cycle regulators, namely the Cdc25A phosphatase, which activates S-phase CDK complexes to enable DNA replication, and Emi1, the F-box protein that inhibits the APC/C by binding to the Cdc20 subunit (see below) [230–232]. Mice that lack β-TrCP-1 are viable due to compensation by β-TrCP-2, but nevertheless have various mitotic and meiotic defects, including accumulation of Emi1 [233]. Further aspects of β-TrCP function and regulation have been reviewed recently [234].

7.2. SCF^{Skp2}

Skp2 is an LRR-containing F-box protein with pleotropic functions in cell cycle control, whose targets include the transcription factor E2F-1, the CDK inhibitors p27, $p21^{Cip1}$ and $p57^{Kip2}$, the origin recognition subunit hOrc1, the replication initiation factor Cdt1, the Rb-related tumour suppressor p130 and the proto-oncogenic transcription factor Myc. E2F-1 is one of several E2F family members that drives gene expression at the G1/S transition [235] and the first potential substrate assigned to Skp2. SCF^{Skp2} binds E2F-1, and moreover forms of Skp2 that lack the F-box stabilize E2F-1, presumably by sequestering it from endogenous Skp2, although indirect effects cannot be ruled out [236]. SCF^{Skp2} activity appears to be a limiting factor in E2F-1 degradation since additional *Cul1* dosage stimulates the formation of ubiquitinated forms of E2F-1 [236]. It appears that E2F-1 stability is determined both by the availability of Skp2, which is itself cell cycle-regulated with a peak in S-phase, and by the binding of E2F-1 to Rb, which may prevent its recognition by SCF^{Skp2} [237–239]. In spite of the above findings, E2F-1 does not accumulate in $Skp2^{-/-}$ mice [240], suggesting other levels of regulation or another pathway may also target E2F-1 for degradation.

The best characterized substrate of SCF^{Skp2} is the CDK inhibitor p27, the degradation of which is critical for entry into S-phase. In fact, p27 is a dosage-dependent barrier for cell cycle progression since it is a haplo-insufficient tumour suppressor [241]. Initial work showed that recognition of p27 by SCF^{Skp2} requires CDK-dependent phosphorylation of p27 on Thr187 [242–245]. p27 is stabilized in $Skp2^{-/-}$ mice and, probably as secondary consequence of p27-dependent inhibition of Cdk2 activity, cyclin E is also stabilized [240]. Unexpectedly, p27 degradation also requires binding to Cks1, an essential component of CDK complexes that binds to the C-terminal lobe of Cdk kinases. Biochemical purification of an activity that potently stimulated p27 ubiquitination in vitro yielded Cks1 [246], while parallel studies of $Cks1^{-/-}$ mice revealed cell cycle misregulation that was traced to accumulation of p27 [247]. Like Skp2, Cks1 is cell cycle-regulated with a peak in G1 phase, such that p27 elimination is not enabled until the G1/S transition [247]. Skp2, Cks1 and phospho-p27 form a trimeric complex, in which Cks1 binds tightly to Skp2, thereby increasing the affinity of Skp2 for the pThr187 epitope, by an allosteric effect [248] and via contributions from an anion-binding pocket on Cks1 [249–251]. p27 degradation entails additional complexities as genetic analysis of cells from mice bearing a Thr187Ala mutant allele of p27 revealed that while p27 degradation in S/G2 phase is indeed Thr187 dependent, its instability in G1 phase is Thr187 independent, though still Skp2- and phosphorylation-dependent [252]. A differentially spliced variant called $p27^{Kip1R}$, in which the C-terminal region containing Thr187 is removed, is stabilized at all points in the cell cycle, suggesting that determinant(s) responsible for Thr187-independent instability also reside in the last 36 amino acids [253]. Further complexities have been revealed by analysis of the *Xenopus* p27 homolog, Xic1, whose degradation depends not on CDK-dependent phosphorylation but on co-recruitment with SCF subunits to pre-replicative origins on chromatin [254]. The related CDK inhibitor $p21^{Cip1}$ is similarly degraded by SCF^{Skp2} in a Cks1- and phosphorylation-dependent manner [255], while genetic evidence suggests that an SCF complex also eliminates a third family member, $p57^{Kip2}$ [59].

SCF^{Skp2} has been implicated in ubiquitination of several other proteins with critical roles in the G1/S transition. hOrc1, a subunit of the origin recognition complex, is degraded via Skp2 after replication initiation as a means to help ensure once and only once per cell cycle replication of the genome [256]. The basis for selective recognition of hOrc1 and not other ORC subunits is not known, but seems likely to depend on CDK-dependent phosphorylation [256]. Another replication factor, called Cdt1, which like Cdc6 helps license origins, also appears to be targeted for degradation by Skp2 upon its phosphorylation by CDK activity [257,258]. Cyclin D-Cdk4/6-mediated phosphorylation similarly targets the Rb-related protein p130 to Skp2, thereby facilitating the onset of S-phase through liberation of E2F activity [235,259]. Finally, cyclin D is targeted for ubiquitin-dependent degradation upon its phosphorylation by Gsk3 [260], in a manner that appears to depend either directly or indirectly on SCF^{Skp2} [261,262]. It is not known if Cks1 or other co-factors are required for recognition of any of these substrates.

Recent findings indicate that Skp2 helps mediate transcriptional activation by Myc, a potent proto-oncogenic transcription factor that is overexpressed or subject to stabilizing mutations in at least 30% of human cancers [263]. Myc modulates expression of some 10% of the genome through binding to E box elements, which are present in many growth responsive genes [264]. In some circumstances, Myc deregulation alone is sufficient to rapidly transform normal cells into full-blown aggressive tumors with near complete efficiency [265]. Oncogenic variants of Myc often bear stabilizing mutations [266,267], while in yeast the SCF system has been linked to

transactivation by synthetic transcription factors [268]. Unexpectedly, SCF^{Skp2} appears to couple Myc turnover at promoter DNA to transcriptional activation, effectively licensing the expression of Myc-responsive genes [269,270]. The Skp2-Myc interaction was arrived at by two means. In a surrogate genetic approach in yeast, Myc was stabilized in *grr1Δ* strains and destabilized by heterologous expression of human Skp2 [269]. In parallel, a candidate survey of Myc binding partners revealed interactions with Cul1 and Skp2 [270]. Skp2 binds the Myc Box II (MBII) and bHLH domains of Myc, apparently in a constitutive manner. As might be expected for a bona fide substrate, interference with Skp2 function by either dominant negative Skp2 or by RNAi partially stabilizes Myc [269,270]. In an analogous manner, instability and activity of a Gal4-MBII hybrid depend on Skp2 and, moreover, overexpression of Skp2 induces Myc-dependent genes [269]. As predicted by this model, Skp2, Cul1 and several proteasome subunits associate with chromatin at Myc-dependent promoters [270]. It appears that coupled activation and degradation of Myc is crucial for the biological activity of Skp2 as overexpression of Skp2 in $Myc^{-/-}$ fibroblasts fails to induce S-phase despite degradation of p27 [270]. Because SCF^{Skp2}-dependent ubiquitination of Myc has not been achieved in vitro, it is possible that Myc recognition may require an unknown cofactor, in the same manner as p27 recognition requires Cks1. It is likely that parallel pathways also contribute to Myc activation as $Skp2^{-/-}$ mice are viable and relatively tumor-free, whereas Myc is highly tumorigenic when fully deregulated [240,263]. Because *Cul1* itself is a Myc-responsive gene [271], it is possible that Myc activity is autoregulated by Cul1 induction, not unlike the Met4-Met30 couple. Finally, Skp2 also appears to target the growth-regulated transcription factor B-Myb in a manner that depends on cyclin A [272].

7.3. SCF^{Fbw7}

The F-box protein Fbw7 is the human ortholog of Cdc4, but due to its simultaneous discovery in several systems, it has several alternate designations including hCdc4, Ago and Sel10. For simplicity, we use the original designation assigned by systematic annotation of mammalian F-box proteins [40,41]. Fbw7 targets a cohort of important substrates, including cyclin E, Notch, and Myc.

Much like G1 cyclins in yeast, cyclin E is largely eliminated through a phosphorylation and ubiquitin-dependent pathway. Initial analysis of cyclin E degradation revealed a requirement for phosphorylation on Thr380, as mutants lacking this site are stabilized [273,274]. In the first genetic analysis of a mammalian SCF core component, it was found that $Cul1^{-/-}$ mice die in early embryogenesis before the onset of gastrulation with high levels of cyclin E protein but not mRNA [275,276]. The role of Fbw7 in cyclin E degradation was discovered in three separate lines of investigation. A surrogate genetics approach in yeast revealed that cyclin E instability depends on SCF^{Cdc4}, thereby prompting analysis of the human homolog of Cdc4, which indeed mediates cyclin E degradation [277]. A systematic survey of mammalian F-box proteins for their ability to bind cyclin E, as well as investigation of yeast F-box proteins capable of targeting cyclin E, also yielded Fbw7 [201]. Finally, a genetic screen in flies for clones that over-proliferate uncovered mutations in a gene called *archipelago* or *ago* [278]. Cyclin E accumulates in cells impaired for Fbw7/hCdc4 function by RNAi, in *ago* mutant cells, and in cell lines derived from $Fbw7^{-/-}$ embryos [201,277–280]. SCF^{Fbw7} binds to and efficiently ubiquitinates phosphorylated forms of cyclin E [201,277]. Intriguingly, although the Cdc4-cyclin E interaction depends heavily on phosphorylation of Thr380, phosphorylation on at least one other site, Thr62, either directly or indirectly influences cyclin E recognition [277]. The relationship between different cyclin E phosphorylation events is far more complex than initially anticipated, as recent evidence suggests that Thr380 is phosphorylated by Gsk3, perhaps in combination with Cdk2 [281]. In addition, Cdk2 phosphorylates Ser384, which may help prime Thr380 phosphorylation by Gsk3, and Ser372, both of which affect cyclin E instability [281]. The requirement for phosphorylation on multiple sites suggests that cyclin E may be recognized in multisite manner, as observed for Sic1 and other substrates, and that multiple signals might converge on cyclin E [281]. In addition to the SCF^{Fbw7} pathway, Cul3 may mediate phosphorylation independent degradation of cyclin E [282], although the presumptive BTB adaptor protein for cyclin E has not emerged.

The primary determinants of Myc instability entail phosphorylation on Thr58 and Ser62 in the Myc Box 1 (MB1) region, which is a hotspot for oncogenic mutations [263]. Phosphorylation of these sites depends on multiple kinases that are in turn governed by Ras activity [283]. Despite compelling effects of Skp2 on transcriptional activation by Myc, recognition of Myc by Skp2 does not depend on MB1 [269,270]. This conundrum was solved with the finding that SCF^{Fbw7} targets Myc for degradation in a phosphorylation- and MB1-dependent manner [284–286]. Myc contains a close match to the consensus CPD around the Thr58 site (LPTPP) and is bound and ubiquitinated by recombinant SCF^{Fbw7} [284,285]. Like cyclin E, Myc is targeted to Fbw7 by Gsk3 [284,285]. Overexpression of Fbw7 destabilizes Myc but not the Thr58Ala Ser62Ala double phosphorylation site mutant, whereas Myc protein and associated transactivation activity are elevated in cells that lack Fbw7 [284,285]. Because Myc is not fully stabilized by loss of Skp2 and Fbw7, it is likely that yet other degradation pathways help dictate Myc abundance.

In flies, *ago* mutations cause dMyc accumulation and induction of a dMyc target gene, while an *ago* hypomorphic allele alleviates proliferative defects of a *dmyc* hypomorph [286]. The outgrowth of *ago* clones may arise in part from

dMyc deregulation, especially given the recent observation that overexpression of *dMyc* confers a "super-competitor" phenotype, in which wild-type clones are rapidly supplanted by dMyc overexpresssing cells [287,288]. Unlike mammalian Myc, which has an optimal CPD site centered on Thr58, dMyc appears to harbor multiple weak CPD sites, analogous to the situation in Sic1 [158,286]. These regulatory differences underscore the plasticity of substrate recognition mechanisms by Cdc4/Fbw7/Ago.

Finally, just as yeast Cdc4 has numerous substrates, other critical regulatory substrates have emerged for Fbw7. The nematode Fbw7 ortholog SEL-10 was recovered genetically as a suppressor of hypomorphic mutations in the LIN-12/Notch transmembrane receptor, which regulates cell fate [289]. Upon ligand binding, Notch family members are cleaved by various proteases, including γ-secretase/presenilin, whereupon the Notch intracellular domain fragment ($Notch^{ICD}$) translocates into the nucleus to activate transcription of target genes [290]. The activity of $Notch^{ICD}$ isoforms is attenuated by Fbw7/Sel10, in the expected phosphorylation-dependent manner [291–293]. Proteasome inhibition increases the protein levels of intracellular Notch1, yet decreases transactivation potential [292], reminiscent of the effects of Skp2 on Myc. SEL-10/Fbw7 is but one of at least four E3 ligases that control Notch activity [294]. The presenilins themselves, which are implicated in some forms of Alzheimer's disease, are also targeted for degradation by SEL-10 [295,296]. In a further transcriptional connection, Fbw7 appears to target phosphorylated forms of Jun, and thereby triggers neuronal apoptosis [297]. By virtue of its cohort of critical substrates, Fbw7 resides at the nexus of growth and cell cycle control.

7.4. SCF^{Fbx2}

In an unexpected variation on substrate recognition, the F-box protein Fbx2 appears to target glycosylated substrates [298]. Fbx2 specifically binds to proteins bearing N-linked high-mannose oligosaccharides, but not to unmodified proteins, and subsequently promotes their ubiquitination [298]. Known targets of the SCF^{Fbx2} complex, including pre-integrin β1, CFTR and the TCR-α subunit, are substrates of the ER-associated degradation (ERAD) pathway suggesting that SCF^{Fbx2} is involved in protein quality control through elimination of cytoplasmic glycoproteins [299]. The polyvalent nature of branched glycosyl moieties bears interesting parallels to the multisite phosphorylation-dependent recognition of Sic1 by Cdc4.

7.5. EC_2S^{VHL}

The VHL tumor suppressor protein has been intensely studied because of its role in angiogenesis and cancer [300]. The hypoxia-inducible transcription factor HIF-1α dimerizes with HIF-1β under low oxygen conditions and thereby induces transcription of hypoxia-inducible genes [300]. Under normoxic conditions, HIF-1α is recognized by the β domain of VHL, rapidly polyubiquitinated by EC_2S^{VHL} and degraded by the 26S proteasome [300–304]. The substrate binding similar to Fbw7, the substrate binding site in the β domain is a hotspot for tumorigenic mutations, which heavily predispose to renal cell carcinoma [116]. Consistently, a variant HIF-1α that is immune to VHL inhibition causes cell transformation [305]. In another twist on substrate recognition, the β domain of VHL only engages HIF-1α that has been hydroxylated on proline residues in a reaction that depends on molecular oxygen [306–308]. The hydroxylprolases that catalyze this reaction, called PHD1, PHD2 and PHD3, recognize a loose sequence element in HIF-1α, LXXLA, which flanks two key targeting proline residues [309]. The hydroxylation reaction appears to be nonreversible because hydroxylated HIF-1α can only be cleared by degradation [310]. Oxygen-sensitive regulation of the system is dictated primarily by the ubiquitin-dependent degradation of PHD1 and PHD3, which appears to be mediated by Siah1/2 containing ubiquitin ligase complexes (see above) [311]. In addition to oxygen-sensitive degradation, the activity of the HIF-1α transactivation domain is inhibited by asparagine hydroxylation [312], by VHL-dependent recruitment of a transcriptional repressor called VHLaK [313] and by VHL-independent pathways [314–316]. Conversely, the CSN5 subunit appears to interact with and partially stabilize HIF-1α under high and low oxygen tension [317]. A second VHL target, the large subunit of RNA polymerase II, has recently been found to be targeted for ubiquitination by VHL in a manner that depends on both proline-hydroxylation and phosphorylation [318]. Other possible substrates of EC_2S^{VHL} include PKCλ and a de-ubiquitinating enzyme called VDU1 [319,320].

7.6. BC_3B^{MEL26}

The best characterized BTB domain adaptor to date is MEL-26. Important genetic evidence in *C. elegans* demonstrates that Cul3 complexes indeed antagonize substrate function in vivo. Loss of either CUL-3 or MEL-26 function causes a failure in mitotic spindle assembly after meiosis because the microtubule severing protein MEI-1/katanin is not degraded [120]. Concordantly, the product of a gain of function *mei-1* allele fails to interact with MEL-26 and defects in microtubule-dependent processes observed in *mel-26* and *cul-3* embryos are suppressed by *mei-1* inactivation [58,97,120]. Moreover, mutations in the MATH protein interaction domain of MEL-26 abrogate the MEI-1 interaction but not the CUL-3 interaction [97]. These observations imply that MEL-26 functions as an adapter that recruits MEI-1 to a CUL-3 based complex. Consistently, the BC_3B^{MEL26} complex can ubiquitinate MEI-1 in vitro [118] and BC_3B^{MEL26}-mediated elimination of MEI-1 requires dynamic neddylation of CUL-3 [321]. MEI-1 may be targeted to MEL-26 upon phosphorylation by the kinase

MBK-2 because *mbk-2* mutants fail to degrade MEI-1 and other proteins at the first mitotic division [322]. Intriguingly, both MEL-26 and the *S. pombe* BTB domain protein Btb3 accumulate when CUL-3/Cul3 are inhibited, reminiscent of the autocatalytic turnover of some F-box proteins [120]. A number of other BTB domain proteins of various functions and potential links to ubiquitin-mediated proteolysis have been reviewed recently [117].

8. Cullin complexes in development

Given the many dozens to hundreds of F-box proteins encoded by model organism genomes (Table 1), and the likelihood that numerous developmental decisions require elimination of pre-existing regulators [323], it is not surprising that SCF complexes have been implicated in many developmental pathways in model organism systems. We elaborate only briefly on some of the best understood examples.

8.1. Plants

Hormone signaling and disease resistance responses in plants rely heavily on SCF-mediated proteolysis, a feature that has proven invaluable for elaboration of the SCF paradigm [324]. In the first such connection established, responses to the plant hormone auxin depend on the elimination of the transcriptional repressors AUX/IAA by the SCF^{TIR1} complex [325,326]. The hormones jasmonate and gibberellin similarly mediate the wounding and growth responses through elimination of repressors by SCF^{COI1} and $SCF^{GID2/SLY1}$, respectively [327–329]. As alluded to above, the regulatory influences of the neddylation and de-neddylation systems were discovered largely though analysis of *Arabidopsis* photomorphogenesis mutants [72]. Recent analysis of the *Arabidopsis* Cand1 ortholog, also called ETA2 (for enhancer of *tir1-1* auxin resistance), has shown that *cand1/eta2* mutations enhance the weak auxin response caused by a *tir1-1* mutation, and correspondingly stabilize AUX/IAA, indicating that CAND1/ETA2 acts a positive regulator of SCF activity in vivo [81,82]. Importantly, because SCF^{TIR1} function is still dependent on the Nedd8 pathway in an *eta2* mutant, neddylation must have an essential function other than dissociation of CUL1 from CAND1 [81]. Different F-box proteins are also implicated in the photo-response and photoperiodic of control plant flowering [330–332]. The evident diversification of the F-box gene family in plants may have been driven by the multitude of adaptive responses necessary for development and survival [333].

8.2. C. elegans

The cullin family was discovered in part through its role in development in the nematode [28]. However, in spite of the phalanx of F-box proteins in *C. elegans*, only a few additional functions have been assigned to date. The potential cadre of SCF complexes in nematodes is enormous because many of the 21 identified SKP-1 homologs, which serve multiple roles in development, interact with CUL-1, and presumably also with hundreds of F-box proteins [44,45]. The worm ortholog of β-TrCP, called LIN-23, has similar role as CUL-1 in restricting proliferation during the later stages of development [334] and also for suppression of neurite outgrowth [335]. The F-box protein FOG-2, one of a large sub-family of nematode F-box proteins that contain a predicted domain of unknown function, appears to recruit a translational repression complex to suppress the *tra-2* mRNA and thereby enable male sex determination [336]. A recently described F-box protein FSN-1 forms a variant SCF complex with Skp1 and a RING domain protein RPM-1 to restrict synapse formation [337]. The *C. elegans* Cul2 ortholog, CUL-2, is in general necessary for G1 phase, mitotic progression and genome stability [338]. Recently, a fascinating role for degradation has emerged in establishment of germ line asymmetry, where a SOCS-box protein called ZIF-1 forms an EC_2S^{ZIF-1} complex that targets multiple CCCH finger proteins for degradation in somatic cells but not in germ cells [339]. CUL-2 is also required for anaphase II in meiosis and polarity establishment, although the putative SOCS-box protein and substrate(s) in this presumptive degradation pathway are unknown [340,341]. Aside from MEL-26, the function of the 11 *C. elegans* BTB domain-containing proteins that interact with CUL-3 in vitro is unknown [97,117].

8.3. D. melanogaster

Genetic analysis in flies has contributed heavily to the understanding of the β-TrCP/Slimb pathway, which interdigitates with the hedgehog (Hh) and wingless (Wg) signaling networks [342]. Slimb was discovered in screen for supernumery limbs [343]. In flies that lack Slimb, the two transcriptional effectors of Hh and Wg pathways, cubitus interuptus (Ci) and armadillo (Arm), respectively, are both stabilized and correspondingly target genes for each pathway are ectopically expressed [343]. The details of SCF^{Slimb}-mediated proteolysis and processing in these pathways are exceedingly complex and have been thoroughly reviewed elsewhere [219]. As noted above, the Rbx1 isoform Roc1a appears to mediate Ci but not Arm proteolysis, suggesting substrate-specific RING domain functions [50]. Slimb also regulates the circadian rhythm in flies by mediating the CK epsilon-dependent elimination of the clock proteins Period and Timeless, which hyper-accumulate in phosphorylated forms when *slimb* mutants are held in darkness [344,345]. In contrast to Skp2-dependent elimination of E2F in mammalian cells, Slimb appears to target dE2F for S-phase destruction in flies [346]. Finally, Slimb also regulates centrosome duplication [347,348]. In another developmental pathway, the F-box

protein Partner of paired (Ppa), expression of which is repressed by the segmentation Eve, targets the Paired transcription factor for degradation in non-Eve expressing cells [349]. Finally, SCF pathways impinge on apoptosis in flies through the F-box protein morgue, which binds and eliminates the inhibitor of apoptosis DAIP1 [350].

8.4. Other metazoans

F-box protein functions have been uncovered in most other eukaryotic model systems. In *Xenopus*, β-TrCP catalyzes the Gsk3-dependent elimination of Xom, a transcriptional repressor of dorsal genes [351]. Numerous other F-box proteins have been identified in *Xenopus* [352], some of which co-localize to the centrosome with SCF core subunits, which are required for centrosome duplication [353]. In *Dictyostelium*, the F-box protein FbxA targets cAMP phosphodiesterase for ubiquitin-dependent degradation and thereby controls cAMP levels and cell aggregation [354]. Interestingly, assembly of the SCF^{FbxA} complex appears to be governed by Erk2, which is itself also required for cAMP-dependent protein kinase activity [354]. Another WD40 repeat containing F-box protein called ChtA suppresses spore formation [355]. An unusual chimera between a MEK kinase and a WD40 F-box protein called MEKKα is necessary for establishing prespore and prestalk compartment boundaries in the slug [356]. The F-box protein portion of MEKKα interacts with both an E2 and a ubiquitin hydrolase, which appear to dictate the stability, and presumably signaling activity, of the kinase [356]. Intriguingly, the *Dictyostelium* Skp1 homolog is heavily modified on a hydroxyproline residue by a novel pentasaccharide [357], although the significance of this modification is unknown.

9. SCF circuits in cell cycle control

Individual biochemical events, including substrate proteolysis, are not isolated reactions but are rather concerted with myriad other events in dynamic networks [358]. Given the preponderance of ubiquitin proteolytic pathways, it is not surprising that interpathway networks have begun to emerge, much as phosphorylation-dependent signaling pathways connect to one another [160]. As described above, both the G1/S and M/G1 transitions, which drive the cell cycle into high and low CDK states, respectively, rely on proteolysis to eliminate key cell cycle regulators that define each preceding state [22]. However, it has recently emerged that the proteolytic effectors are themselves repressed by proteolysis in a complex set of feedback loops (Fig. 7). The G1 state, in which p27 suppresses the emergence of CDK activity, is enforced by the APC^{Cdh1}- and destruction box-dependent degradation of both Skp2 and Cks1, the two key co-factors in p27 degradation [359,360]. Thus, the onset of S phase is accelerated either

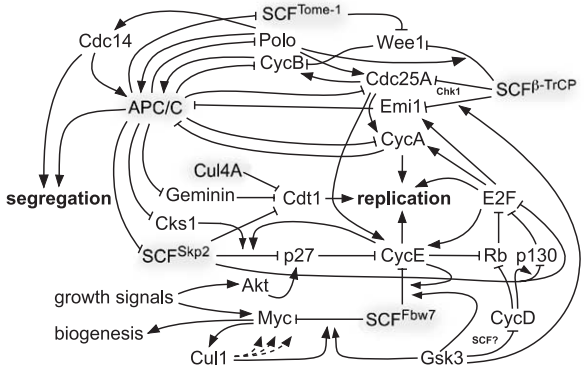

Fig. 7. Proteolytic networks in the mammalian cell cycle. Pointed arrows indicate activation; blunt arrows indicate inhibition. Shaded areas indicate SCF and SCF-like ubiquitin ligase complexes. For clarity not all regulatory pathways are shown, nor are all SCF substrates shown. See text for details.

when a destruction box mutant of Skp2 is introduced or when Cdh1 levels are reduced by RNAi [359,360]. The locked-in G1-phase state begs the question of how p27 degradation is initiated. Much as in yeast [358], extracellular signals appear to trigger a transition causing event. Phosphorylation of a small pool of p27 by the growth factor responsive kinases Akt/PKB and hKIS causes its transport to cytoplasm and thereby liberates an initial active CDK pool in the nucleus [361–364]. In principle, this subtle shift in equilibrium enables parallel positive feedback by rendering p27 susceptible to degradation first through its direct phosphorylation and second by allowing Skp2 and Cks1 accumulation upon CDK-dependent inactivation of APC^{Cdh1}. The APC/C is further repressed by the Cdc20 antagonist Emi1 [134], which is expressed once CDK activity phosphorylates Rb family members and liberates E2F-dependent transcription [365]. Consistently, overexpression of Emi1 causes precocious S phase onset, while flies that lack the Emi1 ortholog Rca1 are unable to elaborate cyclin A activity in G2 phase [366,367]. These parallel mechanisms thus conspire to reverse the low CDK state and establish a high CDK state.

SCF-dependent proteolysis also plays an important role in the onset of mitotic forms of CDK activity needed to initiate mitosis. In S and G2 phase, cyclin A–Cdc2 and cyclin B–Cdc2 complexes are held inactive by Wee1- and Myt1-mediated phosphorylation of tyrosine and threonine residues in the ATP-binding loop of Cdc2 [368]. The Cdc25 phosphatases reverse this inhibition to trigger mitotic onset, through a negative feedback loop in which Cdc2 and Polo phosphorylate and inactivate Wee1, and a positive feedback loop in which Polo and Cdc2 kinases phosphorylate and activate Cdc25 [369]. Cdc25C is further controlled at the level of nuclear localization [370], whereas Cdc25A is regulated by both SCF- and APC/C-dependent proteolysis [230,231,371]. The mitotic transition exhibits a remarkable degree of hysteresis, in that the threshold concentration of cyclin needed to trigger mitosis

is substantially higher than that needed to maintain the active Cdc2 state [372,373]. Proteolysis forms a component of the double negative feedback loop as it has recently been discovered that Wee1 is because, as noted above, Wee1 is targeted to $SCF^{\beta\text{-TrCP}}$ in a co-dependent manner by Polo and Cdc2 [374], a step that probably accounts for earlier observations that Cdc34 is required for mitotic progression [375,376]. Importantly, this switch cannot be thrown until both cyclin and Polo synthesis regenerate pools destroyed at the end of the previous cell cycle by the APC/C [371]. Wee1 may also be targeted by a second F-box protein called Tome-1, which itself is subject to degradation by the APC/C, thereby reinforcing the mutual antagonism between states of high and low CDK activity [377]. The crucial role of the APC/C in maintaining the G1 phase state is illustrated by the finding that conditional inactivation of the APC/C in quiescent cells results in unscheduled DNA replication [378].

In budding yeast, analogous feedback control enables mitosis by inactivation of the Wee1 homolog, Swe1. However, in this case degradation is triggered initially by the PAK-like kinase Cla4 and subsequently the Polo-like kinase Cdc5 [379]. Despite an initial report that Met30, the closest yeast homolog to β-TrCP, might target Swe1 [380], the cognate E3 is now uncertain [381]. The elimination of Swe1 is intimately linked to the assembly of the septin ring at the bud neck, which is essential for proper nuclear transit into the daughter cell and subsequent cytokinesis. In situations where septin assembly is defective, Swe1 is dephosphorylated and stabilized, and mitosis is thereby inhibited until the septin defect is corrected [382]. Consistently, localization of Cdc5 to the bud neck is required for elimination of Swe1 [383]. In addition, Swe1 may establish a minimum size threshold for entry into mitosis, as occurs in fission yeast [384].

Suppression of the APC/C at the G1/S transition and throughout S and G2 phase by Emi1 raises the problem as to how the APC/C is reactivated to initiate anaphase. Here again, a proteolytic switch is at play, this time as a countervailing force against Emi1. Phosphorylation of Emi1 by Polo, and perhaps also by Cdc2, targets Emi1 to $SCF^{\beta\text{-TrCP}}$ for ubiquitination and subsequent degradation [385,386]. In parallel, activation of the Cdc14 phosphatase, probably by Polo [155], triggers dephosphorylation of Cdh1 allowing it to activate the APC/C towards KEN-box containing substrates [387,388]. The two APC/C isoforms are thereby released to destroy cyclin, Polo and Cdc25A, and in so doing restore the cell to a low CDK interphase state that is permissive for loading origins of DNA replication. In addition, the APC/C eliminates geminin, an inhibitor of the replication initiation factor Cdt1, to further prepare cells for a subsequent round of replication [389,390]. The re-entry into G1 phase also has potential switch-like features because of double negative feedback, as the elimination of Cdc2 activity reprieves phosphorylation-dependent inhibition of Cdh1 [391].

Other regulatory schema reinforce and control this core oscillator, as recently summarized in several excellent reviews [232,392,393]. Cell cycle stage-specific transcriptional programs refine the timely appearance and disappearance of regulatory factors such as Skp2, Cks1, Emi1, and cyclin. These programs appear remarkably robust and buffered against genetic perturbation, as for example in the compensatory control of cell cycle duration by the interplay of E2F and CDK activity [394]. Often the same regulatory machinery that controls the cell cycle also controls growth, as noted for the regulation of cyclin E, Notch, Jun and Myc by Fbw7. In addition, growth regulatory pathways may directly control SCF activity, as in the regulation of Skp2 and Cks1 by TGF-β [395]. Growth and division may thus be inextricably linked. The checkpoint pathways that monitor successful completion of key events, including completion of DNA replication, the absence of DNA damage, the attachment of kinetochores to the mitotic spindle and the proper completion of cytokinesis may also engage proteolytic effectors. For example, the mitotic activator Cdc25A is targeted to $SCF^{\beta\text{-TrCP}}$ upon its phosphorylation by Chk1 under conditions of DNA damage [230,231]. In yeast, these self-reinforcing regulatory connections predict a remarkably stable attractor landscape that drains into a large G1 phase basin, as well as smaller basins defined by checkpoint arrests [358]. It seems likely that a similar landscape will dominate the metazoan cell cycle.

10. SCF pathways and disease

10.1. Cancer

Given recent therapeutic successes of general proteasome inhibitors in some cancers, great interest has been stirred in developing specific inhibitors of E3 enzymes [396]. Because critical cell cycle and growth regulators are under SCF control, it is not surprising that perturbation of SCF pathways has been linked to human cancer. Skp2 is often overexpressed, and conversely p27 levels are reduced, in primary breast cancer and other cancer types [397]. Consistently, overexpression of Skp2 cooperates with Ras mutations in cell transformation [398]. The exquisite sensitivity of transformation to p27 dosage may allow initial selection for even modest Skp2 deregulation, followed by gradual progression to more severe phenotypes. That Skp2 licenses Myc for transcription activation, and also eliminates p130, provides even further impetus for Skp2 deregulation during cancer progression. As β-TrCP activates the pro-survival NF-κB pathway, deregulation of β-TrCP might potentially contribute to tumorigenesis. Indeed, β-TrCP levels are elevated in many cancer types [234]. Although β-TrCP also down-regulates β-catenin, this pathway is often inactivated by other means including β-catenin phosphorylation site mutations and inactivation of the targeting kinase Gsk3-β, such that β-TrCP becomes

moribund for this growth inhibitory function. A few β-TrCP mutations have, however, been detected in prostate cancer [399]. Strategies to inhibit substrate interactions with either Skp2 or β-TrCP may selectively retard cancer cell proliferation [396].

Similar striking correlations between Fbw7 mutational status and cancer have begun to emerge. In initial studies, Fbw7 mutations were detected in breast and ovarian cancer cell lines, and in the former case associated with elevated cyclin E levels [277,278]. A strong correlation between reduced *Fbw7* mRNA levels and elevated cyclin E was also observed in breast cancer cells [201]. Significantly, elevated cyclin E activity is the strongest known prognostic indicator of poor clinical outcome in breast cancer [400]. Elevated levels of cyclin E also correlate with Fbw7 mutations in 16% of endometrial cancers [401], and a causal relationship between Fbw7 mutation and cyclin E deregulation in a breast cancer cell has subsequently been established [402]. Recent analysis has uncovered a compelling link between mutations in Fbw7 and colon cancer as 22 of 190 primary tumors were found to bear somatic mutations and, moreover, 4 of 58 benign polyps also contained mutations [403]. Homozygous deletion of *Fbw7* in euploid cell lines causes the formation of micronuclei and rapid progression to aneuploidy, due at least in part to accumulation of cyclin E [403]. Remarkably, many cancer associated mutations in Fbw7 map to residues in the substrate binding pocket [403,404]. Because SCF^{Cdc4} probably functions as dimer, binding pocket mutations might act in a partially dominant negative manner, which may be significant given that a 40% reduction in Fbw7 levels achieved by siRNA treatment recapitulates the chromosome instability phenotype [403]. Recently discovered connections between Fbw7 and Myc remain to be explored in these cancers. The weight of the evidence to date suggests that Fbw7 locus may be a common target for mutational inactivation in human cancer.

10.2. Subversion of host SCF pathways

A number of viral variants that exploit host SCF-like degradation pathways have been uncovered. As noted above, Vpu binds and targets the CD4 membrane protein to $SCF^{β\text{-}TrCP}$, thereby allowing HIV to elude the host immune response [226]. Vpu also appears to competitively inhibit $SCF^{β\text{-}TrCP}$-dependent degradation of IκBα, β-catenin and other substrates [405,406]. A second HIV-encoded protein called Vif interacts with EloB/C, Cul5, and Rbx1 to form an E3 that eliminates the host anti-viral factor CEM15/APOBEC3G [407]. A recently discovered complex formed from the adenovirus encoded proteins E4orf6 and E1B55K and the host Cul5 complex catalyzes ectopic elimination of the tumour suppressor p53 to allow efficient viral replication [408,409]. In yet another example, the parvomyxovirus V protein pirates a host Cul4A–Ddb1 complex to ubiquitinate and eliminate the immune regulators Stat1 and Stat2 [410]. An interaction between SV40 large T antigen and Cul7 is necessary for transformation by TAg, presumably through modulation of an undefined cell growth regulatory pathway [411]. Finally, the neural-specific F-box protein NFB42 targets the HSV replication initiator protein UL9 for proteasome-dependent degradation, thereby promoting viral latency [412]. Many uncharacterized viral open reading frames encode other SCF-like adaptors. For example, cowpox is predicted to produce at least 11 BTB domain proteins (see http://smart.embl-heidelberg.de/). Presumably, each viral analog will re-wire a host SCF-like degradation pathway to facilitate the infection cycle.

10.3. Other genetic disorders

In addition to pathogenic re-wiring of SCF-like pathways, several F-box proteins have also been recently implicated in human disease. The ubiquitin system has long been known to mediate muscle atrophy, which is a severe secondary symptom in many chronic diseases [413]. An F-box protein called MAFbx or Atrogin-1 was identified by transcript profiling of atrophying muscle cells, and found to be necessary and sufficient for induction of muscle atrophy, presumably through ubiquitination of one or more targets [414,415]. The $SCF^{Atrogin\text{-}1}$ complex is thus an attractive target for amelioration of muscle atrophy [413]. In a separate muscle connection, an interaction between Fbx4 and the heat shock protein αB-crystallin has potential links to desmin-related myopathy [416]. Mutations in an F-box protein called dactylin/Fbw4 cause the autosomal dominant disorder splithand/foot malformation [417]. The zebra fish homolog of dactylin, called hagoromo, was identified in a screen for pattern formation mutants and may provide a genetic model for the human disorder [418]. SCF-like pathways also underlie some neurological disorders. A non-canonical SCF-like complex between the RING domain protein Parkin and Sel10 appears to target cyclin E for degradation in post-mitotic neurons, defects in which may contribute to parkinsonism [419]. As noted above, Sel10 is also implicated in Alzheimer's disease [296].

10.4. Therapeutic intervention

The likely influence of SCF-mediated processes in human disease opens possible new avenues for therapeutic intervention. The ubiquitous functions of SCF core components make them less than ideal targets, although this possibility is worth exploring given the remarkable therapeutic window observed for general proteasome inhibitors. The binding pockets of F-box proteins for which structures are known are more attractive targets for design of specific small molecule inhibitors [115,404,420]. To date, however, only a single small molecule inhibitor has been reported in the literature. This compound, called Ro106-9920, has the puzzling property of inhibiting IκBα but not β-catenin ubiquitination in crude extracts, such that the drug may target a pathway that acts in parallel to $SCF^{β\text{-}TrCP}$ [421]. In a

different approach, the plasticity of the SCF system has been exploited to create synthetic E3 enzymes that in principle can target any desired protein for which a protein binding partner exists. In one strategy, the F-box has been fused to heterologous interaction domains to successfully eliminate desired protein targets in cells [422–424]. In a novel variation on this idea, called proteolysis targeting chimeric molecules (Pro-Tacs), the conserved β-TrCP phosphopeptide degron has been linked to a small molecule that binds tightly to the target proteins, thereby bypassing the need for dedicated interaction [425]. In model cell-based studies, specific Pro-Tacs have been used to eliminate methionine aminopeptidase-2, the estrogen receptor and the androgen receptor [425,426]. Conventional strategies to reduce the expression of oncogenic F-box proteins such as Skp2, or perhaps restore function to likely tumour suppressors such as Cdc4, will undoubtedly also be explored [396].

11. SCF structural analysis

Several recent structure determinations of SCF subcomplexes and bound substrate peptides have provided insight into evolutionary relationships, enzymatic mechanism and the basis for substrate recognition.

11.1. Subunit orientation

A model of the human SCF^{Skp2} complex, based on in silico assembly of overlapping subcomplex structures, reveals an elongated C-shaped cradle built on an extended Cul1 scaffold, with the substrate binding domain at one end and the E2 bound at the other [427]. Cul1 consists of an N-terminal domain, which adopts a stalk-like structure and binds the Skp1–Skp2 subcomplex, and a C-terminal globular α/β domain, which interacts with Rbx1–Cdc34 [427]. The neddylation site on Cul1 lies close to the Rbx1 binding site and is thus positioned to influence E2 interactions. These features are fully consistent with previous in vitro assembly data and deletion analysis [29–31]. The N-terminal domain of Cul1 contains three cullin-repeat motifs formed by two short and three long helices, while the C-terminal domain consists of a four-helix bundle, an α/β domain and two copies of the winged-helix motif, and includes the cullin homology region, a stretch of ~200 amino acids found in all cullins [427]. This region of Cul1 appears very rigid and serves to juxtapose the substrate-binding domain of Skp2 and the catalytic center of Cdc34. As in other E3s for which structures are known, the catalytic center and the substrate binding domain face are separated by a gap of some 50 Å [427]. This feature is presumably critical for catalysis, for example in accommodating the substrate and elongating ubiquitin chain. Models of the holo SCF^{Cdc4} and $SCF^{β\text{-}TrCP}$ complexes reveal similar overall properties, including the large gap between the catalytic site and the binding site (Fig. 8) [404,420].

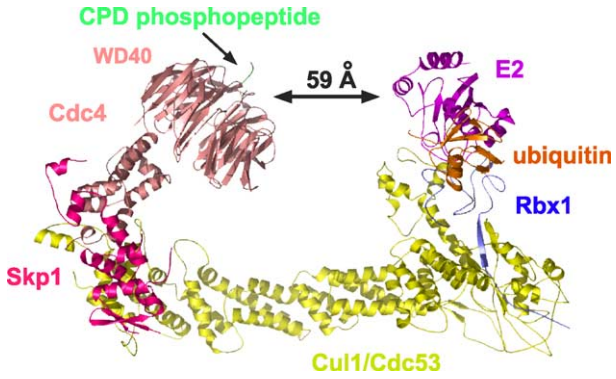

Fig. 8. Model of the holo-SCF^{Cdc4} complex. Ribbons representation was drawn with MacPyMOL (http://www.pymol.org) using coordinates from [404].

11.2. The Rbx1–Cul1 subcomplex

Rbx1 and Cul1 form an intermolecular β-sheet consisting of a combination of β-strands from both proteins [427]. The Rbx1 RING domain contains an insertion that complexes an additional zinc ion, such that in total three zinc ions are bound, as opposed to two in other RING domains (Fig. 3). So far, the Rbx1–E2 interaction has only been modeled on a structure of another RING domain, that of c-Cbl, in complex with UbcH7. The Rbx1 RING domain contains a similar hydrophobic groove as the c-Cbl–RING domain, which forms a loose binding pocket for UbcH7 [428]. The Cbl–UbcH7 interaction does not involve extensive surface contacts, which, if true in SCF complexes, may account for the weak nature of SCF-E2 interactions.

11.3. The Cul1–Skp1 subcomplex

The Skp1-binding surface on Cul1 includes residues that are conserved among Cul1 orthologs but not among other cullins, and the same holds true for the corresponding surface on Skp1 [427]. The resulting specificity of this interaction thus determines that, through Skp1, Cul1 binds only F-box proteins and not other adaptors [110,111]. Mutational analysis has also demonstrated the importance of similarly conserved subfamily-specific residues for the Cul3–BTB domain interaction, and it appears that the same will likely also be true for the Cul2–EloC interaction [117,120]. However, the basis for cross-talk between numerous orthologs and/or isoforms that exist for some cullins and their associated Skp1-like partners in various species is not known. Additionally, the identity of the associated substrate-specific adaptor can influence core interactions. For example, the presence of VHL versus Muf1 can evidently determine whether or not EloC binds to both Cul2 and Cul5 or only to Cul5, respectively [122]. That the associated adaptor can influence the interaction between

the cullin and a Skp1-like linker protein is consistent with the ternary interface observed between Cul1, Skp1 and the Skp2 F-box [427].

11.4. The Skp1–F-box protein subcomplex

The crystal structures of several substrate-specific adapters lend support to the notion that rigidity of the SCF complex is functionally important. In Skp2, the F-box directly abuts the LRRs [115], while in Cdc4 and β-TrCP the linker region between the F-box and the WD40 repeats is both well ordered and necessary for function, presumably because it establishes a particular orientation of the WD40 repeat domain relative to the F-box, and thus to the rest of the SCF complex [404,420]. The VHL structure shows a similar necessity for orienting the substrate-binding β domain relative to the SOCS-box and, indeed, a VHL syndrome mutation hotspot occurs at the interdomain interface [116].

The overall features of the substrate binding domains of adaptor proteins have also yielded several surprises. The WD40 repeats of Cdc4 form an eight-bladed β-propeller structure, unlike the seven-bladed structures determined for other WD40 repeat domains [404,429]. Additionally, the LRRs of Skp2 terminate in an extended loop that returns back across the LRRs and inserts between the F-box and LRRs [115]. This loop has no well-characterized function, but its position across the substrate binding domain and its apparent influence on the F-box/LRR interface are intriguing. One significant feature of the WD40 class of F-box proteins for which structural information is not yet on hand is the essential D-domain motif, which is immediately N-terminal to the F-box and appears to mediate dimerization [208,430–432]. While the sugar recognition domain of Fbx2 forms an anti-parallel β-sandwich that bears structural homology to galectin and xylanase carbohydrate binding domains [433] because this domain was crystallized in isolation, its orientation with respect to the core SCF complex is not known.

11.5. Plasticity of the Skp1–F-box interface

A structural comparison of the F-box-Skp1 and SOCS-box-EloC interfaces together with the BTB domain reveals a striking conserved topology that parallels alignments of primary sequence elements (Figs. 9 and 10). While the F-box and SOCS-box are structurally quite distinct from each other, their interfaces with Skp1 and EloC, respectively, each consist of similar four-helix clusters. The last helix in EloC has a corresponding helix in Skp1, and the second and third helices of the SOCS-box correspond to the first two helices of the F-box. However, the first helix in the SOCS-box (indicated by the yellow arrow) corresponds not to a helix in the F-box but rather to a helix in Skp1. This helix in the SOCS-box, in fact, corresponds to another named motif called the BC-box, identified before the SOCS-box and so named because of its importance in VHL and EloA for binding to the EloB-EloC dimer [434].

Several proteins contain both a BC-box and an F-box. For example, even though EloA binds EloC and not Skp1, EloA was included in the first list of F-box proteins [24]. This contradiction is, however, readily explained by a BC-box in EloA that immediately precedes its divergent F-box (Fig. 9A). The VHL structure strongly suggests that the BC-box in EloA not only contributes to its affinity for EloC but also topologically prevents it from binding Skp1. Other proteins that bind Cul2, including Rad7 and Muf1, similarly possess a BC-box followed by a divergent F-box [122,435]. These proteins may thus be categorized as SOCS-box proteins, which reflect both function (EloC binding) and structure (a putative three helix motif), although their designation as BC-box proteins is in some sense less ambiguous [122].

These evident structural relationships may reflect common evolutionary origins. In perhaps the most plausible model, an early eukaryote may have possessed a protein with an N-terminal BTB domain, an adjacent F-box-like structure and a C-terminal protein interaction domain. The gene encoding this hypothetical protein could have split either just before or just after the BC-box embedded in the BTB fold to create either an ancestral EloC–SOCS-box or Skp1–F-box pair. That is, cleavage of a linear polypeptide with the BTB protein-like structure (Fig. 10) at one of two places could yield modern Skp1–F-box protein and EloC–SOCS-box protein pairs. This type of evolutionary fission is likely to have occurred in numerous other instances [436]. The crystal structure of an intact BTB domain protein should provide a decisive test of this interdomain interface model.

11.6. Structural basis for substrate recognition

Substrate binding pockets have been defined for Cdc4, β-TrCP, VHL and Fbx2 but as yet not for the LRR of Skp2 or other domains. The mode by which Cdc4 recognizes phosphorylated epitopes is revealed by the crystal structure of a Skp1–Cdc4 complex bound to a high-affinity CPD phosphopeptide (LLpTPP) derived from cyclin E [404]. The most conserved residues of the Cdc4 WD-40 repeat domain conjoin to form a complex binding pocket that precisely accommodates all residues of the optimal CPD phosphopeptide. The CPD binding site lies on the top edge of an aqueous central pore that runs through the center of the WD40 toroid. Two hydrophobic pockets house the preferred bulky hydrophobic residues at the −2 and −1 positions of the CPD sequence. A central pocket is formed in part from three Arg residues that directly contact the critical phospho-Thr residue, while in the same physical pocket a conserved Trp residue interacts with the essential +1 Pro residue. Mutation of any of the critical Arg residues or adjacent residues disrupts both Sic1 and cyclin E binding to Cdc4 and Fbw7, respectively [158,201]. The bound peptide is

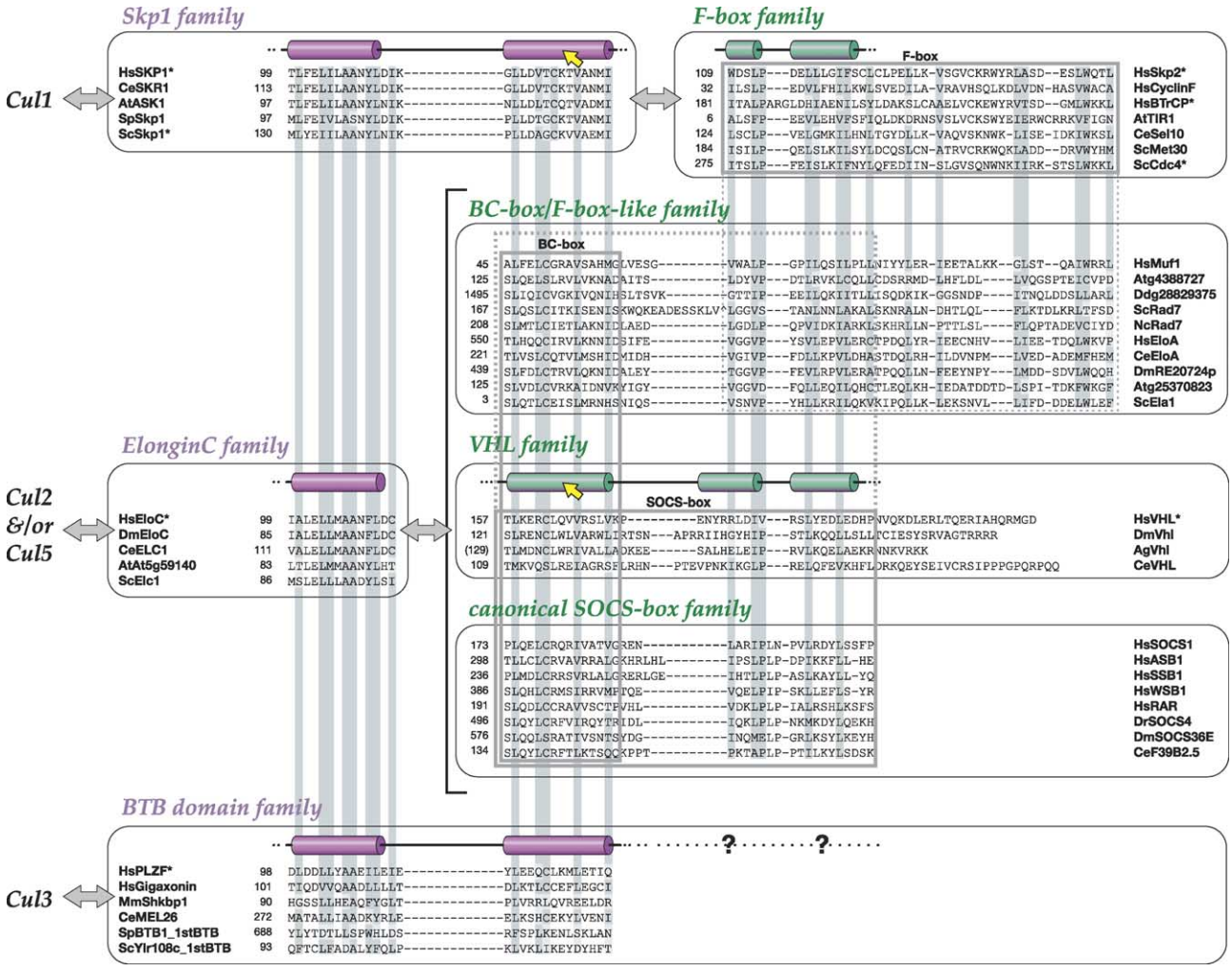

Fig. 9. Alignment of substrate specific adapters. Selected representatives of the indicated Skp1-like linker protein families (showing only a portion of the C-terminal region, left) and substrate specific adaptor families (right) are shown. Sequence alignments are guided by the structural similarities of the Skp1-F-box interface, the EloC-SOCS-box interface and the BTB domain. Helices above the alignment correspond to the four helix clusters that are circled in Figure 10. Yellow arrows indicate the swapped helices that correspond to the BC-box in EloC-interacting proteins (see Fig. 10). Gray arrows indicate protein–protein interactions. Solid boxes denote domains with well-established consensus sequences, while dotted lines indicate possible but more divergent sequence similarities. Not all of the listed proteins have been shown to interact with the indicated partners. Proteins for which crystal structures exist are indicated with an asterisk.

completely disordered beyond the +1 position. However, an important feature is provided by the extended patch of basic residues on the WD40 surface that lie downstream of the binding pocket [404]. That is, electrostatic repulsion between this patch and basic residues that typically occur at the +2 to +5 positions of CDK phosphorylation sites explains why basic residues are disfavored in the CPD consensus (Fig. 6A). As predicted from this model, mutational disruption of the basic patch allows lower phosphorylated forms of Sic1 to bind efficiently to Cdc4 [404]. The architecture of the CPD binding site thus explains the requirement for multiple phosphorylation sites on substrates such as Sic1, while at the same time affording high affinity binding to more optimal CPD sequences that lack antagonistic basic residues, as in cyclin E, Gcn4 or Myc.

Despite these structural insights, it is still a mystery as to why the Sic1–Cdc4 binding transition occurs at between five and six phosphorylation sites and not at fewer sites. Because Cdc4 itself contains only a single dedicated binding site, the usual thermodynamic explanation for cooperativity in protein–protein interactions, namely the existence of two binding sites, may not apply. Dimerization of SCF complex might in principle create two high affinity binding sites, although whether these sites could be in sufficient proximity to allow bound substrate to simultaneously access both sites and also access the catalytic site is unclear. Unfortunately, the D-domain is not included in extant structures so the relative orientation of SCF monomers to each other is unknown. Regardless, to explain the five- to six-site transition on the basis of multiple binding sites alone, six different binding sites would need to be invoked, which seems unlikely given the ability of Cdc4 to recognize different substrates with dissimilar sites and spacing. To address these issues, a kinetic model termed allovalency has

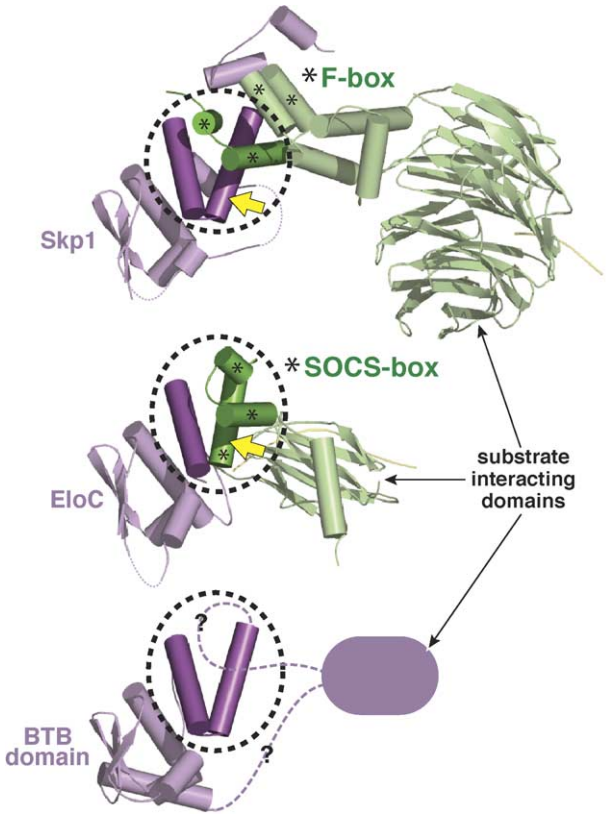

Fig. 10. Structural similarities between substrate specific adapters in cullin-based E3s. The interfaces between Skp1 and the F-box (shown for Cdc4 [404]) and between ElonginC and the SOCS-box (shown for VHL [116]) are comprised of similar four helix clusters (circled), two of which are also present in the structure of the BTB domain from PLZF [448]. The major topological distinction between the helical clusters at the F-box and SOCS-box interfaces arises from the fact that the first helix of the SOCS-box corresponds to a helix not in the F-box but rather in Skp1 (yellow arrows). The BTB domain has a fold similar to Skp1 and EloC. Protein structures were drawn with MacPyMOL (http://www.pymol.org).

been developed that invokes the unstructured nature of Sic1 as a means for multiple ligand binding sites to interact with Cdc4 in rapid equilibrium [437]. The crux of the allovalent model is that as site density increases, rebinding of a site on Sic1 becomes highly favored over diffusion away from the binding site, such that the probability of escape varies with the negative exponential of the number of ligand (i.e., CPD) sites (Fig. 6). It is also possible that rebinding is facilitated by displacement of a bound site by an incoming site. This model awaits definitive biophysical tests, but if correct may be applicable to a wide variety of polyvalent ligand–single receptor site interactions.

A second structurally defined phosphorecognition domain is that of β-TrCP [420]. The structure of β-TrCP bound to a cognate di-phosphorylated peptide derived from β-catenin reveals a similar overall binding mode as for Cdc4 in that the phosphopeptide lies across the top of a conventional seven-bladed WD40 propeller. The specificity for di-phosphorylated peptides is evident in dedicated electrostatic interactions between each phosphate and Arg residues in the binding pocket, as well as several hydrogen bond interactions. In addition, the requirement for the acidic residue at the −1 position is explained by its placement in an extensive hydrogen bond network, while the important glycine residue at the +1 position packs tightly into a small hydrophobic pocket that is unable to accommodate any other residue. Short phosphopeptides are very efficiently ubiquitinated and released by $SCF^{\beta\text{-}TrCP}$, suggesting a high off rate for phosphopeptide interactions, which occur with a K_d in the 0.5 μM range [420]. In contrast, phospho-Sic1 remains tightly bound to Cdc4 even after extensive ubiqutination [96]. The orientation of the β-catenin peptide is such that N- and C-termini are about equidistant from the E2, although precisely spaced N-terminal lysine acceptor residues appear to be favored in natural substrates [420].

Two other substrate interactions have been resolved at the atomic level. A 20-residue HIF-1α peptide bound to a VHL–EloB–EloC complex reveals that the hydroxyproline inserts into a gap of an otherwise hydrophobic stretch in the VHL β domain [438,439]. This binding pocket corresponds precisely to the mutational hotspot in renal cell carcinomas [438,439]. Similar to SCF models, the binding site on the β domain of VHL is on the order of 75 Å away from the predicted E2 catalytic site [420,438,439]. Recent structure determination of a complex between the sugar binding domain of Fbx2 and a key binding determinant in *N*-glycan called chitobiose reveals that a methyl group of chitobiose inserts into a hydrophobic pocket of Fbx2, in addition to other distal contacts [433]. Because amino acid residues around the N-glycosylation site would normally interact with the carbohydrate moiety, it is likely that Fbx2 recognizes unfolded glycoproteins [433].

11.7. SCF catalytic mechanism

The ubiquitin transfer reaction imposes stringent steric constraints in that the protein substrate, the ubiquitin moiety and the elongating ubiquitin chain together must occupy a much larger volume than typical enzyme substrates [2]. This requirement probably explains the large catalytic cleft found in all E3 structures solved to date. The mechanism of E3-mediated catalysis, however, remains a mystery. Recently, a localized diffusion model has been proposed, based on the rather weak association of the ubiquitin charged Cdc34 with the SCF^{Cdc4} complex [440]. This mechanism might explain how multiple substrate lysine residues are accessed by Cdc34 in the context of the rigid SCF structure. Puzzlingly though, it appears that free Cdc34 has a very high affinity for SCF^{Cdc4} and, moreover, once bound to SCF, Cdc34 seems not to readily form a ubiquitin thiolester, yet can be displaced by the lower affinity thiolesterified form of Cdc34 [440]. Superficially, both of these features would seem to suggest that uncharged Cdc34 might dominantly interfere with SCF ubiquitination cycles, unless very little Cdc34 is in uncharged form in the cell. In addition, the observed selective ubiquitination of specific subunits with protein

complexes, specific lysines within substrates and even specific lysines on ubiquitin itself is difficult to explain by unrestricted diffusion. For example, $SCF^{\beta\text{-}TrCP}$ strongly selects for lysines within 10–15 residues of the binding consensus [420] and a similar distance relationship may hold for the multiple CPD sites in Sic1 [441]. Thus, while the weak association of Cdc34 with the SCF core complex may be important for catalytic activity, other influences are undoubtedly at play.

Rather than a purely diffusion-based model, substrate–E2 interactions may be facilitated by tethered distance constraints that increase effective local concentrations. The potential contribution of these effects has been carefully assessed for lysine utilization in a series of β-catenin peptides bound to SCF^{TrCP} [420]. Insertion or deletion of residues between the lysine acceptor and the consensus recognition motif altered reaction efficiency in a manner remarkably consistent with average length distributions predicted by polymer theory [420]. As many, if not all, SCF recognition sites reside in unstructured or low complexity regions, as in β-catenin or Sic1 [158,442], the polymer length model may be generally applicable.

Another constraint that may modulate reaction mechanism is the apparent requirement for Cdc34 dimerization for function in vivo and in vitro. Cdc34 dimerization does not depend on the Cdc34–SCF interaction, but instead correlates strongly with thiolester formation [443]. If the thiolesterified form of Cdc34 is indeed a dimer, then it is possible that one Cdc34 molecule binds to the SCF complex, while a second Cdc 34 binds to the first. This configuration would help bridge the large gap in the catalytic cradle and effectively concentrate the E2 loaded next to the substrate. As one solution to the problem of chain extension, it has also been suggested that preassembled polyubiquitin chains on the E2 may be transferred en masse to the substrate [443]. The obligatory dimerization of the WD40 class of F-box proteins discussed above may also orient the SCF catalytic cradle so as to geometrically constrain substrate and/or E2 diffusion, again increasing the local concentration of reactants.

Substrate flexibility probably also enables the catalytic site to access and ubiquitinate multiple lysines in spite of the rigid cullin backbone. Significantly, the multiple low affinity CPD sites in Sic1 in principle allow it to interact with Cdc4 in a number of different orientations [158], which would by definition present different lysine residues to the catalytic site. Consistently, at least six lysines on Sic1 can ubiquitinated in vitro [441]. The site preference for ubiquitin conjugation on Sic1 may parallel the hierarchy of genetic effects associated with elimination of CDK phosphorylation sites on Sic1 [158]. As noted above, many other SCF substrates appear to bear disordered targeting regions, including Cln2, p27, cyclin E, Myc and β-catenin, so this feature may be a requirement for efficient ubiqutination.

It is also clear that the SCF system is quite plastic in its ability to dictate the extent of substrate ubiquitination. Sic1 is very heavily ubiquitinated on at least six sites [441], whereas β-TrCP substrates are usually ubiquitinated on two closely spaced sites adjacent to the phospho-dependent recognition motif [234], and in the extreme, Met4 appears to be ubiquitinated on a single lysine [198]. Such effects presumably reflect the particular attributes of any given substrate and E3 combination. The intrinsic recognition properties of an SCF complex can obviously be modified, as exemplified by the use of auxiliary recruitment factors such as Cks1 in SCF^{Skp2} or Vpu in $SCF^{\beta\text{-}TrCP}$. In another example, a factor that co-purifies with the $SCF^{\beta\text{-}TrCP}$ complex, HnRNP-1a, appears to act as a pseudosubstrate inhibitor of the IkBα reaction, even though HnRNP-1a does not contain even loose matches to the β-TrCP consensus sequence [444]. Finally, at least in the instance of Met4, the extent of ubiquitin chain elongation by SCF^{Met30} complex is modified by growth conditions [195]. The basis for this effect is not understood but might be accounted for by an E4-like elongation activity in minimal media or by substrate de-ubiquitination in rich medium. Selective substrate degradation may also depend on the precise context of polyubiquitin conjugates, as it has recently emerged that the multi-ubiquitin chain binding proteins (MCBPs) associated with the proteasome, Rad23 and Rpn10, specifically influence the degradation of some substrates [445].

The proteasome–SCF interaction may be crucial for catalytic efficiency as at least some substrates, such as Sic1, are not released even after extensive polyubiquitination [96]. This observation is perhaps not surprising since it is difficult to imagine how a distant ubiquitin conjugate might alter the affinity of a substrate–E3 interaction. The ability of the proteasome to selectively strip the ubiquitinated substrate from multiprotein assemblies, first observed in the selective destruction of a single ubiquitinated subunit in a β-galactosidase tetramer [95], is thus critical for regeneration of competent SCF complexes. This feature of the proteasome is critical not only for the release of SCF^{Cdc4} from polyubiquitinated Sic1, but also for the liberation of active Clc5–Cdc28 complexes, which remain inactive when bound to polyubiquitinated Sic1 [96]. Whether or not proteasome-mediated recycling of SCF complexes is a general principle remains to be determined. However, at least for model peptide substrates derived from β-catenin, a high off rate enables efficient catalytic turnover in the absence of the proteasome [420].

12. Current issues

Intense efforts over the past few years have quickly driven the SCF filed to maturity, yet several recalcitrant problems remain unsolved. The most important general question is the nature of the ubiquitin transfer mechanism itself. How does the catalytic cradle accommodate the ever extending ubiquitin chain? Might there be separate initiation and elongation steps either of which may be limiting under

different contexts? What role does substrate flexibility play in lysine selection and reaction efficiency? What role might SCF dimerization play in catalysis? From the regulatory point of view, while known SCF-dependent pathways have more than delivered on their regulatory promise, the functions of literally hundreds F-box proteins, SOCS box proteins and BTB domain proteins still remain to be discerned. A particular puzzle is the anomalous expansion of the F-box protein family in nematodes and plants. Can each of several hundred F-box proteins possibly have a specific set of substrates? Or might some Byzantine regulatory balance be at play, in which excess non-functional F-box proteins attenuate the activity of a modest number of biologically more salient complexes? Do the solo F-box or BTB domain proteins that lack apparent interaction domains recruit substrates by a different mechanism or do they compete with active complexes? And just how many substrates are there for any given F-box protein, particularly those targeted by degenerate phosphodegrons generated by abundant kinase activities, such as the CDKs or Gsk3? Unexpectedly, considerable overlap between parallel degradation pathways appears to be an emerging theme, which, when combined with regulatory interconnections, will make it problematic to discern direct from indirect effects. Answers to these and other questions will elaborate the already rich SCF tapestry and perhaps open windows of therapeutic opportunity for cancer and other diseases.

Acknowledgements

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